Guiding Cell Function at the Cell-Material Interface

Guiding Cell Function at the Cell-Material Interface

Jordi Cabanas Danés

**Reversibly Tethering
Growth Factors to Surfaces:**
Guiding Cell Function at the
Cell-Material Interface

Jordi Cabanas Danés

Members of the committee:

Chairman:	Prof. Dr. G. van der Steenhoven	(University of Twente)
Promotor:	Prof. Dr. Ir. J. Huskens	(University of Twente)
Assistant promotor:	Dr. Ir. P. Jonkheijm	(University of Twente)
Members:	Dr. A. van Amerongen	(Wageningen University)
	Prof. Dr. D. W. Grijpma	(University of Twente)
	Prof. Dr. H. B. J. Karperien	(University of Twente)
	Dr. P. M. Mendes	(University of Birmingham)
	Prof. Dr. B. J. Ravoo	(University of Münster)
	Prof. Dr. V. Subramaniam	(University of Twente)

The research described in this thesis was performed within the laboratories of the Molecular Nanofabrication (MnF) group, the MESA+ Institute for Nanotechnology and the Department of Science and Technology of the University of Twente. This research forms part of the Project P2.02 OAcontrol of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

Reversibly Tethering Growth Factors to Surfaces: Guiding Cell Function at the Cell-Material Interface

Copyright © 2013, Jordi Cabanas Danés, Enschede, The Netherlands.

All rights reserved. No part of this thesis may be reproduced or transmitted in any form, by any means, electronic or mechanical, without prior written permission of the author.

ISBN: 978-90-365-3486-4

DOI: 10.3990/1.9789036534864

Cover art: Calvin Dexter (www.calvindexter.com)

Printed by: CPI-Wöhrmann Print Service – Zutphen (The Netherlands)

REVERSIBLY TETHERING
GROWTH FACTORS TO SURFACES:
GUIDING CELL FUNCTION AT THE
CELL-MATERIAL INTERFACE

PROEFSCHRIFT

ter verkrijging van
de graad van doctor aan de Universiteit Twente,
op gezag van de rector magnificus,
Prof. Dr. H. Brinksmā,
volgens besluit van het College voor Promoties
in het openbaar te verdedigen
op donderdag 31 januari 2013 om 16.45 uur

door

Jordi Cabanas Danés
geboren op 28 januari 1985
te Vic, Catalonië, Spanje

Dit proefschrift is goedgekeurd door:

Promotor: Prof. Dr. Ir. J. Huskens

Assistant promotor: Dr. Ir. P. Jonkheijm

"Well, I must endure the presence of a few caterpillars if I wish to
become acquainted with the butterflies"
[Antoine de Saint-Exupéry, *The Little Prince*]

"Bé, he de suportar dues o tres erugues si vull conèixer les papallones."
[Antoine de Saint-Exupéry, *El Petit Príncep*]

A la meva família i a en Danny

Table of Contents

Chapter 1: General Introduction	1
1.1 References.....	4
 Chapter 2: Strategies for the Delivery and Presentation of Growth Factors	5
2.1 Introduction	6
2.2 Non-covalent GF immobilization	6
2.2.1 Nitrilotriacetic acid – Ni(II) – hexahistidine interactions	7
2.2.2 Biotin – streptavidin interactions	9
2.2.3 Peptide amphiphiles (PAs)	13
2.2.4 Heparin-based systems.....	15
2.3 Covalent GF immobilization	26
2.3.1 Covalent attachment through amines	26
2.3.2 Covalent attachment through carboxylic acids.....	31
2.4 Physical entrapment	33
2.5 Conclusions and outlook.....	37
2.6 References.....	38
 Chapter 3: Controlling the Orientation of Self-assembled Proteins at Surfaces.....	43
3.1 Introduction	44
3.2 Results and discussion	45
3.2.1 Assembly of His ₆ -tagged TagRFP variants on Ni(II)·NTA monolayers	45
3.2.1.1 TagRFP mutants	45
3.2.1.2 SPR studies.....	46
3.2.1.3 Fluorescence anisotropy experiments	50
3.2.1.4 Infrared experiments	51
3.2.1.5 Fluorescence microscopy experiments.....	53
3.2.2 Orthogonal assembly of proteins	55
3.3 Conclusions.....	60
3.4 Acknowledgements.....	60
3.5 Experimental section.....	60
3.6 Appendix	72
3.7 References.....	72

Chapter 4: A Supramolecular Approach to Growth Factor Immobilization Using His₆-tagged VHH Single-domain Antibodies.....75

4.1 Introduction	76
4.2 Results and discussion	78
4.2.1 Selection and production of VHH binder to hBMP-6	78
4.2.2 Immobilization of His ₆ -VHH onto β CD surfaces	79
4.2.3 Functional assembly and delivery of hBMP-6 using supramolecular interactions.....	85
4.3 Conclusions	89
4.4 Acknowledgements.....	90
4.5 Experimental section.....	90
4.6 Appendix	97
4.7 References.....	102

Chapter 5: A Fluorogenic Monolayer to Detect the Immobilization of Peptides that Combine Cartilage Targeting and Regeneration..... 105

5.1 Introduction	106
5.2 Results and discussion	107
5.2.1 Immobilization strategy of peptides.....	107
5.2.2 Immobilization of CGRGDS for localized cell adhesion.....	109
5.2.3 Detected co-immobilization of CLPLGNSH and CLRGRYW and binding of TGF β -1 and collagen type II	110
5.2.4 Regenerative potential of platforms functionalized with CLPLGNSH ..	114
5.3 Conclusions	117
5.4 Acknowledgements.....	117
5.5 Experimental section.....	117
5.6 References.....	122

Chapter 6: Spatiotemporal Regulation of hBMP-6 Growth Factor by Reversible Covalent Surface Tethering 123

6.1 Introduction	124
6.2 Results and discussion	125
6.2.1 hBMP-6 immobilization on siloxane surfaces.....	125
6.2.2 Biological activity of hBMP-6 using siloxane surfaces.....	128
6.2.3 Release and local confinement of hBMP-6	131

6.2.4 Spatiotemporal bioactivity of released hBMP-6	135
6.2.5 hBMP-6 on Polyactive™ films.....	136
6.3 Conclusions.....	142
6.4 Acknowledgements.....	142
6.5 Experimental section.....	142
6.6 References.....	149
 Chapter 7: Cell Studies with Arrays of Multiple Proteins Fabricated Using a Hydrogel-Filled Silicon Stamp	151
7.1 Introduction	152
7.2 Results and discussion	153
7.2.1 Preparation, design and printing procedure of hydrogel-filled Si stamps	153
7.2.2 Printing fibronectin arrays with different geometries on PTMC ₃₇₃	157
7.2.3 Cell adhesion and spreading on printed fibronectin arrays on PTMC ₃₇₃	159
7.2.4 Printing an hBMP-6 array for early cellular differentiation studies.....	164
7.3 Conclusions.....	168
7.4 Acknowledgements.....	169
7.5 Experimental section.....	169
7.6 References.....	174
 Summary	177
 Samenvatting.....	179
 List of publications	181
 Acknowledgements.....	183
 About the author	189

Chapter 1

General Introduction

Since the late 1980s tissue engineering has evolved into an interdisciplinary field of research combining expertise from materials science and technology, cell biology and chemical biology in order to develop materials that can be used to restore, maintain or improve tissue or organ function.^[1]

Growth factors (GFs) are naturally occurring substances that regulate a variety of cellular processes such as stimulating cellular growth, proliferation and differentiation, and therefore GFs are central to many therapies in the regeneration of damaged tissue but also in the treatment of many diseases such as cancer.^[2] Crucial to the success of such GF-therapy is the mode of GF delivery. Strategies relying on bolus drug delivery or systemic administration are of limited use because the inherent instability of many GFs requires very high levels of protein for a measurable effect and the potential exists for unwanted activity at distant sites. GFs are endogenously primarily present as proteins attached to the extracellular matrix (ECM).^[3] This natural attachment strategy takes care of several aspects, such as GF stability while preventing endocytosis and degradation of GF receptors, the presentation of highly localized signaling by the proteolytical activation of specific doses and duration of the stimulation. The ability to mimic such a microenvironment would create a perfect tool to control the spatiotemporal delivery of GFs. This challenge has spurred scientists to develop various strategies which are reviewed in Chapter 2. Although the latest developments of biomaterials are based on *immobilized* GFs,^[4] up to now however, not all design criteria are met.

To date, a variety of methodologies for the presentation and recruitment of GFs on materials has been reported that ranges from covalently^[5] and non-covalently^[6] tethering GFs onto materials to tethering peptides that either mimic the GF activity^[7] or recruit GFs to the desired place.^[8]

The research described in this thesis introduces novel reversible GF immobilization methodologies for the functionalization of biomaterials exploiting both covalent and non-covalent chemistry. The work presented here covers the chemical synthesis of building blocks that are used to tether GFs to surfaces, surface chemistry and characterization, biochemistry and cell biological experiments to demonstrate the functional immobilization of the GFs.

In **Chapter 2** an overview of the literature is presented of current immobilization strategies for the tethering and delivery of GFs. Emphasis is put on the design criteria for an optimal GF activity in the cellular microenvironment.

In **Chapter 3**, two different supramolecular interactions, histidine-nickel(II)-nitrilotriacetate and adamantane- β -cyclodextrin are presented for the site-specific binding of model fluorescent proteins. In this chapter, fluorescent proteins are used to give a fundamental perspective on the usage of such methodologies for achieving control over orientation and packing density while it opens a discussion about the competition of strong and weak binding affinities. Such properties would be useful for the reversible presentation and delivery of GFs.

In **Chapter 4**, a combination of the supramolecular interactions presented in Chapter 3 is employed for the immobilization of histidine-tagged single-domain fragments derived from camelid antibodies, which were screened against human bone morphogenetic protein 6 (hBMP-6) with micromolar affinity. The system is successfully explored for the delivery of hBMP-6 to mouse progenitor cells marking an increase in osteogenic activity.

In **Chapter 5** a fluorogenic platform is introduced for detecting the immobilization of non-labeled cysteine-terminated peptide sequences. The immobilized peptides possess relevant known biological properties such as cell adhesion properties,^[9] binding affinity for transforming growth factor (TGF) – $\beta 1$ ^[10] as well as for collagen type II.^[11] By co-immobilizing peptides with the latter two sequences, a bifunctional platform is fabricated that incorporates both chondrogenic differentiation and collagen II targeting properties at the same time.

In **Chapter 6** a strategy is presented for the reversible covalent immobilization of hBMP-6 with a time-specific release upon hydrolysis of the siloxane and imine bonds. Differences in early osteogenic cell differentiation are analyzed when presenting the GF by using linkers with different release kinetics under physiological conditions. In addition, our findings are extrapolated onto films of a model biomaterial such as Polyactive™.

Finally, in **Chapter 7** a novel hydrogel-filled silicon stamping device is used to print for the first time protein arrays with multiple proteins or with multiple features. The printed arrays are used for : i) the study of the effect of geometrical micro-cues on the adhesion and spreading of C2C12 cells (mouse myoblast cell line), and ii) the investigation of the effect of the surface dose of hBMP-6 on the early osteogenic differentiation of C2C12.

1.1 References

- [1] R. Langer, J. Vacanti, *Science* **1993**, 260, 920.
- [2] a) P. Tayalia, D. J. Mooney, *Advanced Materials* **2009**, 21, 3269. b) S. Aaronson, *Science* **1991**, 254, 1146.
- [3] R. O. Hynes, *Science* **2009**, 326, 1216.
- [4] T. Dvir, B. P. Timko, D. S. Kohane, R. Langer, *Nature Nanotechnology* **2011**, 6, 13.
- [5] K. S. Masters, *Macromolecular Bioscience* **2011**, 11, 1149.
- [6] G. A. Hudalla, W. L. Murphy, *Advanced Functional Materials* **2011**, 21, 1754.
- [7] M. J. Webber, J. Tongers, C. J. Newcomb, K.-T. Marquardt, J. Bauersachs, D. W. Losordo, S. I. Stupp, *Proceedings of the National Academy of Sciences of the United States of America* **2011**, 108, 13438.
- [8] L. Li, J. R. Klim, R. Derda, A. H. Courtney, L. L. Kiessling, *Proceedings of the National Academy of Sciences* **2011**, 108, 11745.
- [9] D. Liu, Y. Xie, H. Shao, X. Jiang, *Angewandte Chemie International Edition* **2009**, 48, 4406.
- [10] R. N. Shah, N. A. Shah, M. M. Del Rosario Lim, C. Hsieh, G. Nuber, S. I. Stupp, *Proceedings of the National Academy of Sciences* **2010**, 107, 3293.
- [11] D. A. Rothenfluh, H. Bermudez, C. P. O'Neil, J. A. Hubbell, *Nature Materials* **2008**, 7, 248.

Chapter 2

Strategies for the Delivery and Presentation of Growth Factors

Since the first demonstration of employing growth factors (GFs) to control cell behavior in vitro, one characteristic feature has received continuous attention for the translation of these strategies in vivo. Namely, the properties resulting from a physically confined mobility of the GF have been used for various tissue engineering applications, such as stents, orthopaedic implants, sutures and contact lenses. The lack of control over the mobility of GFs in biomaterials hampers the performance of these materials in vivo. In this chapter an overview is given of the presentation of GFs to cells with a strong focus on the importance of the strategy of tethering them to surfaces. In the first part, non-covalent strategies are described covering interaction motifs that are generic to direct GF immobilization. In the second part, covalent strategies are described emphasizing the introduction of reactive groups in existing biomaterials. This chapter ends with describing strategies based on the physical entrapment of growth factors with delivery profiles regulated by mechanisms such as diffusion, swelling or by external stimuli.

2.1 Introduction

Growth factors (GFs) are a powerful class of signaling molecules capable of regulating cellular functions and processes, including proliferation, differentiation, migration, adhesion and gene expression, and thus offering the potential to coordinate events like tissue formation, maintenance or regeneration.^[1] Although GF signaling is initiated directly upon forming stable complexes with GF receptors, which reside on the cell surface, complete gene expression is a much slower process. Therefore, control over the presentation of GFs in biomaterials is required not only in terms of retained biological activity upon inclusion of GFs into these materials, ideally with optimized accessibility to and orientation of the GFs, but also in terms of extended longevity of the presence of GFs to obtain efficient cell response.

Endogenously, the mobility of GFs is confined by trapping them in the extracellular matrix (ECM). Throughout the last decades sophisticated approaches have been developed incorporating features derived from the ECM.^[2] Many of these approaches consist of tethering GFs onto the surface of a (bio)material to achieve control over their spatial distribution. Other approaches rely on blending GFs into biopolymers to achieve temporal control over the GF delivery. Notwithstanding the progress in the development of employing GFs in biomaterials, the *in vivo* performance of biomaterials in tissue engineering applications, such as stents, orthopaedic implants, sutures and contact lenses, is still challenged by the necessary control over the mobility of growth factors in biomaterials.

In this chapter an overview is given of the methodologies presented in literature for the presentation of GFs to cells at biosurfaces. The discussion will be centered around selected examples emphasizing the different types of strategies irrespective of the type of GF, (bio)material or application involved. This chapter continues with highlighting examples ranging from the use of non-covalent interactions (Section 2.2), covalent attachment (Section 2.3) and the use of matrices (Section 2.4) to confine GFs with the common goal of controlling the spatiotemporal evolution of the GFs.

2.2 Non-covalent GF immobilization

The strategy used by the ECM to control the mobility of GFs and thereby to ensure proper cell functioning is based on non-covalent interactions between different parts of the ECM and GFs. When non-covalent interactions are non-directional, including for example ionic bonds, hydrophobic and polar

interactions, typically GFs are physisorbed. However non-covalent interactions exist that are directional, including for example hydrogen bonds and host-guest interactions.^[3] The advantage over non-directional interactions lies in the specificity and directionality of the supramolecular interaction and the tunability of the type and number of host-guest interactions. In addition to an homogeneous and oriented attachment, the reversibility of immobilization can be very attractive to tune the extent of delivery in time. Typically the ECM binds GFs through a combination of directional and non-directional interactions to ensure optimal orientation and temporal availability of the GFs.

In recent years, researchers have adopted affinity tags for immobilizing GFs onto surfaces. Many of the interactions currently used for this purpose have been originally developed for protein applications such as purification, biocatalysis and drug targeting.^[4]

2.2.1 Nitrilotriacetic acid – Ni(II) – hexahistidine interactions

Currently, recombinant proteins bearing an engineered hexahistidine (His₆) tag are produced by genetic engineering, thus enabling site-specific immobilization of His₆-tagged proteins on Ni(II)-nitrilotriacetic acid (Ni(II)-NTA)-functionalized surfaces. NTA is a tetradentate ligand that forms an octahedral complex with divalent metal ions, such as Ni(II), Co(II), Cu(II) and Zn(II), leaving two binding sites available for binding to a His₆-tag. The binding affinity is usually in the range of 10^6 - 10^7 M⁻¹.^[5] This immobilization can be easily reversed by the addition of a competitive metal binding agent (e.g. imidazole or ethylenediaminetetraacetic acid (EDTA)). Many researchers have taken advantage of this system for the binding of fluorescent proteins, antibodies, virus proteins, and GFs to surfaces for a variety of applications.^[5-6]

Iwata and co-workers employed the recombinant epidermal growth factor (EGF) carrying a C-terminal His₆-tag (EGF-His), allowing it to be immobilized onto surfaces presenting Ni(II)-NTA.^[7] First, the authors described a method to build arrays of Ni(II)-NTA on gold-coated glass substrates for the construction of EGF-His microarrays.^[7a, 7b] Briefly, a 1-hexadecanethiol self-assembled monolayer (SAM) was formed that covered the entire surface. Then, 1-hexadecanethiol molecules were photolytically removed in a pre-defined dot pattern and these bare gold areas were subsequently functionalized with 11-mercapto-1-undecanoic acid. Further derivatization into active succinimidyl esters was achieved upon reaction with *N*-hydroxysuccinimide (NHS) in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC). Reaction with *N*-(5-amino-1-carboxypentyl)

iminodiacetic acid (NTA) and incubation with NiSO_4 yielded a microarray of $\text{Ni(II)}\cdot\text{NTA}$ -terminated spots (Scheme 2.1).

Scheme 2.1 – Immobilization strategy of EGF-His onto $\text{Ni(II)}\cdot\text{NTA}$ -terminated SAMs.

After confirming the presence of EGF within the array spots, neural stem cells (NSCs) were cultured on the platforms. While NSCs seeded on chelated EGF-His spots adhered and proliferated to a substantial number, NSCs seeded on controls of covalently immobilized EGF spots, were deprived of aggregation. These results demonstrated that cell aggregation, proliferation and phenotype maintenance were mediated more efficiently on chelated EGF-His surfaces as compared to those with covalently tethered EGF. Most likely favorable interaction between the EGF and the specific EGF receptors (EGFR) on the cell surface takes place on EGF-His surfaces. In a follow-up study the authors were able to relate cell activity with the control over orientation, conformation and surface stability when immobilizing EGF via His-tag technology in comparison with covalently bound EGF via NHS-chemistry.^[7c] Multiple internal reflection-infrared absorption spectroscopy (IR-IRAS) analysis of EGF-His anchored to the surfaces suggested that chelated EGFs retain the same conformation both in solution as well as for physically adsorbed EGF-His (through ionic bonds). Contrarily, covalently immobilized EGF exhibited an altered spectrum being indicative of protein denaturation. In addition, NSCs cultured on immobilized EGF-His presented negligible expression of the β III neuronal marker and astrocytic GFAP marker indicating that on these regions the pluripotent phenotype is maintained. In contrast, cells outside the pattern expressed high levels of both β III and GFAP

while the expression of nestin (a stem cell marker) was reduced. Interestingly, when the activity of chelated human EGF (hEGF) was compared to the covalent immobilization of mouse EGF (mEGF) similar biological activities were found in terms of proliferation. The authors attributed this to the fact that mEGF contains a single primary amine at the N-terminus which will result in a single covalent linkage that affects the natural conformation of the protein to a lesser extent. Taken together, these results showed a technology to create microarray surfaces to study protein-based cell function and that immobilizing EGFs employing directional interactions is advantageous over physisorption and (random) covalent chemistry.

2.2.2 Biotin – streptavidin interactions

Similarly to the previous strategy, the interaction between biotin and streptavidin (SAv) has been broadly used to specifically bind proteins to materials.^[5-6] This interaction leads to highly stable and nearly irreversible complexes with a binding affinity ranging between $10^{13} - 10^{15} \text{ M}^{-1}$. An example of the use of this strategy to immobilize GFs was presented by Groves et al. in an attempt to understand ligand-receptor interactions.^[8] Their approach consisted of the use of a fluid-supported lipid bilayer (SLB) for displaying soluble ligands to cells. In this manner, the authors claimed to obtain a system combining a solution behavior (local concentration can be enriched by reaction-diffusion processes) and a solid behavior (with control over the spatial location of the ligands). To prove the concept, 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (DMOPC) was used with or without biotinylated EGF. The EGF was then bound to the SLB doped with 3% biotin-modified dipalmitoyl-phosphatidylethanolamine (DPPE) by means of interaction with SAv (Figure 2.1).

Figure 2.1 – Fabrication of the SLB on glass substrates. Biotinylated-EGF could be tethered by means of Alexa 647-labeled SAV to the biotinylated SLB and fluorescence recovery after photobleaching (FRAP) experiments were performed to determine the fluidity. SLBs were used to study the interaction between EGF presented to specific EGFR on the cell membrane and cellular signaling. The Attofluor cell chamber was used to maintain the SLB stable while immersed in a NaCl solution.^[8] Copyright © 2006 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

EGF was conjugated to Alexa 647 via labeled SAV and the SLB contained 2% 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) for visualization purposes. This facilitated the possibility to regioselectively photobleach both bare lipids and EGF-containing layers in order to study fluorescence recovery. Their results pointed to a reduced fluidity for SLB containing EGF in comparison to bare lipid layers, indicating the success of the functionalization. Cells from the human breast epithelial cell line (MCF-10a cells) were used to assess the biological activity of the platforms. Cells seeded on a EGF-SLB were incubated for 20 h. After this time, cell attachment was only visible on EGF-modified SLB but not on those excluding EGF. Moreover, when a competing antibody for EGF-receptor tyrosine kinase (EGFR) was added, cell attachment was reduced in the same manner as for platforms in the absence of EGF. These results suggested that cell attachment is mediated by binding of EGF to EGFR on those platforms. To verify these results, cells were treated with Tarceva, a kinase inhibitor of EGFR, resulting in a similarly poor cell attachment, thus corroborating that activation of EGFR kinase activity is required for cell attachment. EGF clustering was observed 100

min after plating as reported by Alexa 647 fluorescence and EGF clusters were enlarged in time corresponding to focal adhesions required for cell attachment. Additionally, endocytosis of the complex EGF-EGFR was found (since fluorescence was observed at different planes) indicating the progress of cell signaling. The authors found as well that when reducing the system mobility by replacing DMOPC by 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) while maintaining the same surface concentration of GF, cell spreading was also reduced accordingly. Moreover, fewer EGF clusters were observed for the DPPC-EGF-SLB compared to DMOPC-based EGF-SLB. These results indicated that the layer mobility facilitates clustering of EGF and ultimately cell adhesion and spreading.

In another example given by Park and co-workers the biotin-SAv strategy was employed for cell transfection purposes.^[9] Briefly, the primary amine groups of human EGF were used to couple biotin-derivatized PEG via the NHS moiety present in the biotin-PEG. This yielded mono-, di-, and tri-pegylated EGF species. Then, polyethyleneimine (PEI) and luciferase plasmid DNA were mixed to form positively charged polyelectrolyte complex particles (PEI-DNA) of around 90 nm in diameter. These particles could be coated electrostatically with SAv yielding an effective diameter from 100 to 200 nm for a SAv-DNA molar ratio of 100. SAv-PEI-DNA complexes with a molar ratio of 100 (SAv-DNA) were used to form supramolecular complexes with EGF-PEG-biotin (mono- and multi-pegylated) conjugates. However, only when mono-pegylated EGF was bound to the complexes, stable nanoparticles were produced while multi-pegylated EGF led to abrupt aggregation at a biotin-SAv molar ratio of 4. This DNA delivery platform represented an optimal alternative to overcome DNA enzymatic degradation when incubated with nuclease, suggesting that entrapped plasmid DNA was effectively protected. Finally, the particles were used to successfully transfect A431 human epidermoid carcinoma cells which over-express EGF receptors. The transfection efficiency of PEI-DNA complexes was dependent on surface charge. When the surface charge became less positive, for example by the interaction with SAv, adsorptive endocytosis decreased resulting in a reduced transfection efficiency. When mono-pegylated EGF-PEG-biotin was conjugated to streptavidin-PEI-DNA a maximized transfection efficiency was found since in this case, transfection was mediated by the specific interaction between EGF and EGF receptors.

In another recent example exploiting the interaction between biotin and SAv, M13 phages were modified to express biotin-like peptide sequences (HPQ) and/or integrin binding sequences (RGD) on their coat proteins for the immobilization of SAv-conjugated basic fibroblast growth factor (FGF-2) and

nerve growth factor (NGF).^[10] Some of the advantages of presenting binding points for GFs on phages is that identical copies of the phage can be easily produced on large scale via bacterial amplification, and the resulting phage can be used to build nanofibrous networks without using additional fabrication techniques. FGF-2 and NGF could then be bound to the phage in order to successfully regulate proliferation and differentiation of hippocampal neural progenitor cells (NPCs) in a synergistic manner together with RGD.

Shoichet's group developed an approach for the efficient spatially controlled immobilization of sonic hedgehog (SHH) and ciliary neurotrophic factor (CNTF) to promote differentiation of retinal precursor.^[11] In their case a three-dimensional thiol-agarose scaffold was protected with the photolabile 6-bromo-7-hydroxycoumarin moiety which upon two-photon irradiation could be cleaved yielding exposed thiol groups only in the illuminated areas. Those thiol groups could be further modified through the Michael addition of maleimide-terminated SAv and barnase to take advantage of the orthogonal non-covalent binding pairs barnase-barstar ($K_d = 10^{-14}$ M) and SAv-biotin ($K_d = 10^{-15}$ M). In this way, once the hydrogel was functionalized with both units, barstar-SHH and biotin-CNTF could self-sort upon supramolecular interaction with their binding partners. (Figure 2.2a).

Figure 2.2 – a) Strategy to simultaneously immobilize SHH and CNTF. Maleimide-barnase (black) is immobilized by locally irradiating the hydrogel in the presence of the compound and allowing it to react with the deprotected thiol groups in the matrix. In a similar way maleimide-streptavidin (orange) can be immobilized in another desired location. Incubation with barstar-SHH and biotin-CNTF leads to the self-sorting of each factor with its complementary binding partner. b) Loss of the coumarin protection by two-photon irradiation and maleimide functionalization. While the large broken circle corresponds to maleimide-barnase, the smaller oval corresponds to maleimide-SAv. c-e) Confocal images corresponding to different views of the two-regions functionalized with barstar-SHH-488 (green) and biotin-CNTF-633 (red). Adapted by permission from Macmillan Publishers Ltd: *Nature Materials*, Shoichet et al.^[11] Copyright © 2011.

After analyzing the relationship between scan number and concentration of immobilized GF in independent experiments for each binding pair, the technique was used to simultaneously immobilize the two proteins following the process in Figure 2.2a. Confocal microscopy was used to sequentially irradiate two different regions while functionalizing them with either barnase or SAV. Since the coumarin protective group has an intrinsic fluorescence, functionalization could be followed by loss of fluorescence (Figure 2.2b). Co-functionalization could be observed by using two different Alexa fluorescent dyes for labeling the GFs (Figure 2.2 c-e). Finally, the bioactivity of the scaffolds was confirmed *in vitro* with retinal precursor cells (RPCs) derived from the ciliary margin of the adult mouse retina since expression of relevant markers was found while the platforms were perfectly non-cytotoxic.

2.2.3 Peptide amphiphiles (PAs)

Peptide amphiphiles (PAs) combine the amphiphilic features from surfactants with peptide sequences with relevant biological functions to self-assemble into one-dimensional nanostructures (mostly cylindrical nanofibers) resulting in a gel under physiological conditions.^[12] Moreover, they represent a highly robust construction since differences in peptide sequence have a minimum impact on the self-assembly process.

Throughout the last decades, the group led by Stupp has been a pioneer in the development of supramolecular PA nanostructures in the tissue regeneration field.^[13] One of the PA-fibers was employed for direct binding and delivery of transforming growth factor $\beta 1$ (TGF- $\beta 1$)^[14]. In this example, the researchers presented the co-assembly of two PAs: i) one PA bearing at the N-terminus the HSNGLPL epitope (identified by phage display) with a high binding affinity to TGF- $\beta 1$ ^[15] and ii) a biologically passive sequence that acts as filler peptide to control the distribution and accessibility of the binding epitopes (Figure 2.3), with the purpose of supporting the viability and chondrogenic differentiation of mesenchymal stem cells (MSCs).

Figure 2.3 – Design of PAs with chondrogenic potential. Chemical structure of A) TGF-β1-binding PA and B) filler PA. C) Illustration of the resulting self-assembled nanofibers displaying the accessible TGF-β1-binding sequences. D) TGF-β1 release profile for nanofibers composed of only filler PA or filler PA containing a 10% of TGF-β1-binding PA (TGFβPA). Stupp et al. [14] Copyright © 2010.

First, the authors demonstrated a slower release of TGF-β1 in the case pre-loaded nanofibers containing 10 mol% of TGF-β1-binding PA were used in comparison to TGF-β1 supplemented to fibers assembled from only filler PA. MSCs were not only viable within the PA gel *in vitro* but they also showed an increased expression of cartilage markers in the presence of TGF-β1 for TGF-β1-binding PA fibers compared to fibers of filler PA after 4 weeks of culture. The *in vivo* potential of the fibers was evaluated in full thickness chondral defects in a rabbit model. The defects were filled with the PA fibers and after 12 weeks of treatment, macroscopic differences were observed for defects treated with TGF-β1-binding PA both with and without TGF-β1 compared to those treated either with TGF-β1 alone or with non-bioactive filler PA. For the TGF-β1 loaded as well as unloaded TGF-β1-binding PAs, the defect was nearly filled by new tissue similar in color and texture to the surrounding cartilage. The fact that unloaded TGF-β1-binding PA was able to regenerate the tissue in the defects as effectively as in the presence of exogenous TGF-β1 was explained by the ability to bind endogenously present TGF-β1 (i.e. from the bleeding marrow or from the surrounding synovial fluid). The use of a supramolecular material promoting specific biological response without the need for exogenous GFs or transplanted cells, inspired others to explore the potential of PA as a scaffold to bind platelet-derived growth factor BB (PDGF-BB),

vascular endothelial growth factor A (VEGF-A), FGF-2 and angiopoietin-1 (Ang-1).^[16] A prolonged PDGF-BB delivery was found for up to 14 days when delivered together with PA gel with potential applications in the preservation of myocardial function. Moreover, FGF-2 was also bound to the PA matrix by mixing the GF with a PA aqueous solution resulting in the *in situ* formation of a 3D scaffold inducing angiogenesis *in vivo* and *in vitro*.^[17]

The group of Lee was able to decorate the periphery of the peptide-based fibers with biotin for the specific interaction with SAV and biotinylated insulin-like growth factor 1 (IGF-1).^[18] The "biotin-sandwich" approach was used to deliver IGF-1 to the myocardium. The authors designed biotinylated peptide sequences that can self-assemble into nanofibers at physiological conditions. After SAV was attached to the fibers, biotinylated IGF-1 was complexed to the SAV bound to the fibers. The presence of IGF-1 bound to peptide fibers was 5-fold higher than for peptide fibers in the absence of biotin. The construct was then used to treat rat neonatal cardiac myocytes and Akt phosphorylation was analyzed as it represents a downstream target of IGF-1 signaling. Fibers loaded with biotinylated IGF-1 induced Akt phosphorylation 5-fold after prolonged delivery for 14 days compared to either peptide fibers alone or untethered IGF-1. When the IGF-1 loaded fibers were delivered *in vivo* to the myocardium of rats, an enhanced GF retention was observed up to 28 days in comparison to the soluble one, which was rapidly eliminated. Additionally, after 14 days Akt activation was detected in tissues with tethered IGF-1 but not with the controls without tethered IGF-1. Tethered IGF-1 further reduced implanted cardiomyocyte apoptosis while increasing cell growth was observed.

2.2.4 Heparin-based systems

In the late 1990s an increasing interest in the interactions between proteins and glycosaminoglycans (GAGs) arose. In particular, heparin and heparan sulfate interactions with proteins with relevant biological functions have been exhaustively studied to date, resulting in the appearance of several reviews on the topic.^[19] Heparin and heparan sulfate, both present in the ECM, are sulfated, linear, unbranched polysaccharides structurally composed of disaccharide repeat units.^[19b] They contain dimers of uronic acid and 1-4 linked glucosamine (Figure 2.4a). While the major occurring disaccharide sequences in heparin contain three sulfonate groups, heparan sulfate contains only an average of less than 1 per disaccharide.^[19a] O-sulfated saccharides have been found both in heparin and heparan sulfate for the specific interaction with various members of the FGF family.^[20]

Figure 2.4 – a) Structure of heparin (top) and heparan sulfate (bottom) consisting of major and minor variable disaccharide repeating units. ($X = H$ or SO_3^- , $Y = Ac$, SO_3^- , or H). b) Helical conformation of a heparin dodecasaccharide with the major sequence in a). Sulfur atoms are yellow, oxygen atoms red, nitrogen atoms blue and hydrogen atoms cyan. Adapted from Capila and Linhardt. ^[19b] Copyright © 2002 Wiley-VCH Verlag GmbH, Weinheim.

Heparin exists primarily as a helical structure (Figure 2.4b). The key of the specificity of the interaction of heparin with proteins is suggested to rely on a defined orientation and distribution pattern of the charges of both the sulfonate and carboxyl groups at the exterior of the helix. ^[19b] Several consensus sequences including basic and hydroapathic (neutral and hydrophobic) amino acid residues with turns in the secondary structure (which bring basic amino acid residues into proximity), have been frequently reported for the interaction with a multitude of GFs. ^[19a] As an example, Lindhart and co-workers presented a collection of studies regarding the interaction of acidic FGF-1, basic FGF-2 and TGF- β 1 with heparin. ^[19a] In summary, after structural analysis of the three GFs a common motif was found: TXXBXXTBXXBTBB (where T defines a proline turn, B a basic aminoacid residue such as arginine or lysine (or occasionally a hydrogen-bonding glutamine) and X a hydroapathic residue). This interaction resulted in a complex with a dissociation constant in the 10^{-9} M range for FGF-2 complexed with heparin. ^[21] Moreover, competitive binding studies in the presence of different concentrations of NaCl served to determine that only 30% of the binding free energy is caused by pure electrostatic interactions while the rest of the contributions rely mostly on hydrophobic interactions and hydrogen bonding through the hydroxyl groups present in heparine. ^[21]

As seen above, one of the most well studied heparin-binding protein is FGF with a high affinity for heparan sulfate proteoglycans on the cell surface. To deploy their potential, FGF binds specifically to cell surface receptors called fibroblast growth

factor receptors (FGFRs). Interestingly, FGFRs are also heparin-binding proteins with multiple interacting points. Therefore, for the success of the interaction a simultaneous ternary complex formation is required.^[22] Within this context, heparan sulfate mediates FGFR dimerization, necessary to initiate signal transduction, by binding several FGF next to each other. Depending on the FGF and FGFR pairs, the complex will be 1:1:1 interacting with another 1:1:1 and thus resulting in a 2:2:2 complex for the pair FGF-2 and FGFR-1 or 2:2:1 for FGF-1 and FGFR-2 with heparin as reported by Schlessinger et al.^[23] and Pellegrini et al.^[24] respectively and presented in Figure 2.5.

Figure 2.5 – Structures of FGF-FGFR-heparin complexes for FGF-2:FGFR1:heparin (left) and FGF-1:FGFR2:heparin (right). FGFR is gold, FGF green and heparin molecules are shown as sphere models with sulfur in yellow, oxygen in red and nitrogen in blue.^[19b] Copyright © 2002 Wiley-VCH Verlag GmbH, Weinheim.

Examples describing the interaction of heparin with isoforms of the vascular endothelial growth factor (VEGF), TGF- β 1, PDGF and EGF have been presented by Capila and Lindhart as well.^[19b] These invaluable efforts in exploring and characterizing in great detail interactions between several growth factors and heparin gave origin to a multitude of applications in the tissue engineering field.^[2d, 4] Some studies used approaches to incorporate heparin to a broad range of existent biocompatible materials in order to improve their GF retention, presentation and delivery properties. In one example heparin was modified with methacrylate groups in order to be co-polymerized with dimethacrylated PEG yielding a hydrogel for the localized delivery of biologically active FGF-2 for up to 5 weeks. The complexed FGF-2 was able to promote adhesion, proliferation and osteogenic differentiation of human mesenchymal stem cells (hMSCs).^[25] Bone morphogenetic protein 2 (BMP-2) and RGD were also presented to hMSCs by this type of hydrogel resulting in the production of increased levels of osteogenic markers.^[26] In another example, hyaluronic acid, gelatin and heparin were

modified with thiol groups and co-crosslinked with poly(ethylene glycol) diacrylate (PEGDA). These hydrogels containing only 0.3% of heparin in its composition showed sustained release of either VEGF or FGF-2 and improved *in vitro* neovascularization properties, when compared to hydrogels without co-crosslinked heparin.^[27]

Titanium surfaces were also functionalized with heparin for the immobilization of BMP-2^[28] and VEGF.^[29] In a recent example,^[29b] the activity of heparin-bound VEGF was compared to the one of VEGF tethered covalently to the same type of surface. VEGF was covalently immobilized on Ti foils coated with hyaluronic acid-catechol (HAC) or non-covalently on heparin-catechol (HepC). The Ti surfaces were used to evaluate cell response using endothelial cells (ECs) and osteoblasts. Although similar surface densities of immobilized GF were achieved following both the covalent and non-covalent strategies, the EC response of the covalently immobilized VEGF was significantly reduced when compared to the heparin-bound VEGF. In addition, the latter case led to enhanced mineralization in osteoblast/EC co-cultures. Moreover a reduced bacterial infection was observed in the studies which could be related to the highly hydrophilic and negatively charged nature of the heparin-bound Ti surfaces.

A range of biomaterials has been covalently cross-linked with heparins. For example alginate^[30] and poly(lactic-co-glycolic acid) (PLGA)^[31] are covalently cross-linked with FGF-2 binding heparin and these materials showed improved *in vivo* and *in vitro* angiogenesis properties when compared to the materials without heparin. A dendrimer modified with EGF-binding heparin was cross-linked with a collagen gel and successfully used for inducing the proliferation of human cornea epithelial cells (HCECs).^[32] The surface of electrospun fibers of poly(ϵ -caprolactone) (PCL)/gelatin was covalently modified with heparin for the binding of PDGF-BB. The fibers showed prolonged proliferation and smooth muscle cells (SMCs) could infiltrate extensively into the heparin-modified scaffold.^[33] Polymeric micelles of a block copolymer of Tetronic®-PCL-heparin were prepared by an emulsion and solvent evaporation method as an injectable vehicle for long-term delivery of FGF-2 showing an excellent performance of GF delivery properties.^[34]

An elegant example based on the use of natural matrices functionalized with heparin was presented by Hubbel's group for the controlled delivery of FGF-2^[35] or beta-nerve growth factor (β -NGF) for nerve regeneration technology making use of heparin-GF interactions.^[36] β -NGF is known for its weak interaction with heparin. In fact, this GF has even been used as a negative control in experiments concerning GF binding to heparin.^[37] However, the authors postulated that a basic domain present in β -NGF or other neurotrophins such as brain-derived

neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) could actually interact with heparin while slowing down the diffusion-based protein release from a fibrin matrix. In order to demonstrate it, fibrin was decorated with heparin-binding peptides covalently cross-linked to the matrix by the enzymatic activity of factor XIIIa. Those peptides could subsequently sequester heparin within the fibrin matrix. After loading the matrix with β -NGF, its release was studied and compared with a case with both heparin-binding peptides and heparin being absent. Without the heparin-binding and heparin components present in the matrix, the majority of the GF was released within a day, whereas in the presence of the components, only 50% of the initial amount of β -NGF was released within a day while 30% of the initial GF remained stagnant in the matrix after 15 days. In addition, a neuronal cell culture model was used to assess the performance of β -NGF, BDNF, or NT-3 presented via the heparin-based delivery system resulting in a significant enhancement of neurite extension only when heparin-binding peptide, heparin and GF were present in comparison to unmodified fibrin with β -NGF in the cell culture medium.

Another biomimetic anchoring method was recently presented for the immobilization of FGF-2 and FGF-8 that enables a switchable GF bioavailability.^[38] Here conducting poly(3,4-ethylenedioxythiophene) (PEDOT) films were formed on poly(ethyleneterephthalate) (PET) substrates by the oxidative electropolymerization of 3,4-ethylenedioxythiophene (EDOT), resulting in a net positively charged polymer. This property was used by Teixeira and co-workers to form a stable electrostatic complex between negatively charged heparin and the positively charged polymer backbone. The electrochemical responsiveness of the system was described (Figure 2.6). When an electrochemical reduction process was applied to the system, PEDOT became nearly neutral decreasing the ionic binding of heparin to PEDOT and when the system was electrochemically oxidized, fully oxidized PEDOT restored the tight original complex with heparin.

Figure 2.6 – Schematic representation of the two redox states of the electro-responsive GF presentation system. a) When reduced, PEDOT becomes neutral while weakening the electrostatic interaction with heparin which gains then an increased freedom of movement for the interaction of the heparin-immobilized GF with specific cell receptors. b) Contrarily, when oxidized, PEDOT becomes positively charged with a high affinity for the anionic sulfonate groups of heparin resulting in a tight structure that hampers the interaction of heparin-bound GFs with cells. The two states have a clear impact in NSCs differentiation. c) While interaction with FGF-2 prevents cell differentiation while cells remain proliferative, d) a restricted contact with the GF leads to astrocytic differentiation.^[38] Copyright © 2011 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

FGF-2 could be bound to the negatively charged heparin and upon applying the PEDOT reductive potential the heparin complexation to the PEDOT film was disrupted, thereby releasing FGF-2. The authors found significant stabilization of FGF-2 against enzymatic degradation when compared to soluble FGF-2, which represents a clear advantage to a daily soluble dose required in cultures of NCs. In addition the authors found that the control over the bioavailability of the GF via an electrochemical stimulus resulted either in undifferentiated (Figure 2.6c) or differentiated (Figure 2.6d) NSC cells.

Stupp and co-workers have been exploring the potential of heparins by decorating the periphery of their PAs with heparins as pro-angiogenic matrixes.^[39] After mixing two aqueous solutions: i.e. one solution containing a PA with a positively charged peptide sequence (i.e. LRKKLGKA), which is able to bind to heparin chains ($K_a = \sim 10^7 \text{ M}^{-1}$)^[39a] and another solution of heparin with or without FGF-2 and VEGF, PA-heparin fibers were formed. These PA-heparin fibers were reported to bind FGF-2 and delay its release in comparison to PA in absence of heparin. The FGF-2 loaded PA fibers resulted in enhanced

vascularization of a rat cornea in comparison with samples using PA without heparin or using a PA fiber made of a scrambled version of the heparin-binding PA sequence (i.e. LLGARKKK) (Figure 2.7).^[39b]

Figure 2.7 – Schematic representation of heparin-nucleated HBPA fibers (blue). Heparin chains (red) interact with VEGF (purple), FGF-2 (yellow) and FGF receptor (green). The authors attribute an extra stabilization of the complex heparin-FGF-2-FGF receptor due to hydrophobic interactions of the GF with another HBPA fiber (upper part). They hypothesize that the absence of hydrophobic interactions for the scrambled sequence might lead to a decreased GF activity. Reprinted from Stupp et al.^[39b] Copyright © 2008, with permission from Elsevier.

HBPA fibers were also used by the authors to deliver angiogenic GFs to extrahepatic islet isografts in diabetic mice while increasing vascular density in the transplant site, thereby improving islet engraftment and insulin production and reducing the time required to achieve normoglycemia.^[39c, 39d] Finally, in a more recent example these authors combined the use of HBPA fibers with hyaluronic acid to create a membrane at their interface.^[39e] This membrane has three regions: an amorphous layer, a region with PA fibers parallel to the contact interface and a zone with fibers aligned perpendicular to the interface.^[40] These membranes, that can form *in situ*, were successfully used to deliver VEGF and FGF-2 *in vitro* and promote angiogenesis *in vivo*.

Recently, Tekinay, Guler and co-workers designed PA fibers that contain carboxylic acid, hydroxyl and sulfonate groups to mimic the binding function of heparin.^[41] A binding constant of $K_D = 10^6 \text{ M}^{-1}$ was found for VEGF binding to the PA fibers containing all three charged groups which compares favorably to the VEGF binding constant with heparin. $\text{SO}_3\text{-PA}$ nanofibers did not reveal any VEGF binding but the PA fibers with SO_3^- , COOH and OH groups exhibited slow VEGF

release rates within the narrow VEGF therapeutic region. Culturing human umbilical vein endothelial cells (HUVECs) on such multi-charged PA fibers led to the formation of capillary-like structures without the presence of any exogenous GF. *In vivo* neo-vascularization of rat cornea was successfully achieved with GFs amounts several times lower than the ones used in literature when in combination with HMPA providing new opportunities for angiogenesis and general tissue regeneration. The usability of the system was extended lately to binding other GFs such as hepatocyte growth factor (HGF), BMP-2 and NGF with different affinities ^[42]

As described above, the interaction between heparin and GFs can be used to synthesize heparin-mimicking materials in which heparin is eventually absent. ^[43] In particular, the example presented by the Maynard's group ^[43b] shows a method to create micro- and nanoarrays of FGF-2 and VEGF by using electron beam (e-beam) lithography. First, the authors designed a novel synthetic polymer which mimicked heparin to overcome the limitations of costumed heparin synthesis. Poly(sodium 4-styrenesulfonate)-co-poly(ethylene glycol methacrylate) (PSS-co-PEGMA) was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization yielding sulfonate groups, which can be used to mimic heparin. These sulfonate groups are more stable towards hydrolysis than the sulfonate groups present in the natural polysaccharides. Moreover, the PEG units rendered the material biocompatible. After polymerization, *n*-butylamine was used to reduce the dithioester groups found at the end of the polymer to create thiol groups for stably coating gold substrates. Subsequently SPR experiments revealed that both VEGF and FGF-2 can bind to PSS-co-PEGMA in a specific and dose-dependent manner. Moreover, the GF-PSS-co-PEGMA complex was stable at physiological salt concentrations. Using e-beam lithography allowed the creation of microarrays on polymer films that were spin-coated onto silicon substrates. The e-beam was used to regioselectively cross-link the PEG block of the polymer to the substrate. After washing with water and methanol the non-cross-linked polymer was removed rendering polymer patterns surrounded by background areas. Within these patterns VEGF or FGF-2 could be specifically immobilized through interactions with the sulfate groups (Figure 2.8).

Figure 2.8 – Strategy to create GF nanopatterns on a heparin mimicking polymer. a) Films of PSS-co-PEGMA are deposited on Si substrates and e-beam treated yielding b) a size-tunable micro or nanoarray of heparin mimicking polymer. The platform can then be used for binding of c) VEGF or d) FGF-2. Reprinted with permission from Maynard et al. [43b] Copyright © 2008 American Chemical Society.

This technology was recently implemented by these authors for the co-immobilization of FGF-2 by electrostatic interaction with the sulfonate groups while ketone-functionalized RGD was bound covalently through the formation of an oxime bond with 8-armed aminooxy-terminated PEG that was co-cross-linked to the substrates together with PSS-co-PEGMA. The platforms contributed synergistically in the spreading of HUVECs in comparison to controls. [44]

Another recent example using heparin-GF interactions was presented by Lahann and co-workers. [45] The authors presented the synthesis of a novel polymer coating (poly[4-formyl-*p*-xylylene-co-4-ethynyl-*p*-xylylene-co-*p*-xylylene]) bearing two orthogonal functional groups i.e. aldehydes and alkynes. Aldehyde-functionalized heparin was attached to the aldehyde functional group in the polymer through the hydrazide-aldehyde reaction using a bis-hydrazide crosslinker. Azide-functionalized cyclic RGD (cRGD) was attached to the alkyne functional groups in the polymer using the click reaction. FGF-2 was subsequently immobilized on the heparin-presenting surfaces while the RGD could lead to better cell adhesion properties.

In a study presented by Segura et al. heparin was immobilized covalently to a SAM on gold. [46] Their strategy consisted of using the heparin-binding domain of VEGF to orient the molecule and a secondary functional group in the SAM to mediate covalent bonding, yielding VEGF that is simultaneously covalently as

well as non-covalently bound to the surface.^[46] This bind-and-lock approach aimed to homogenize GF orientation prior to covalent reaction which stabilizes the GF layers (Figure 2.9).

Figure 2.9 – Bind-and-lock immobilization strategy for VEGF. A) VEGF electrostatic interaction with a heparin-modified surface via the heparin binding domain of the GF. B) When heparin is modified with *p*-azidobenzoyl, VEGF electrostatically interact with the polysaccharide to form a covalent bond with the *p*-azidobenzoyl moiety upon UV illumination for 10 min. Reprinted from Segura et al.^[46] Copyright © 2009, with permission from Elsevier.

First, mixed SAMs were formed on gold substrates consisting of (1-mercapto-11-undecyl) tetra(ethylene glycol) (EG-OH, 99%) and (1-mercapto-11-undecyl) hepta(ethylene glycol) amine (EG-NH₂, 1%). Second, heparin was modified with the photoreactive group *p*-azidobenzoyl (Heparin-ABH). The reaction between the aldehyde groups in heparin and the amine groups on the SAM formed a Schiff base which after a reduction step yielded an irreversible bond. VEGF could be immobilized yielding a GF density of around 200 pg/cm² similarly on both heparin-ABH and oxidized heparin without the photoactive group. A GF surface density on EG-NH₂ was found to be around 120 pg/cm² and it was released up to 80% during 2 days in PBS resulting in 20 pg/cm² while for the other surfaces, 40% was released throughout the first 3 days resulting in a plateau at 100 pg/cm². These differences in retention demonstrated the specific interaction of the GF with both heparin or heparin-ABH. However upon covalent binding a much reduced release was found for VEGF bound to heparin-ABH compared to the one electrostatically interacting with heparin. Upon contact between the platforms and porcine aortic endothelial (PAE) cells overexpressing KDR (PAE/KDR), similar VEGFR-2 phosphorylation was found for

cells in contact with both electrostatically and covalently immobilized VEGF. Nevertheless, when HUVECs (endogenously presenting VEGFR-2) were used instead, phosphorylation of the receptor was found for both covalent and non-covalent immobilization approaches again, but in this case a cut-off was observed after some time for the phosphorylated receptor for VEGF delivered in a soluble format which was not found for the immobilized one. Those results indicated different phosphorylation kinetics for immobilized and soluble VEGF. The fact that phosphorylation occurred for covalently immobilized VEGF indicated that phosphorylation can occur without internalization of the ligand-receptor complex. In addition, the different release properties observed for electrostatically and covalently bound VEGF converts these platforms in excellent surfaces for further studying VEGF-VEGFR-2 signaling.

Kiick and co-workers are intensively involved in the use of low-molecular-weight heparin-modified star polymers (PEG-LMWH) that are assembling into a physically crosslinked hydrogel network upon addition of VEGF.^[47] This hydrogel presented a higher elastic modulus when crossed-link by VEGF than upon the addition of a control protein not interacting with heparin such as BSA. This confirms that the cross-linking is mediated by the addition of VEGF. 30% of the VEGF was released over a 10-day period when incubated in PBS, however, when incubated in the presence of VEGF receptor 2 (VEGFR-2), the release was increased to 80% for the same period of time while the hydrogel completely disappeared. Maintained VEGF bioactivity was demonstrated since an enhancement in the proliferation of PAE/KDR was observed for cells cultured in the presence of the hydrogel. The authors used this novel system for the presentation of other GFs such as FGF-2^[47b] or they used sulfated peptides with binding affinities ($\sim 10^6 \text{ M}^{-1}$) for both heparin-binding peptides (HBPs)^[48] and VEGF165. (Scheme 2.2).^[49]

Scheme 2.2 – Non-covalently assembled hydrogels for the delivery of GFs. Hydrogels can be formed either by mixing PEG-LMWH or PEG-[heparin mimic] such as a four-armed PEG modified with sulfated peptides with either GF alone or together with four-armed PEG decorated with PEG-HBP in order to obtain hydrogels with different release and mechanical properties. Reprinted from Kiick et al.^[49] Copyright © 2007, with permission from Elsevier.

2.3 Covalent GF immobilization

2.3.1 Covalent attachment through amines

A groundbreaking contribution was presented by Kuhl and Griffith-Cima in 1996 in which a GF was covalently tethered to a surface.^[50] In this example, the authors hypothesized that delivery of non-endocytosible and non-diffusible (i.e. tethered to an insoluble substrate) EGF can ensure appropriate numbers of GF-receptor complexes during the necessary period of time for signaling in comparison to soluble factors. To explore that, star poly(ethylene oxide) (PEO) tethers (40-80 nm when fully extended) were utilized as tethering units. Two strategies were used for the immobilization of the GF (Scheme 2.3): i) in the "surface-first" approach (Scheme 2.3a), PEO star was first attached to the surface in order to subsequently immobilize EGF and ii) in the "solution-first" approach (Scheme 2.3b), the conjugation between PEO star and GF was performed in solution and the complex was then immobilized.

Scheme 2.3 – EGF immobilization strategies. a) "Surface-first method and b) "solution-first method". Reprinted by permission from Macmillan Publishers Ltd: *Nature Medicine*. Kuhl and Griffith-Cima. [50] Copyright © 1996.

Briefly, via the "surface-first" methodology, an amino-terminated silane-based SAM was built on glass, then star PEO hydroxyl termini were activated with tresyl chloride in order to render them reactive to conjugate with primary amine groups. Afterwards, native murine EGF could be covalently immobilized in a single conformation through the terminal amine, which is its only primary amine. In parallel, when the authors omitted the tresyl chloride activation step, EGF could only be physisorbed onto the background as a control. In the "solution-first" method, conjugation of tresylated star PEO and EGF was performed in the presence of ethylenediamine (EDA) in order to attach the GF to the star PEO and to attach the GF loaded star PEO through remaining amine groups to the glass slides. In this case, aldehyde-terminated SAMs were formed on glass for the formation of an imine bond with the GF carrier construct. For both strategies, DNA synthesis was stimulated in primary rat hepatocytes in a similar manner as using a comparable concentration of soluble EGF. Additionally, no biological response was found by EGF that was non-specifically absorbed on the surfaces which the authors related to a protein conformation unsuited for complexation with EGFR. The bioactivity of the tethered GF was also assessed by analyzing morphological changes and it was observed to inhibit cell spreading as efficiently as GF delivered in solution in a similar concentration after 3 days of cell culture.

Following a similar approach EGF was presented to MSCs while tethered on thin films (100 nm) of poly(methyl methacrylate)-*graft*-poly(ethylene oxide) (PMMA-g-PEO). Such films exhibited an excellent ability to promote cell spreading and survival for this cell type in comparison to soluble EGF, even in the presence of FasL, a potent death factor for human MSCs, ^[51] while controlling cell migration. ^[52] In another example, scaffolds of PMMA-g-PEO were used to tether EGF to achieve an enhanced osteogenic colony formation of connective tissue progenitors (CTPs) when compared to soluble EGF. ^[53]

Sako and co-workers used N-(6-maleimidocaproyloxy)sulfo-succinimide (sulfo-EMCS) to cross-link the terminal and only primary amine of murine EGF to thiol-modified glass surfaces while preventing lateral diffusion and internalization of EGF receptors. ^[54] Briefly, amine-terminated SAMs on glass were reacted with succinimidyl 6-[3'-(2-pyridyldithio)-propionamido]hexanoate (LC-SPDP) to further reduce it with dithiothreitol (DTT) to yield a thiol-terminated layer. EGF could then be coupled by the reaction with sulfo-EMCS. Up to 1 EGF molecule/nm² was found with uniform density. However, the density could be tuned by changing the concentration of maleimide-modified EGF dramatically affecting cellular response. To assess the biological activity of the layers, the authors cultured A431 cells (epidermoid carcinoma) on the EGF-modified coverslips. After immunofluorescently staining phosphotyrosine, the fluorescence intensity was found much higher in cells cultured on the EGF substrates in comparison to unstimulated cells. This indicates that the EGF remained active for successful interaction with EGFR and it induced dimerization and autophosphorylation of the tyrosine residues of the receptor. Additionally, single-molecule observation of the dissociation events of Grb2, an adaptor protein that binds to the phosphorylated EGF receptor, was analyzed for living A431 cells that were stimulated with tethered EGF resulting in a dissociation rate of 0.37 s⁻¹ and a dissociation constant of $K_d = 100$ nM, demonstrating that the turnover time scale in living cells falls in the range of seconds.

In another example, Cavalcanti-Adam and co-workers recently used NHS-functionalized SAMs on gold for the covalent random immobilization of BMP-2 via the primary amine groups of the protein structure leading to surface concentrations of around 70-80 ng/cm². ^[55] Since there are a number of lysine residues present on the exterior of BMP-2, attachment may occur simultaneously through several residues, potentially creating heterogeneity in the population of immobilized proteins. Nevertheless, the BMP-2 remained active upon immobilization while inducing cellular responses on C2C12 myoblasts, such as phosphorylation of Smad and induction of Smad-dependent transcription of BMP-2 target genes, while osteogenic differentiation was reported.

A sophisticated recent example is presented by the group of Zandstra.^[56] The authors investigated three approaches for the presentation of leukemia inhibitory factor (LIF) from poly(octadecene-*alt*-maleic anhydride) (POMA) (Figure 2.10).^[56a]

Figure 2.10 – LIF immobilization strategies. a) covalent attachment of the factor to POMA. b) covalent binding to flexible PEG7 spacer arm tethered to POMA and c) non-covalent binding to ECM coating deposited on top of hydrolyzed POMA. Reprinted by permission from Macmillan Publishers Ltd: *Nature Methods*. Zandstra et al. ^[56a] Copyright © 2008.

Two of the approaches are based on the covalent attachment of the factor either directly to POMA (Figure 2.11a) or to POMA functionalized with a flexible PEG7 spacer (POMA-PEG7) (Figure 2.11b) while the third approach takes advantage of the non-covalent interaction of LIF to POMA pre-coated with ECM components (POMA-Matrix) (Figure 2.11c). To prepare the immobilization platforms, POMA was first bond to amino-functionalized glass substrates and the factor was then immobilized either via direct reaction with the anhydride groups of freshly annealed polymer or by water-soluble carbodiimide chemistry (WSC) to the free carboxylic acid groups of the PEG spacer in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and sulfo-NHS. For the non-covalent approach, all the anhydride groups were deliberately hydrolyzed and the polymer was coated either with native collagen type I and fibronectin or gelatin and LIF was then let to physisorb.

¹²⁵I-radiolabeled factor was used to quantify the amount immobilized in each case leading to different amounts: while saturation was reached for LIF covalently immobilized on POMA-PEG7 with a maximum surface density of around 90 ng/cm², incubation with a solution of the same concentration did not saturate POMA with covalently immobilized LIF. This observation is attributed to the fact that POMA-PEG7 reduces binding since: i) it presents a higher hydrophilic interface for pre-concentrating the protein and ii) it has a lower density of binding sites. LIF physisorbed to ECM protein coatings led to a similar density as for LIF that was covalently immobilized on POMA. Nevertheless, comparable surface densities could be achieved by using solutions of different concentrations for incubation. Additionally, a maximized retention was found for LIF covalently attached to POMA whereas accessibility to LIF was maximized for LIF on POMA-PEG7 and POMA-matrix in comparison to POMA. When mouse embryonic stem cells (mESCs) were seeded on immobilized LIF platforms, a dose-dependent activation of STAT3 signaling was found for covalently immobilized LIF as well as of phosphorylated MAPK in an equal manner as diffusible LIF. Additionally, immobilized LIF supported mESC pluripotency for at least 2 weeks in the absence of added diffusible LIF.

Using a similar chemical strategy, Milica Radisic and co-workers presented an approach to decorate collagen scaffolds with VEGF and angiopoietin-1 (Ang1).^[57] The authors followed a *step immobilization* in which collagen scaffolds were first incubated with EDC, sulfo-NHS and subsequently VEGF was coupled or a *bulk immobilization* in which VEGF was pre-mixed with EDC/sulfo-NHS in order to incubate the scaffolds. Moreover, different reaction media were used to perform the reaction (i.e. PBS, water or 2-(N-morpholino)ethanesulfonic acid (MES)). The authors found that PBS resulted in the reaction buffer in which higher GF amounts were immobilized as well as higher proliferation rates of different endothelial cell lines were observed. Additionally, *step immobilization* was more effective than *bulk immobilization* in the immobilization of active forms of the GF. Nevertheless, in any case, immobilized VEGF or Ang1 resulted in an enhanced cell proliferation and lactate metabolism when compared to soluble GFs in a similar concentration. The authors corroborated recently their results and extended the application of the functionalized scaffold to an *in vitro* ventricular free wall defect in rat hearts.^[58]

Similarly, Shin and co-workers reported recently the functionalization of poly(L-lactide-co-ε-caprolactone) (PLCL) with tethered VEGF^[59] or the co-immobilization of an RGD-containing peptide and FGF-2 for their synergistic activity.^[60] In both cases, polydopamine was deposited on the surface of PLCL by simply dipping it in a solution of the compound.^[61] VEGF could then be

tethered by the reaction of its primary amine or thiol groups via imine formation or Michael addition reaction. In both cases, immobilized GF enhanced adhesion and spreading of HUVECs while improving migration, suggesting the cells maintain their biological activity on these platforms.

Another common technique to render material surfaces reactive for the binding of GFs is the use of plasma polymerization. For example, Sheardown and co-workers showed a case in which PDMS was functionalized with amine groups with plasma polymerization of allylamine.^[62] EGF could be then coupled to the reactive primary amine groups on the surface by the homo-bifunctional N-hydroxysuccinimide ester of PEG-butanoic acid resulting in the immobilization from 40 to 90 ng/cm² of EGF while remaining active to significantly promote epithelial cell coverage when compared to unfunctionalized negative controls.

West and co-authors have frequently reported the use of acryloyl-PEG-N-hydroxysuccinimide to couple GFs to PEGDA.^[63] For example, TGF- β 1 was tethered following this approach and it was reported to enhance the matrix production by vascular smooth muscle cells in comparison to the same amount of soluble GF.^[63a] This strategy was also used to create gradients of FGF-2 by photopolymerization as seen before to observe that cells align and migrate in the direction of increasing tethered FGF-2,^[63b] as well as to tether VEGF that increased endothelial cell tubulogenesis on the surface of the non-degradable hydrogel 4-fold compared to the negative control while being further increased in the presence of a bound RGDS peptide sequence.^[63c] In a more recent example, the authors showed the simultaneous immobilization of PDGF-BB and FGF-2 to promote angiogenesis *in vitro* with co-cultures of endothelial cells and mouse pericyte precursor 10T1/2 cells.^[63d]

Another strategy based on WSC was presented for the immobilization of nerve growth factor (NGF) on gelatin-tricalcium phosphate membranes by the group of Su.^[64] The membranes with tethered GF showed a sustained release of bioactive NGF for up to two months.

2.3.2 Covalent attachment through carboxylic acids

The group of Ito presented a strategy to immobilize EGF with photoreactive polyallylamine coated on a polystyrene substrate.^[65] In short, polyallylamine was functionalized with N-[4-(azidobenzoyl)oxy]succinimide, yielding a photoreactive polymer (AzPhPAAm). Mouse EGF was then conjugated with the modified polymer by means of WSC using the carboxylate groups of the protein structure (Figure 2.11a) resulting in 1.4 molecules of GF per molecule of AzPhPAAmEGF as determined by elemental analysis.

Figure 2.11 – a) Synthetic scheme of photo reactive polyallylamine conjugated to EGF and b) substrate preparation and GF patterning strategy on a polystyrene substrate.

Subsequently, the authors coated a polystyrene substrate with AzPhPAAm and upon UV irradiation this AzPhPAAm was grafted onto the polystyrene plate via the reaction of the highly reactive photogenerated nitrenes with neighboring hydrocarbons. Subsequently, AzPhPAAmEGF was deposited and a photomask was used to selectively crosslink the EGF-containing material to the surface into patterns relying on the same reactive nitrenes. Although the strategy is subject to randomly orienting the EGF to the photoprecursor and prone to denaturation of the EGF, fluorescence immunostaining demonstrated that a substantial part of the EGF retained its native conformation. When Chinese hamster ovary cells overexpressing EGF receptors (CHO-ER cells) were cultured on the patterned substrate, cell adhesion was similar as without immobilized EGF. However, the intensity of immunofluorescently labeled phosphorylated tyrosine was visible only in cells adhered on EGF-immobilized areas. Patterned EGF induced also cell proliferation in contrast to platforms in the absence of GF. This represented one of the first examples in which EGF was immobilized on the surface of a polymeric

matrix with an impact on the study of EGF signaling transduction and to the manipulation of cells using artificial substrates.

Another GF immobilization strategy requiring UV light or a pulsed infrared laser to enable immobilization was presented by the group of Molly Shoichet.^[66] Their approach was based on the example presented in Figure 2.2. However, in this case, VEGF165 was randomly modified with 4-(4-N-maleimidophenyl) butyric acid hydrazide in the presence of EDC and coupled to agarose-thiol via a Michael addition reaction. Fluorescence visualization served the authors to quantify around $900 \text{ ng} \cdot \text{mL}^{-1}$ of VEGF immobilized within the hydrogel while only $80 \text{ ng} \cdot \text{mL}^{-1}$ was physically adsorbed. VEGFR2+ bran-derived endothelial (bEnd3) cells were used to assess the bioactivity of covalently bound GF in comparison to soluble VEGF in a similar concentration. In both cases, similar proliferation profiles were found indicating that covalent VEGF remains active upon immobilization. More interestingly, gradients of immobilized VEGF could be created within the hydrogel to analyze the guiding capacity of the GF to tubule-like formation mimicking an *in vivo* VEGF gradient regulated by interstitial flow. Gradients were created taking advantage of the photolabile coumarin-protected agarose-sulfide groups. Therefore, controlling the scanning number and scanning regions of a confocal laser, gradients were observed upon immobilization of fluorescently labeled VEGF. Three gradients were created with variations of $2.48, 1.65$ and $1.00 \text{ ng} \cdot \text{mL}^{-1} \cdot \mu\text{m}^{-1}$ and after 3 days of culture cells were observed to have penetrated to a depth of more than $200 \mu\text{m}$ and to form tubule-like structures for gradients of 1.65 and $1.00 \text{ ng} \cdot \text{mL}^{-1} \cdot \mu\text{m}^{-1}$. However, cells cultured on the steeper gradient (i.e. $2.48 \text{ ng} \cdot \text{mL}^{-1} \cdot \mu\text{m}^{-1}$) showed no evidence of tubular formation in the gel. The authors attributed this affect to saturation of VEGFR2 receptors limiting the cells to sense the gradient. In fact, the authors argued that as important as the gradient steepness is the starting concentration of the GF enhancing or limiting tubule extension. VEGF was also successfully attached to resorbable PLLA and PCL surfaces through WSC as presented by Albertsson et al.^[67]

2.4 Physical entrapment

Traditionally, polymeric matrixes and scaffolds have been used to deliver GFs that were entrapped inside. In this manner, release is controlled by: i) diffusion, ii) swelling, iii) erosion or by iv) external stimuli (Figure 2.12).^[4]

Figure 2.12 – Mechanisms of drug release for drugs entrapped in a polymeric matrix or scaffold.

Either way, control over release rate, orientation and effective dose is limited. These and new findings about the mechanisms that control GF delivery in organisms and successful efforts to reproduce those from a synthetic point of view, have reduced the interest for the delivery of physically entrapped GFs. However, still several interesting examples are found to date.

For example Mooney and co-workers designed a system in which VEGF delivery is based on the compressive stimulation of an alginate hydrogel.^[68] Without mechanical stimulation, the release rate appeared to be constant (due to other mechanisms presented in Figure 2.12) and the cumulative release was increasing linearly with time. When mechanical stimuli were applied, the release rate increased five times compared to the control and depending on mechanical input the cumulative release could be up to the double compared to the one for the control without mechanical stimuli. Alginate hydrogels both loaded and unloaded with VEGF were implanted *in vivo* into the dorsal region of mice. While no vascularization was observed without VEGF both with or without mechanical stimuli, the neighboring tissue of the implants showed enhanced vascularization for hydrogels loaded with VEGF. Nevertheless, mechanically stimulated hydrogels showed a significant increase in vascularization compared to non-stimulated gels.

The group of Picart is actively involved in the presentation of diffusive BMP-2 from different material supports coated with polyelectrolyte matrixes.^[69] Their strategy consists of the layer-by-layer (LbL) deposition of poly(L-lysine) (PLL) and hyaluronic acid (HA). After covalently cross-linking the films using carbodiimide

chemistry, these films were soaked in acidic solutions of BMP-2 to load the films with GF (Figure 2.13a).^[69b]

Figure 2.13 – a) Stiffness control and functionalization of polyelectrolyte LbL-deposited films with BMP-2. 1) the film is formed by alternating the deposition of PLL and HA. 2) EDC concentration and WSC reaction time can be used to tune the stiffness of the films. 3) incubation with BMP-2 is used to trap the GF within the film matrix. b) Differences between the delivery of soluble or matrix-bound BMP-2. While crosstalk between BMP and adhesion receptors is allowed when BMP-2 is retained by the matrix thus creating a high local concentration of BMP receptors complexed to the GF in the vicinities of adhesion receptors, when BMP-2 is presented in solution, receptor diffusion to interact with soluble factor decreases this cross-talk. Adapted from Picart et al.^[69b] Copyright © 2011 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

After GF loading for 1h under optimized conditions, the local concentration of BMP-2 could be increased up to 500-fold in comparison to the concentration of the loading solution and it was dependent both on the deposition conditions and the film thickness. The thickness of the polyelectrolyte layers was also shown to have an effect on the diffusion of the GF. While 12 bilayers allowed diffusion, BMP-2 entrapped within 24 bilayer matrices showed a limited capability to diffuse. Release was demonstrated to follow an initial "burst" during the first 5h while reaching a steady state which prolonged further release for several days. Additionally the authors suggested a non-covalent interaction between BMP-2

and HA gels that could be used to tune the release properties. Finally, they tested this material for the culture of C2C12 myoblast cells. When C2C12 were seeded on films without BMP-2 normal differentiation into myotubes was observed. However, when cells were seeded on loaded films a higher activity of alkaline phosphatase (ALP) was observed as an indication of osteogenic differentiation while troponin T expression (a marker of myogenic differentiation) decreased in a dose-dependent manner.^[69a] Interestingly, those films could be used for three consecutive culture sequences while BMP-2 remained bioactive, which confirms the protective role of the polyelectrolyte films. These initial findings spurred the interest of the group to apply these layers for example, as a coating of macroporous tricalcium phosphate (TCP)/hydroxyapatite (HAP) ceramic scaffolds.^[69c] This resulted in significant amounts of BMP-2 loaded within the materials with controlled release properties compared to bare scaffolds and enhanced osteogenic differentiation remaining bioactive for long periods of time.

As mentioned before, the stiffness of the films can be modulated by tuning the cross-linking via WSC (Figure 2.13a). Using this property, the authors showed that large cell adhesion, spreading and mobility differences were found between the presentation of bound and soluble BMP-2 depending on the material stiffness.^[69b] For example, no difference in cell spreading and adhesion was observed for cells cultured on stiff films both with bound BMP-2 or BMP-2 delivered in a soluble format. In contrast, when cells were seeded on soft films a significant increase in cell number, area and spreading velocity was observed in the case BMP-2 was loaded in the polyelectrolyte matrix, whereas no difference was found between BMP-2 delivered in solution or in the absence of BMP-2. As depicted in Figure 2.13b, the authors hypothesized that there must be a cooperation between BMP receptors and adhesion receptors when BMP receptors interact with highly localized BMP-2, that might affect cell shape and cytoskeletal dynamics in cases in which cell adhesion is initially poor (i.e. non-functionalized soft films). In contrast, when BMP-2 is presented in solution, BMP receptors diffuse for the complexation with soluble BMP-2 reducing the cross-talk with adhesion receptors.

BMP-2 was also delivered within a nanostructure made of high-purity carbon-fiber web (TCFW) in an approach presented by Saito et al.^[70] TCFWs with a diameter of 250 or 1000 nm were obtained by electrospinning polymer solutions showing good mechanical properties to function as implants. The scaffolds could entrap BMP-2 resulting in induction of ectopic new bone formation *in vivo* and they could repair large bone defects orthotopically.

Another interesting way to present GFs is by entrapping them in cubic inclusion bodies such as polyhedra. Inclusion bodies are proteinous crystals found for example in *Bombyx mori* cytoplasmatic polyhedrosis virus (BmCPV).^[71] Hiraki et al. have presented the co-expression of the FGF-2 encoding a sequence fused to the one for the virion outer capsid protein VP3 of BmCPV gene together with BmCPV polyhedron yielding around 10 μm large polyhedra containing FGF-2. The advantage of these protein crystals is that while they are stable and insoluble at physiological pH, release is triggered at high pH. That was demonstrated by observing FGF-2 released in cell culture medium containing 5% FBS while no release was observed in PBS. Mouse chondrogenic ATDC5 cells and mouse NIH3T3 fibroblast were cultured in the presence of FGF-2 polyhedra resulting in a higher proliferation when compared with normal polyhedra which was proven to be regulated by the FGF receptors. The FGF-2 polyhedra were also shown to inhibit chondrogenic differentiation of ATDC5 as expected resulting in micron-sized bioactive substances.

Recently, Park et al. presented an example of a potential multiple GF delivery system.^[72] Their approach consisted of constructing a matrix of PLGA microspheres coated with nanospheres of the same material via electrostatic interactions. Using preloaded nanospheres with dexamethasone (DEX), negatively charged GFs (i.e. BMP-7, IGF/FGF-2 and TGF- β 3) were complexed with positively charged heparin, which allowed to coat the nanospheres. By embedding human mesenchymal stem cells (hMSCs) onto the nanosphere-coated microspheres containing the various GFs, osteogenic, adipogenic and chondrogenic differentiation were observed. After four weeks of culturing hMSCs embedded onto the nanospheres, the expression levels of GAG, ALP and Oil red-O were evaluated for the determination of their differentiation. These results demonstrated the specificity of each coated GF towards each kind of differentiation compared to a control in which hMSCs were cultured on bare nanospheres or nanospheres loaded with the complementary GFs. In addition, results obtained via reverse transcription polymerase chain reaction (RT-PCR) and Western blot of different genes related with the different differentiation pathways as well as histology and immunohistochemistry were in agreement. Finally, the transplantation of the scaffolds *in vivo* into mice showed that the factors remained in the scaffold matrix for up to 3 weeks, thereby enabling prolonged stimulation of the differentiation of the embedded hMSCs.

2.5 Conclusions and outlook

To date, the variety and number of methodologies to present GFs from different materials have shown an exponential growth. Most of the strategies presented in

this discussion demonstrated enhanced protein stability upon immobilizing the GFs. Enhanced *in vitro* and *in vivo* performances were observed in the case of immobilized GFs when compared to traditional regenerative medicine treatments relying on the use of soluble GFs. In addition, a common feature found in all presented strategies is the creation of high local concentrations of GFs which favor i) the formation of complexes with specific cell receptors in order to initiate a cascade of reactions, regulating the signal transduction to coordinate cell processes as: tissue formation, maintenance or regeneration^[73] and ii) multivalent complexes and hampered internalization, thus resulting in special mitogenic effects.^[74] However, even though benefits have been found in systems with immobilized GF over soluble doses in terms of regenerative properties, still a need for controlling the spatiotemporal GF presence in the targeted site is required in many cases.

This control is endogenously found in living organisms by mechanisms occurring by elements in the ECM.^[75] That explains why every time, a larger number of examples to deliver GF are based on interactions found in that microenvironment.^[2a, 2c, 76] In those bioinspired systems, issues as interaction affinity, GF stability, orientation or release rates are known to be optimal in advance. Nevertheless, the demand of GF might vary from case to case and individual to individual which is why research interest is directed to the development of stimuli-responsive materials in order to customize those needs.^[77] Further progress in this field can only take place if different disciplines join forces both to elucidate living biological systems and reproduce them from a synthetic perspective while incorporating attributed features for an improved performance in an *on demand* basis.

2.6 References

- [1] a) P. Tayalia, D. J. Mooney, *Advanced Materials* **2009**, 21, 3269. b) F.-M. Chen, M. Zhang, Z.-F. Wu, *Biomaterials* **2010**, 31, 6279.
- [2] a) F. Rosso, A. Giordano, M. Barbarisi, A. Barbarisi, *Journal of Cellular Physiology* **2004**, 199, 174. b) N. Huebsch, D. J. Mooney, *Nature* **2009**, 462, 426. c) E. S. Place, N. D. Evans, M. M. Stevens, *Nature Materials* **2009**, 8, 457. d) G. A. Hudalla, W. L. Murphy, *Advanced Functional Materials* **2011**, 21, 1754.
- [3] E. Frieden, *Journal of Chemical Education* **1975**, 52, 754.
- [4] N. X. Wang, H. A. von Recum, *Macromolecular Bioscience* **2011**, 11, 321.
- [5] P. Jonkheijm, D. Weinrich, H. Schröder, C. M. Niemeyer, H. Waldmann, *Angewandte Chemie International Edition* **2008**, 47, 9618.
- [6] a) M. J. W. Ludden, D. N. Reinhoudt, J. Huskens, *Chemical Society Reviews* **2006**, 35, 1122. b) D. Weinrich, P. Jonkheijm, C. M. Niemeyer, H. Waldmann, *Angewandte Chemie International Edition* **2009**, 48, 7744.
- [7] a) K. Kato, H. Sato, H. Iwata, *Langmuir* **2005**, 21, 7071. b) T. Nakaji-Hirabayashi, K. Kato, Y. Arima, H. Iwata, *Biomaterials* **2007**, 28, 3517. c) T. Nakaji-Hirabayashi, K. Kato, H. Iwata, *Biomaterials* **2008**, 29, 4403.

- [8] J.-M. Nam, P. M. Nair, R. M. Neve, J. W. Gray, J. T. Groves, *ChemBioChem* **2006**, *7*, 436.
- [9] H. Lee, T. H. Kim, T. G. Park, *Journal of Controlled Release* **2002**, *83*, 109.
- [10] S. Y. Yoo, A. Merzlyak, S.-W. Lee, *Soft Matter* **2011**, *7*, 1660.
- [11] R. G. Wylie, S. Ahsan, Y. Aizawa, K. L. Maxwell, C. M. Morshead, M. S. Shoichet, *Nature Materials* **2011**, *10*, 799.
- [12] a) H. Cui, M. J. Webber, S. I. Stupp, *Peptide Science* **2010**, *94*, 1. b) T. Aida, E. W. Meijer, S. I. Stupp, *Science* **2012**, 335, 813.
- [13] J. B. Matson, R. H. Zha, S. I. Stupp, *Current Opinion in Solid State and Materials Science* **2011**, *15*, 225.
- [14] R. N. Shah, N. A. Shah, M. M. Del Rosario Lim, C. Hsieh, G. Nuber, S. I. Stupp, *Proceedings of the National Academy of Sciences* **2010**, *107*, 3293.
- [15] H. A. Behanna, J. J. J. M. Donners, A. C. Gordon, S. I. Stupp, *Journal of the American Chemical Society* **2005**, *127*, 1193.
- [16] P. C. H. Hsieh, M. E. Davis, J. Gannon, C. MacGillivray, R. T. Lee, *The Journal of Clinical Investigation* **2006**, *116*, 237.
- [17] H. Hosseinkhani, M. Hosseinkhani, A. Khademhosseini, H. Kobayashi, Y. Tabata, *Biomaterials* **2006**, *27*, 5836.
- [18] M. E. Davis, P. C. H. Hsieh, T. Takahashi, Q. Song, S. Zhang, R. D. Kamm, A. J. Grodzinsky, P. Anversa, R. T. Lee, *Proceedings of the National Academy of Sciences* **2006**, *103*, 8155.
- [19] a) R. E. Hileman, J. R. Fromm, J. M. Weiler, R. J. Linhardt, *Bioessays* **1998**, *20*, 156. b) I. Capila, R. J. Linhardt, *Angewandte Chemie International Edition* **2002**, *41*, 391. c) A. K. Powell, E. A. Yates, D. G. Fernig, J. E. Turnbull, *Glycobiology* **2004**, *14*, 17R. d) J. Kreuger, D. Spillmann, J.-P. Li, U. Lindahl, *The Journal of Cell Biology* **2006**, *174*, 323.
- [20] S. Ashikari-Hada, H. Habuchi, Y. Kariya, N. Itoh, A. H. Reddi, K. Kimata, *Journal of Biological Chemistry* **2004**, *279*, 12346.
- [21] L. D. Thompson, M. W. Pantoliano, B. A. Springer, *Biochemistry* **1994**, *33*, 3831.
- [22] M. Fannon, K. E. Forsten, M. A. Nugent, *Biochemistry* **2000**, *39*, 1434.
- [23] J. Schlessinger, A. N. Plotnikov, O. A. Ibrahim, A. V. Eliseenkova, B. K. Yeh, A. Yayon, R. J. Linhardt, M. Mohammadi, *Molecular Cell* **2000**, *6*, 743.
- [24] L. Pellegrini, D. F. Burke, F. von Delft, B. Mulloy, T. L. Blundell, *Nature* **2000**, *407*, 1029.
- [25] D. S. W. Benoit, K. S. Anseth, *Acta Biomaterialia* **2005**, *1*, 461.
- [26] D. S. W. Benoit, A. R. Durney, K. S. Anseth, *Biomaterials* **2007**, *28*, 66.
- [27] D. B. Pike, S. Cai, K. R. Pomraning, M. A. Firpo, R. J. Fisher, X. Z. Shu, G. D. Prestwich, R. A. Peattie, *Biomaterials* **2006**, *27*, 5242.
- [28] S. E. Kim, S.-H. Song, Y. P. Yun, B.-J. Choi, I. K. Kwon, M. S. Bae, H.-J. Moon, Y.-D. Kwon, *Biomaterials* **2011**, *32*, 366.
- [29] a) C. Wolf-Brandstetter, A. Lode, T. Hanke, D. Scharnweber, H. Worch, *Journal of Biomedical Materials Research Part A* **2006**, *79A*, 882. b) X. Hu, K. G. Neoh, J. Zhang, E.-T. Kang, W. Wang, *Biomaterials* **2012**, *33*, 8082.
- [30] M. Tanihara, Y. Suzuki, E. Yamamoto, A. Noguchi, Y. Mizushima, *Journal of Biomedical Materials Research* **2001**, *56*, 216.
- [31] J. J. Yoon, H. J. Chung, H. J. Lee, T. G. Park, *Journal of Biomedical Materials Research Part A* **2006**, *79A*, 934.
- [32] M. A. Princz, H. Sheardown, *Journal of Biomaterials Science, Polymer Edition* **2008**, *19*, 1201.
- [33] J. Lee, J. J. Yoo, A. Atala, S. J. Lee, *Biomaterials* **2012**, *33*, 6709.
- [34] J. S. Lee, D. H. Go, J. W. Bae, S. J. Lee, K. D. Park, *Journal of Controlled Release* **2007**, *117*, 204.
- [35] S. E. Sakiyama-Elbert, J. A. Hubbell, *Journal of Controlled Release* **2000**, *65*, 389.
- [36] S. E. Sakiyama-Elbert, J. A. Hubbell, *Journal of Controlled Release* **2000**, *69*, 149.
- [37] M. K. Lee, A. D. Lander, *Proceedings of the National Academy of Sciences* **1991**, *88*, 2768.
- [38] A. Herland, K. M. Persson, V. Lundin, M. Fahlman, M. Berggren, E. W. H. Jager, A. I. Teixeira, *Angewandte Chemie International Edition* **2011**, *50*, 12529.

- [39] a) K. Rajangam, H. A. Behanna, M. J. Hui, X. Han, J. F. Hulvat, J. W. Lomasney, S. I. Stupp, *Nano Letters* **2006**, 6, 2086. b) K. Rajangam, M. S. Arnold, M. A. Rocco, S. I. Stupp, *Biomaterials* **2008**, 29, 3298. c) J. C. Stendahl, L. J. Wang, L. W. Chow, D. B. Kaufman, S. I. Stupp, *Transplantation* **2008**, 86, 478. d) L. W. Chow, L. J. Wang, D. B. Kaufman, S. I. Stupp, *Biomaterials* **2010**, 31, 6154. e) L. W. Chow, R. Bitton, M. J. Webber, D. Carvajal, K. R. Shull, A. K. Sharma, S. I. Stupp, *Biomaterials* **2011**, 32, 1574.
- [40] a) R. M. Capito, H. S. Azevedo, Y. S. Velichko, A. Mata, S. I. Stupp, *Science* **2008**, 319, 1812. b) D. Carvajal, R. Bitton, J. R. Mantei, Y. S. Velichko, S. I. Stupp, K. R. Shull, *Soft Matter* **2010**, 6, 1816.
- [41] R. Mammadov, B. Mammadov, S. Toksoz, B. Aydin, R. Yagci, A. B. Tekinay, M. O. Guler, *Biomacromolecules* **2011**, 12, 3508.
- [42] a) R. Mammadov, B. Mammadov, M. O. Guler, A. B. Tekinay, *Biomacromolecules* **2012**, 13, 3311. b) B. Mammadov, R. Mammadov, M. O. Guler, A. B. Tekinay, *Acta Biomaterialia* **2012**, 8, 2077.
- [43] a) I. Freeman, A. Kedem, S. Cohen, *Biomaterials* **2008**, 29, 3260. b) K. L. Christman, V. Vázquez-Dorbatt, E. Schopf, C. M. Kolodziej, R. C. Li, R. M. Broyer, Y. Chen, H. D. Maynard, *Journal of the American Chemical Society* **2008**, 130, 16585.
- [44] C. M. Kolodziej, S. H. Kim, R. M. Broyer, S. S. Saxer, C. G. Decker, H. D. Maynard, *Journal of the American Chemical Society* **2012**, 134, 247.
- [45] X. Deng, J. Lahann, *Macromolecular Rapid Communications* **2012**, 33, 1459.
- [46] S. M. Anderson, T. T. Chen, M. L. Iruela-Arispe, T. Segura, *Biomaterials* **2009**, 30, 4618.
- [47] a) N. Yamaguchi, L. Zhang, B.-S. Chae, C. S. Palla, E. M. Furst, K. L. Kiick, *Journal of the American Chemical Society* **2007**, 129, 3040. b) T. Nie, A. Baldwin, N. Yamaguchi, K. L. Kiick, *Journal of Controlled Release* **2007**, 122, 287. c) S. H. Kim, K. L. Kiick, *Macromolecular Rapid Communications* **2010**, 31, 1231.
- [48] K. L. Kiick, *Soft Matter* **2008**, 4, 29.
- [49] S. H. Kim, K. L. Kiick, *Peptides* **2007**, 28, 2125.
- [50] P. R. Kuhl, L. G. Griffith-Cima, *Nature Medicine* **1996**, 2, 1022.
- [51] V. H. Fan, A. Au, K. Tamama, R. Littrell, L. B. Richardson, J. W. Wright, A. Wells, L. G. Griffith, *Stem Cells* **2007**, 25, 1241.
- [52] S. Wu, A. Wells, L. G. Griffith, D. A. Lauffenburger, *Biomaterials* **2011**, 32, 7524.
- [53] N. A. Marcantonio, C. A. Boehm, R. J. Rozic, A. Au, A. Wells, G. F. Muschler, L. G. Griffith, *Biomaterials* **2009**, 30, 4629.
- [54] J. Ichinose, M. Morimatsu, T. Yanagida, Y. Sako, *Biomaterials* **2006**, 27, 3343.
- [55] T. L. M. Pohl, J. H. Boergemann, G. K. Schwaerzer, P. Knaus, E. A. Cavalcanti-Adam, *Acta Biomaterialia* **2012**, 8, 772.
- [56] a) K. Alberti, R. E. Davey, K. Onishi, S. George, K. Salchert, F. P. Seib, M. Bornhauser, T. Pompe, A. Nagy, C. Werner, P. W. Zandstra, *Nature Methods* **2008**, 5, 645. b) T. Pompe, K. Salchert, K. Alberti, P. Zandstra, C. Werner, *Nature Protocols* **2010**, 5, 1042.
- [57] L. L. Y. Chiu, R. D. Weisel, R.-K. Li, M. Radisic, *Journal of Tissue Engineering and Regenerative Medicine* **2011**, 5, 69.
- [58] Y. Miyagi, L. L. Y. Chiu, M. Cimini, R. D. Weisel, M. Radisic, R.-K. Li, *Biomaterials* **2011**, 32, 1280.
- [59] Y. M. Shin, Y. B. Lee, S. J. Kim, J. K. Kang, J.-C. Park, W. Jang, H. Shin, *Biomacromolecules* **2012**, 13, 2020.
- [60] Y. B. Lee, Y. M. Shin, J. H. Lee, I. Jun, J. K. Kang, J.-C. Park, H. Shin, *Biomaterials* **2012**, 33, 8343.
- [61] H. Lee, S. M. Dellatore, W. M. Miller, P. B. Messersmith, *Science* **2007**, 318, 426.
- [62] B. J. Klenkler, M. Griffith, C. Becerril, J. A. West-Mays, H. Sheardown, *Biomaterials* **2005**, 26, 7286.
- [63] a) B. K. Mann, R. H. Schmedlen, J. L. West, *Biomaterials* **2001**, 22, 439. b) S. A. DeLong, J. J. Moon, J. L. West, *Biomaterials* **2005**, 26, 3227. c) J. E. Leslie-Barbick, J. J. Moon, J. L. West, *Journal of Biomaterials Science, Polymer Edition* **2009**, 20, 1763.

- d) J. E. Saik, D. J. Gould, E. M. Watkins, M. E. Dickinson, J. L. West, *Acta Biomaterialia* **2011**, 7, 133.
- [64] P.-R. Chen, M.-H. Chen, F.-H. Lin, W.-Y. Su, *Biomaterials* **2005**, 26, 6579.
- [65] Y. Ito, G. Chen, Y. Imanishi, *Bioconjugate Chemistry* **1998**, 9, 277.
- [66] Y. Aizawa, R. Wylie, M. Shoichet, *Advanced Materials* **2010**, 22, 4831.
- [67] U. Edlund, T. Sauter, A. C. Albertsson, *Polymers for Advanced Technologies* **2011**, 22, 166.
- [68] K. Y. Lee, M. C. Peters, K. W. Anderson, D. J. Mooney, *Nature* **2000**, 408, 998.
- [69] a) T. Crouzier, K. Ren, C. Nicolas, C. Roy, C. Picart, *Small* **2009**, 5, 598. b) T. Crouzier, L. Fourel, T. Boudou, C. Albigès-Rizo, C. Picart, *Advanced Materials* **2011**, 23, H111. c) T. Crouzier, F. Sailhan, P. Becquart, R. Guillot, D. Logeart-Avramoglou, C. Picart, *Biomaterials* **2011**, 32, 7543. d) V. Gribova, R. Auzely-Velty, C. Picart, *Chemistry of Materials* **2011**, 24, 854.
- [70] K. Aoki, Y. Usui, N. Narita, N. Ogiwara, N. Iashigaki, K. Nakamura, H. Kato, K. Sano, N. Ogiwara, K. Kametani, C. Kim, S. Taruta, Y. A. Kim, M. Endo, N. Saito, *Small* **2009**, 5, 1540.
- [71] H. Mori, C. Shukunami, A. Furuyama, H. Notsu, Y. Nishizaki, Y. Hiraki, *Journal of Biological Chemistry* **2007**, 282, 17289.
- [72] J. S. Park, H. N. Yang, D. G. Woo, S. Y. Jeon, K.-H. Park, *Biomaterials* **2011**, 32, 28.
- [73] M. P. Lutolf, J. A. Hubbell, *Nature Biotechnology* **2005**, 23, 47.
- [74] Y. Ito, *Soft Matter* **2008**, 4, 46.
- [75] R. O. Hynes, *Science* **2009**, 326, 1216.
- [76] G. A. Hudalla, W. L. Murphy, *Soft Matter* **2011**, 7, 9561.
- [77] P. M. Mendes, *Chemical Society Reviews* **2008**, 37, 2512.

Controlling the Orientation of Self-assembled Proteins at Surfaces

This chapter describes the decoration of fluorescent proteins with supramolecular motifs and their study to probe the orientation of these proteins upon immobilization on planar surfaces. TagRFPs (a red fluorescent protein) were produced with different numbers of hexahistidine (His₆) tags for their binding to Ni(II)-complexed nitrilotriacetic acid (NTA) monolayers. TFP (a cyan fluorescent protein) was modified with a trivalent adamantyl-modified peptide for its interaction with β -cyclodextrin (β CD) monolayers.

Surface plasmon resonance spectroscopy, fluorescence microscopy and anisotropy measurements, and infrared reflection-absorption spectroscopy were used to characterize the stability, reversibility, density and orientation of the proteins at the surface. Stability and orientation can be correlated with the number and position of the His₆-tags at the protein structure. An increased number of His₆-tags led to complexes of higher stability, His₆-tags positioned at the same side of the protein structure resulted in densely-packed protein layers with a preferred face-on orientation whereas positioning of the tags at opposite sides led to a more loose protein packing with a preferred side-on orientation. In addition, nanoimprint lithography (NIL) was utilized to create bifunctional patterns to study sequential co-immobilization of fluorescent proteins carrying orthogonal supramolecular elements. Crucial for successful co-immobilization was the order in which the proteins were immobilized regarding their binding affinity.

3.1 Introduction

Proteins immobilized on solid substrates play an increasingly important role in a variety of biomedical and biotechnological applications, biomaterials and cell biology research. Specific properties of surface-based diagnostic assays and cell culture supports are often determined by the site-selective attachment of proteins to solid supports. Controlling the site-specific immobilization and thus orientation of proteins is particularly important because, as opposed to non-specific adsorption, it generates homogeneous surface coverage and, in particular, accessibility to the protein's active site. Consequently, different types of bio-orthogonal reactions, either non-covalent or covalent, have been developed to site-specifically attach proteins to surfaces.

Few reports exist that prove the fabrication of arrays displaying proteins uniformly oriented on the surface. Saavedra's group reported the determination of the distribution of orientations of cytochrome c attached site-selectively to glass substrates.^[1] Tampé and coworkers attached 20 S proteasome (*Thermoplasma acidophilum*) to substrates functionalized with chelated metal complexes using the His₆-tag attached to α - or β -subunits of the proteasome. Placing His₆-tags on the α -subunits resulted in a face-on orientation of the barrel-shaped enzyme while His₆-tags attached to the β -subunits resulted in enzymes attached side-on to the substrate.^[2] Proteolytic activity was determined for both orientations of the proteasome on the surface and found to be twice as high for the side-on compared to the end-on configuration. Moreover, details on the mechanism of proteolysis by the 20 S proteasome were revealed this way. The above examples clearly show that precise control over protein orientation and geometry is crucial for the construction of uniform functional protein arrays. However, to date, no systematic study on the influence of full control over the geometry has been carried out. The aim of this study is to construct such arrays in which the effect of different degrees of control over the geometry of proteins on their function will be studied. The change in geometry of protein immobilization to the surface will be studied with respect to the exact positioning such as localization, orientation and number of binding sites. Precise localization of proteins is an essential prerequisite for creating reproducible arrays of single proteins for multiplexed single-molecule spectroscopy experiments.

In this chapter, a collection of mutants of red fluorescent protein (TagRFP)^[3] and teal fluorescent protein (TFP)^[4] is produced with either a number of hexahistidine-tags or with a trivalent adamantyl-modified peptide. The model fluorescent proteins were used for studying their binding to either a Ni(II)-NTA or a β CD surface. To obtain a detailed insight in the influence of supramolecular elements

on the protein's orientation and packing densities upon surface immobilization various analytical surface techniques have been employed. These results are described in the first section of this chapter. In the subsequent section work is described that was devoted to assemble proteins carrying two orthogonal supramolecular elements on bifunctional surfaces.

3.2 Results and discussion

3.2.1 Assembly of His₆-tagged TagRFP variants on Ni(II)-NTA monolayers

3.2.1.1 TagRFP mutants

Fluorescent proteins were used in this study as they provide an intrinsic probe for the orientation of the proteins upon immobilization, as their fluorescence characteristics are highly sensitive to the orientation of the chromophore with respect to the surface. Five different variants of a monomeric red fluorescent protein generated from the wild-type RFP from sea anemone *Entacmaea quadricolor*,^[3] TagRFP, were recombinantly produced differing in their number and position of His₆-tags (Figure 3.1).

Figure 3.1 – Schematic representation of the five TagRFP proteins with different numbers and positions of His₆-tags used in this study.

To introduce one of the His-tags the chemoselective reaction between a Cys-residue and a maleimide-modified His₆ peptide was employed. To this end, site-directed mutagenesis of TagRFP was used to introduce a single Cys residue at position 128, which is located in a flexible loop on top of the β -barrel opposite of the N- and C-terminus. In addition, to ensure the conjugation reaction of the maleimide-modified peptide proceeds site-selectively, the two other accessible Cys residues on the surface of the β -barrel (positions 114 and 222) were changed

to Ser residues, yielding $^{10}\text{TagRFP}$. Mutating TagRFP only at positions 114 and 222 from Cys to Ser yielded $^{14}\text{TagRFP}$. Of both mutants $^{10}\text{TagRFP}$ and $^{14}\text{TagRFP}$, a variant with a single His-tag at the N-terminus ($^{\text{N}}\text{His}_6\text{-}^{10}\text{TagRFP}$ and $^{\text{N}}\text{His}_6\text{-}^{14}\text{TagRFP}$ (**1H**)) as well as one with two His-tags at N- and C-terminus ($^{\text{N}}\text{His}_6\text{-}^{\text{C}}\text{His}_6\text{-}^{10}\text{TagRFP}$ and $^{\text{N}}\text{His}_6\text{-}^{\text{C}}\text{His}_6\text{-}^{14}\text{TagRFP}$ (**2H**)) were made. The maleimide-modified His₆-peptide was conjugated to $^{\text{N}}\text{His}_6\text{-}^{10}\text{TagRFP}$ and $^{\text{N}}\text{His}_6\text{-}^{\text{C}}\text{His}_6\text{-}^{10}\text{TagRFP}$ to yield after purification $^{\text{N}}\text{His}_6\text{-}^{\text{S}128}\text{CHis}_6\text{-}^{10}\text{TagRFP}$ (**1+1H**) and $^{\text{N}}\text{His}_6\text{-}^{\text{C}}\text{His}_6\text{-}^{\text{S}128}\text{CHis}_6\text{-}^{10}\text{TagRFP}$ (**2+1H**), respectively. The His₆-tag-free variant TagRFP (**0H**) was produced employing Enterokinase to cleave off the N-terminal His-tag from wild-type TagRFP (Figure 3.1). The resulting proteins were characterized using SDS gel electrophoresis and mass spectrometry. All variants were found to have unchanged photophysical properties when compared to wild-type TagRFP verifying their structural integrity (Table 3.1 in the Appendix).

3.2.1.2 SPR studies

His-tagged TagRFP proteins were immobilized on Ni(II)-NTA SAMs on gold as shown in Scheme 3.1 following a protocol adapted from literature.^[5]

Scheme 3.1 – Gold substrate functionalization and protein immobilization process by assembling 11-mercaptoundecanoic acid (MUA) on gold, followed by NHS/EDC activation, subsequent immobilization of *N,N*-bis(carboxymethyl)-*L*-lysine (NTA-NH₂), complexation with Ni²⁺ ions and self-assembly of His-tagged proteins.

SPR studies were performed to evaluate the interaction of each TagRFP with the Ni(II)-NTA surface. To this end, SPR titrations were carried out by flowing solutions with different concentrations of each TagRFP over Ni(II)-NTA monolayers, while recording the changes in intensity at a fixed angle. In all SPR experiments, 0.01% Tween-20 was added to the buffer to suppress any flow-induced physisorption of the proteins to the Ni(II)-NTA surfaces. No significant binding was observed in the case of using **0H**, without His-tag, at a concentration in the highest concentration range used for the experiments with His-tag-functionalized proteins. Clear binding events in the SPR signal were observed for all four His-tagged TagRFP constructs as shown in Figure 3.2. These results indicate that the assembly of TagRFP occurs via the specific interaction between the His₆-tag and the Ni(II)-NTA surfaces as envisioned.

Figure 3.2 – SPR titration data (markers) and corresponding Langmuir fits (lines) of TagRFP with different number of His₆-tags (concentrations ranging from 0 to 22 μM) to Ni(II)-NTA monolayers.

As expected, the higher the number of His-tags attached to the protein the lower the concentration of protein at which binding could still be observed. Notably, the apparent affinities of **2H** and **1+1H** are identical, and the differences in affinities between 1 and 2 His₆-tags and between 2 and 3 His₆-tags are the same. When comparing the SPR titration curves of **1H** (Figure 3.2, red curve) and **2H** (Figure 3.2, blue curve), a similar maximum change in SPR signal was observed, indicative of similar amounts of protein adsorbing onto the surface. In strong contrast, when TagRFPs **1+1H** and **2+1H** were assembled, a lower maximum adsorption of proteins to the surface was observed when compared to **1H** and **2H**. Taken together, when the proteins have His-tags attached to both sides of

the β -barrel the maximum amount of adsorbed protein is on average 30% lower than when compared to proteins with one or two His-tags attached to only one side of the β -barrel. These observations may indicate that there are two ways for the proteins to cover the surface depending on the spatial arrangement of their His-tags. Presumably, variants with His-tag(s) on one side might be oriented with the axis of the β -barrel normal to the surface, i.e. face-on or upright, while variants with His-tags on either side might be oriented with the axis of the β -barrel parallel to the surface, i.e. side-on.

The SPR data were first fitted to a 1:1 Langmuir-type model assuming that the His-tags of the TagRFPs interact with the Ni(II)·NTA surface as one entity. As shown in Figure 3.2a, the SPR data were satisfactorily fitted to yield an overall binding constant of $K_{LM} = 2.7 \times 10^6 \text{ M}^{-1}$ for **1H**. In the case of **2H** a K_{LM} of $3.8 \times 10^7 \text{ M}^{-1}$ was found while for **1+1H** $4.7 \times 10^7 \text{ M}^{-1}$ and for **2+1H** $2.7 \times 10^8 \text{ M}^{-1}$ was found. These increasing values are in agreement with the increasing number of His₆-tags of the TagRFPs and are indicative of an increase in the thermodynamic stability of the surface complexes. In a quantitative manner, each additional His₆-tag appears to lead to an affinity increase of a factor 10, regardless the point of attachment of the His₆-tag.

As a next step a model was constructed to predict and determine the thermodynamic stability parameters of the protein complex in relation with SPR experimental data according to a multivalency model as previously reported in literature.^[6] When considering the His₆-tag-(Ni(II)·NTA)₃ interaction as a single binding motif, binding of consecutive His₆-tags of a protein will be then controlled by the effective concentration (C_{eff}) of Ni(II)·NTA at the surface. As an example the equilibrium (1) and mass balance (2) for a double His₆-tagged TagRFP (**2H**) protein are given:

$$\theta_{\text{Ni(II)} \cdot \text{NTA}_{\text{tot}}} / \theta_{\text{tot}} = \theta_{\text{Ni(II)} \cdot \text{NTA}_{\text{free}}} / \theta_{\text{tot}} + \theta_{\mathbf{2H} \cdot \text{Ni(II)} \cdot \text{NTA}} / \theta_{\text{tot}} + \theta_{\mathbf{2H} \cdot (\text{Ni(II)} \cdot \text{NTA})_2} / \theta_{\text{tot}} \quad (2)$$

And the corresponding binding constant expressions are:

$$K_1 = \frac{\theta_{\mathbf{2H} \cdot \text{Ni(II)} \cdot \text{NTA}} / \theta_{\text{tot}}}{[\mathbf{2H}] \cdot \theta_{\text{Ni(II)} \cdot \text{NTA}_{\text{free}}} / \theta_{\text{tot}}} = 2K_{i, \text{His}_6} \quad (3)$$

$$K_2 = \frac{\theta_{\mathbf{2H} \cdot (\text{Ni(II)} \cdot \text{NTA})_2} / \theta_{\text{tot}}}{\theta_{\mathbf{2H} \cdot \text{Ni(II)} \cdot \text{NTA}} / \theta_{\text{tot}} \cdot \theta_{\text{Ni(II)} \cdot \text{NTA}_{\text{free}}} / \theta_{\text{tot}}} = \frac{1}{2} C_{\text{eff}} K_{i, \text{His}_6} \quad (4)$$

Substituting the equilibrium constant equations (3 and 4) into the mass balance (equation 2) (not shown) yields a set of numerically solvable equations with $\theta_{\text{Ni(II)}\cdot\text{NTA}}/\theta_{\text{tot}}$ as the variable. θ corresponds to the absolute surface coverage of either a certain surface species or the total surface coverage and it is expressed in mol/surface area units, while species in solution are expressed as concentration in mol/L units. Since the experiment was carried out under continuous flow conditions, $[\mathbf{2H}]$ is assumed to be $[\mathbf{2H}]_{\text{tot}}$. C_{eff} was approximated to $10/K_{i,\text{His}_6}$ since each additional His₆-tag leads to an affinity increase of a factor 10 (Figure 3.2, see above) indicating that the multivalency factor $K_{i,\text{His}_6} \times C_{\text{eff}} = 10$. The equations were solved in a spreadsheet approach, [6a] in which initially K_i and the maximum SPR intensity change (ΔI_{max}) were iteratively optimized to fit the theoretical with the experimentally obtained SPR data. The experimental data (Figure 3.2) indicate that TagRFP binding is determined by the valency of binding at low TagRFP concentrations, while at high concentrations TagRFP binding is limited by steric effects, as indicated by the different maximum coverages which hint at different orientations. When assuming a face-on orientation for **1H** and **2H** and a side-on orientation for **1+1H** and **2+1H**, as was postulated above, the percentage of Ni(II)·NTA units at the surface that are covered as a result of such binding orientations were estimated to be 2.8% (face-on) and 1.5% (side-on) per protein using the TagRFP crystallographic data and assuming an NTA coverage comparable to that of thiols on gold (4.5 molecules/nm²). [7] Using these values, the ΔI_{max} values obtained from the Langmuir fits were divided by these fractions to obtain the values for ΔI_{max} that would correspond to a complete usage of all NTA units and which are assumed to hold in the lower concentration ranges in which the steric effects are still negligible. As a result, K_{i,His_6} values were found (Table 3.1) of ca. 10^4 M^{-1} for all TagRFP mutants in agreement with literature data. [8] From these values and using equation (6), a corrected overall stability constant could be deduced for each TagRFP mutant. [6b]

$$K_{\text{corr}} = K_{i,\text{His}_6}^n \cdot C_{\text{eff}}^{n-1} \quad (6)$$

TagRFP	$K_{LM} (M^{-1})$	Corr. %	Corr. ΔI_{max} (a.u.)	$K_{i,His_6} (M^{-1})$	$C_{eff} (M)$	$K_{corr} (M^{-1})$
1H	$2.7 \cdot 10^6$	0.93	32387	$1.8 \cdot 10^4$	N/A	$1.8 \cdot 10^4$
2H	$3.8 \cdot 10^7$	0.47	63511	$3.5 \cdot 10^3$	$2.9 \cdot 10^{-3}$	$3.5 \cdot 10^4$
1+1H	$4.7 \cdot 10^7$	0.25	75480	$2.1 \cdot 10^4$	$4.8 \cdot 10^{-4}$	$2.1 \cdot 10^5$
2+1H	$2.7 \cdot 10^8$	0.17	123900	$2.4 \cdot 10^3$	$4.1 \cdot 10^{-3}$	$2.4 \cdot 10^5$

Table 3.1 – Thermodynamic parameters determined by fitting the experimental data to theoretical models (see text for details). K_{LM} is the apparent overall binding constant for each mutant from the Langmuir fit, K_{i,His_6} is the intrinsic binding constant for a single His₆-tag to the Ni(II)-NTA monolayer, while K is the real overall stability of the multivalent complex, found by fitting the data to a model accounting for the effect of the effective concentration (C_{eff}) and using only the data points in which steric effects can be ignored, while ΔI_{max} was corrected with the correction % values presented in the table resulting in ΔI_{max} values corrected for steric effects.

These results confirm the increasing stability when increasing the number of His₆-tags attached to the TagRFPs. Moreover, the K_{corr} values represent a more realistic picture in comparison to the values found from the Langmuir fitting, since the latter overestimates the multivalent binding affinities by two to three orders of magnitude.

3.2.1.3 Fluorescence anisotropy experiments

To corroborate the observations made using SPR on the amount and orientation of adsorbed TagRFP proteins on the surface, fluorescence anisotropy experiments were performed.^[9] The steady state anisotropies, r_{ss} , were measured of monolayers of immobilized proteins in a closed cell filled with buffer. For a cell filled with a solution of rhodamine an r_{ss} value close to zero was found indicative of freely diffusing molecules as expected. Similarly, free protein in an unfunctionalized cell showed an r_{ss} value of 0.36 in agreement with literature for a mutant of the green fluorescent protein (GFP).^[10] These reference measurements show that the configuration used for the determination for r_{ss} values is viable. In agreement with the SPR results, two statistically different regimes were found in the observed fluorescence anisotropy results for immobilized TagRFP: **1H** and **2H** have higher r_{ss} values than **1+1H** and **2+1H** (Figure 3.3).

Figure 3.3 – a) Fluorescence steady-state anisotropy values (r_{ss}) at 585 nm (TagRFP emission maximum) or 605 nm (rhodamine emission maximum) for individual measurements both in solution or in a protein monolayer on Ni(II)-NTA SAMs as indicated. b) Average values for each case.

Higher anisotropies of **1H** and **2H** (0.49 and 0.51, resp.) could be related to a restricted freedom of movement of the protein fluorophore (i.e. rotation and bending) while the comparatively lower r_{ss} values for **1+1H** and **2+1H** (0.40 and 0.36, resp.) could be due to less restriction of movement. A stronger restriction of movement in the case of **1H** and **2H** could be the result of a tighter packing of proteins while more loosely packed **1+1H** and **2+1H** are configured at the surface. This interpretation would be in agreement with the SPR data assuming that a face-on orientation leads to tighter packing than a side-on orientation. However, it should be mentioned that there is another effect influencing the steady state anisotropy, which could lead to an underestimation of the observed differences in anisotropy values. Energy transfer between neighboring chromophores should lead to lower r_{ss} values than for isolated chromophores. However, this effect should lead to a lower r_{ss} value in the case of **1H** and **2H** when compared with **1+1H** and **2+1H**, and is the opposite to the observed case.

3.2.1.4 Infrared spectroscopy experiments

To analyze the orientation of the immobilized proteins, polarization modulation infrared reflection-absorption spectroscopy (PM-IRRAS) was used. Spectra for monolayers of **1H** and **2+1H** bound to Ni(II)-NTA as well as for a bare Ni(II)-NTA monolayer are shown in Figure 3.4.

Figure 3.4 – PM-IRRAS spectra of **1H** and **2+1H** bound to Ni(II)-NTA surfaces. Colored bands correspond to regions associated to specific protein secondary structure elements, within the amide I region, used to elucidate protein orientation. Intensities are giving as dichroic ratios.

When comparing the intensity of the IR signal at 1660 cm^{-1} (amide I band) with the IR signal at 1740 cm^{-1} (COOH band) ^[11] a ratio of about 1 was found for the Ni(II)-NTA surface while ratios of 4.5 and 2.3 were found in the case of immobilized **1H** and **2+1H** on Ni(II)-NTA surfaces, respectively. This observation indicates that in the case of **1H** more protein is present at the surface when compared to **2+1H**. The amide I band between 1600 and 1700 cm^{-1} can be assigned to C=O stretch vibrations and its shape is correlated with the secondary structure of proteins.^[14] For example, the band at 1654 cm^{-1} can be assigned to α -helices while the band at 1633 cm^{-1} can be assigned to the main band of β -sheets.^[12] When comparing the ratios between the α -helix and the main β -sheet bands, values of 1.3 and 1.1 were found for **1H** and **2+1H**, respectively. Since **1H** and **2+1H** are structurally identical, this remarkable observation indicates a difference in orientation. From the TagRFP crystallographic data it can be seen that the only substantial α -helical structural element is oriented along the axis of the β -barrel. Therefore, the α -helix C=O stretch vibration, which is nearly parallel to the axis of the α -helix, should be observable in PM-IRRAS for a high angle of incidence of the polarized light on a metal surface only in the case the protein is oriented face-on. The IR

spectrum of **1H** indicates that the β -barrel and thus, the α -helix are oriented normal to the surface, while the IR spectrum of **2+1H** indicates that the β -barrel is oriented parallel to the surface. Taken together, the results from PM-IRRAS are in agreement with the results from both SPR and fluorescence anisotropy measurements indicating a tightly packed layer of face-on oriented TagRFPs in the case of **1H** and **2H** and a less tightly packed layer of side-on oriented TagRFPs in the case of **1+1H** and **2+1H**.

3.2.1.5 Fluorescence microscopy experiments

To visualize the stability and reversibility of the binding of TagRFPs to Ni(II)-NTA surfaces as a function of the number and spatial arrangement of His-tags, fluorescence micrographs were recorded of line patterns of TagRFP fabricated employing nanoimprint lithography (NIL) (Scheme 3.1).

Scheme 3.1 – Nanoimprint lithography (NIL) was used to generate patterns on poly(methyl methacrylate) (PMMA)-coated glass substrates. Upon NIL and removal of the residual PMMA layer, the exposed glass areas were first functionalized with N-[3-(trimethoxysilyl)propyl]ethylenediamine (TPEDA) followed by the reaction with *p*-phenylene diisothiocyanate (DITC). Afterwards, *N,N*-bis(carboxymethyl)-*L*-lysine hydrate (NTA-NH₂) was coupled to the surface. Then remaining PMMA was stripped and the exposed areas of bare glass were functionalized with 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (PEG-silane) to prevent non-specific protein interactions. Finally, the platforms were complexed with Ni(II) ions in order to immobilize His-tagged TagRFPs.

After incubating the Ni(II)-NTA patterns with TagRFPs the patterns were washed overnight with phosphate buffered saline (PBS) containing 5% of Tween-20, whereafter the fluorescence images showed a high contrast between the functionalized areas and the ones passivated with PEG-silane (Figure 3.5). No

apparent fluorescence intensity differences were found between the four proteins. However, upon washing for another 36 hours the fluorescent patterns of **1H** could not be observed anymore, while patterns of **1+1H** and **2H** showed reduced intensities by ca. 73% and 37%, respectively. In contrast, the intensity of patterns of **2+1H** was unchanged. These observations are in agreement with the observed trend in binding strengths as found using SPR.

Figure 3.5 – Fluorescence microscopy images corresponding to substrates patterned via NIL with Ni(II)-NTA and PEG-silane regions after incubation with a-d) **1H**, e-h) **2H**, i-l) **1+1H** and m-p) **2+1H** and the subsequent washing with PBS containing 5% of Tween-20, overnight, after two days and after washing during 24 h with either saturated imidazole or EDTA solutions in PBS as indicated in the figure. Insets provide the intensity profiles. Images were recorded at 2000 ms.

To investigate the reversibility of TagRFP binding, patterns of **2H**, **1+1H** and **2+1H** were washed with either imidazole or ethylenediaminetetraacetic acid (EDTA). While washing with monovalent imidazole, which can bind to Ni(II)-NTA, did not change the fluorescence intensity of the patterns significantly, washing with EDTA, which can complex Ni^{2+} , showed a decrease in fluorescence intensity in the case of **1+1H** and **2H** up to 80% and in the case of **2+1H** of 50%. The observations can be attributed to the enhanced stability of the multivalent

interaction of the multiple His₆-tags on the TagRFPs. The SPR, fluorescence anisotropy, IR and microscopy experiments show that stability and orientation can be attributed to the number and position of the His₆-tags at the protein structure. Namely, while an increased number of His₆-tags will lead to complexes of higher stability, His₆-tags positioned at the same side of the protein structure will result in densely-packed protein layers with a preferred face-on orientation whereas when the tags were introduced at opposite sides, less dense protein packing was found with a preferred side-on orientation.

3.2.2 Orthogonal assembly of proteins

After having demonstrated the orientationally controlled immobilization of TagRFPs, in this section orthogonal assembly of two proteins on specific regions of one substrate will be shown. To this end, a single-cysteine-containing mutant of a monomeric variant of *Clavularia* cyan fluorescent protein was recombinantly expressed and purified (G¹⁷⁴CTFP). A peptide was synthesized containing glutamic acid residues functionalized with adamantyl moieties (G^{ad}ER^{ad}ED^{ad}EK) for the supramolecular host-guest interaction with β -cyclodextrin (β CD) surfaces. The peptide was N-capped with maleimido caproic acid for the subsequent conjugation to the Cys residue of TFP to yield a trivalent adamantyl protein guest (**Ad₃-TFP**) (Figure 3.6a).

Figure 3.6 – a) Structure of TFP covalently coupled to a trivalent peptide construct carrying three adamantyl moieties for interaction with β CD platforms. b) Schematic representation of the procedure to functionalize glass substrates both with Ni(II)-NTA and β CD lines, which were produced employing NIL.

β CD SAMs were prepared on gold substrates, as described in literature,^[13] and used in SPR experiments to determine the binding strength of **Ad₃-TFP** with the β CD surface. To this end, solutions containing different concentrations of **Ad₃-TFP** were flown over a β CD surface while monitoring the change of resonance angle upon binding. The β CD surfaces had been previously blocked by flowing 0.1 mM of a mono-adamantyl hexaethylene glycol linker (HEG-Ad) to prevent non-specific protein interactions.^[14] Increasing concentrations of **Ad₃-TFP** resulted in increasing equilibrium angle shifts reaching surface saturation as shown in Figure 3.7.

Figure 3.7 – Equilibrium values (squares) of the SPR titration experiment of **Ad₃-TFP** to a βCD SAM after saturating the surface with the monovalent HEG-Ad and corresponding fit (solid line) using a multivalent binding model.

The experimental data could be fitted using a multivalent binding model as reported previously.^[6b] The competitive equilibrium for the formation of the surface complex of HEG-Ad was taken into account for the calculation of the theoretical maximum SPR angle shift for each concentration. C_{eff} , accounting for the immobilization of a second and third additional adamantane groups once the first was immobilized, was set at 0.1 M.^[6b] Using a binding constant of $2.6 \cdot 10^4 \text{ M}^{-1}$ for Ad-HEG^[14] a value of $K_{i,TFP} = 1.8 \cdot 10^3 \text{ M}^{-1}$ was determined and an overall stability constant for the trivalent surface complex of $K = 5.4 \cdot 10^7 \text{ M}^{-1}$. Both constants are lower than for small trivalent guest molecules for which an apparent overall constant in the order of 10^{10} M^{-1} was reported.^[15] This phenomenon can presumably be attributed to the fact that the guest moiety of **Ad₃-TFP** is attached to a large water-soluble protein affecting the binding to the surface.^[16]

In order to study the specificity of the immobilization of **Ad₃-TFP** to βCD surfaces on glass, patterns of βCD were created employing NIL as shown in Scheme 3.1 by replacing NTA-NH₂ by βCD-heptaamine. Adjacent lines of PEG-silane to prevent non-specific protein interactions and βCD were incubated overnight with **Ad₃-TFP** and a control TFP, which was functionalized with an adamantane free peptide (GEREDEK), using PBS containing 5% Tween-20 and 5 mM of HEG-Ad. This incubation buffer was found the best after several series of optimization experiments for reducing non-specific interactions at βCD SAMs. Fluorescence microscopy was used to visualize the fabricated patterns (Figure 3.8).

Figure 3.8 – Fluorescence images corresponding to substrates patterned via NIL with β CD and PEG-silane regions after incubation with a) 1 μ M TFP functionalized with GEREDEK, b) **Ad₃-TFP** and subsequent washing overnight with incubation buffer. c and d) correspond to the consecutive washing of the samples with a solution containing 10 mM β CD in incubation buffer for a) and b) respectively. Insets provide the intensity profiles. Images were recorded at 2000 ms.

Upon protein incubation and overnight washing, a 2.7-fold increase in fluorescence intensity was found for **Ad₃-TFP** in comparison to the control. After overnight washing the samples with a high concentration of β CD the fluorescence intensity was highly reduced for the control TFP when compared to the fluorescence intensity of **Ad₃-TFP**. This results shows that for **Ad₃-TFP**, the largest contribution on the protein immobilization was of supramolecular, multivalent nature. The enhanced stability of the multivalent complex (reported before from the SPR results) caused that, under competition conditions with β CD host molecules in solution, the surface trivalent complex can still remain while removing the weakly bound protein either non-specifically or forming complexes with lower valences as reported before in literature. [14]

As a next step, the orthogonal assembly of (His)₆-TagRFP and **Ad₃-TFP**, was attempted. To this end, bifunctional surfaces were prepared according to Figure 3.6b. Briefly, NIL was used to create t-butyl ester protected NTA patterns following Scheme 3.1. After stripping the remaining PMMA, β CD was attached to these areas following Scheme 3.6b. Finally, NTA was deprotected by incubating the samples in trifluoroacetic acid (TFA) overnight to yield the bifunctional line patterns of NTA/ β CD. After complexing Ni(II) to NTA and HEG-Ad to β CD protein

solutions were applied to the surfaces. From a series of incubation conditions and sequences only a few yielded successful orthogonal protein assembly. Figure 3.9 shows the microscopy images corresponding to the successful cases. As shown before (Figure 3.8), when incubating patterns of adamantane-functionalized TFP in the presence of β CD in the incubation buffer, the specificity of the interaction with the β CD lanes was enhanced.

Figure 3.9 – Fluorescence images corresponding to substrates patterned via NIL with Ni(II)NTA and β CD regions after incubation with a) **2+1H**, b,d) **Ad₃-TFP** and e) **1H** accompanied by their respective intensity profile plots c) for a and b) and f) for d and e) respectively. While in the upper line the sequence of incubations was **2+1H** and **Ad₃-TFP** in the line below the bifunctional sample was first incubated with **Ad₃-TFP** followed by incubation with **1H**. Images were recorded at: a and e) 1000 ms and b and d) at 3000 ms.

After incubating the bifunctional platforms sequentially with one protein at a time and systematically exploring all possible combinations only two cases were consistently successful in the co-localization of the proteins to their respective complementary functionality on the surface. On the one hand only when **2+1H** was first immobilized on lines of Ni(II)·NTA (Figure 3.9a) followed by the subsequent immobilization of **Ad₃-TFP** on the complementary thin lines of CD, clear images were recorded with both TFP and TagRFP present. On the other hand, when **Ad₃-TFP** was first immobilized on CD, only **1H** could be immobilized on the Ni(II)·NTA region. Considering the overall stability constants of 5.1×10^4 and $2.4 \times 10^6 \text{ M}^{-1}$ as estimated for **1H** and **2+1H**, respectively, while $5.4 \times 10^7 \text{ M}^{-1}$ was found for **TFP-Ad₃**, these observations can presumably be attributed to the individual thermodynamic stability of each of the guest proteins. Namely, when the protein having the strongest affinity is assembled first, a protein with lower affinity to the surface was preferentially immobilized on its complementary

surface bound functionality. When this order was reversed or the platforms were stepwise incubated with protein of similar stability mixed cases were found.

3.3 Conclusions

TagRFP mutants presenting different numbers and distribution of His₆-tags were successfully generated to study the influence of the site-specific modifications on the stability and orientation of surface complexes on Ni(II)·NTA. First, the binding constant was found to increase for (His₆)_x-tagRFP with the number of His₆-tags. Moreover, differences in surface coverage were found specially for **1+1H** and **2H** that have roughly the same stability, indicating two orientation regimes dictated by the position of the His₆-tags within the protein structure: face-on and side-on respectively, which was also observed for **1H** and **2+1H**. The latter, being supported by surface plasmon resonance, fluorescence anisotropy measurements and infrared reflection-absorption spectroscopy data, resulted in a practically unique finding. In agreement with their stability, surface complexes could be reversibly disassembled when washing with a NTA competitor for the Ni(II) complexation such as EDTA but not when imidazole was used to compete against histidine due to the enhanced stability of the intrinsic multivalent stability of bound His₆-tags.

Second, a mutant of TFP was modified to present a trivalent adamantyl linker (**TFP-Ad₃**). The mutant was successfully immobilized via its specific interaction with a βCD SAM when compared to TFP modified with the control peptide sequence mic-GEREDEK. Moreover, the specificity of the interaction was improved when adding a competitive host such as βCD in the incubation buffer.

Finally, sequential protein co-functionalization of bifunctional substrates fabricated by NIL was successful though with a strong influence on the stability of the first interaction for the specific binding of the second.

3.4 Acknowledgements

The work presented in this chapter was performed entirely in collaboration with Dr. D. Wasserberg. C. Stoffelen and D. te Brake are gratefully acknowledged for the synthesis of the mic-G^{ad}ER^{ad}ED^{ad}EK and mic-GEREDEK sequences.

3.5 Experimental section

Materials

Fmoc-L-glutamic acid *α*-tBu-ester, Fmoc-protected amino acids and *N,N,N',N'*-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate

(HBTU) were purchased from MultiSynTech (Witten, Germany) and Novabiochem (Darmstadt, Germany), 1-hydroxy benzotriazole (HOBt) and maleimide caproic acid-hexa(histidine) (mic-His₆) were purchased from JPT Peptide Technologies, Germany and all solvents were of p.a. quality and purchased from Biosolve (Valkenswaard, The Netherlands). All other starting components were purchased from Acros (Geel, Belgium) and Sigma Aldrich (Zwijndrecht, The Netherlands). All compounds were used as received unless stated otherwise. Deuterated solvents used for NMR spectroscopy were purchased from Cambridge Isotope Laboratories and the water used was of MilliQ quality (Millipore, R = 18.2 MΩcm).

Synthesis of NTA(*OfBu*)₃-NH₂

NTA(*fBu*)₃-NH₂ was synthesized according to a protocol adapted from literature.^[17] H-Lys(Z)-*OfBu*-HCl (4.0 g, 10.7 mmol) was dissolved in 100 mL of dry dimethylformamide (DMF). Subsequently, *fBu*-bromoacetate (6.36 mL, 43.1 mmol) and diisopropyl ethyl amine (DIPEA) (9.2 mL, 52.8 mmol) were added sequentially. After purging the flask with N₂, the solution was stirred overnight at 55 °C under inert N₂ atmosphere. The volatiles were removed *in vacuo* at 60 °C and the partially solidified reaction mixture was resuspended in 60 mL of cyclohexane:ethylacetate (3:1) and filtered over a sintered glass funnel. The precipitate was washed with the same solvent mixture (3 x 40 mL), concentrated under reduced pressure and purified by silica gel chromatography with cyclohexane:ethylacetate (3:1) as the mobile phase to quantitatively yield 5.95 g (10.5 mmol) Lys(Z)-NTA(*OfBu*)₃ (R_f = 0.50). Then, Lys(Z)-NTA(*OfBu*)₃ (6.0 g, 10.5 mmol) was dissolved in methanol (300 mL) and the solution was purged with three vacuum-nitrogen cycles. Subsequently, 10% Pd/C (0.120 g) was added under N₂. The solution was mixed vigorously and stirred for 6 h under H₂ atmosphere at room temperature. After the H₂ gas had been allowed to slowly leak from the reaction mixture while being replaced by nitrogen, the Pd/C catalyst was removed by filtration over celite and the solvent was removed from the filtrate under reduce pressure to yield (*OfBu*)₃NTA-NH₂ (3.68 g, 8.5 mmol, 81%). ¹H NMR (300 MHz, CDCl₃, 20 °C, TMS) δ: 3.47 (dd, 4H, ((CH₃)₃COCOCH₂)₂N-), 3.31 (t, 1H, ((CH₃)₃COCOCH₂)₂NCH), 2.72 (t, 2H, NH₂CH₂CH₂CH₂CH₂CHN-), 2.28 (bs, 2H, NH₂CH₂CH₂CH₂CH₂CHN-), 1.65 (m, 2H, NH₂CH₂CH₂CH₂CH₂CHN-), 1.50 (m, 4H, NH₂CH₂CH₂CH₂CH₂CHN-), 1.45 (s, 9H, (CH₃)₃COCOCH-), 1.44 (s, 18H, ((CH₃)₃COCOCH₂)₂N-); MS (ESI): m/z = 432.3 [M+H]⁺, (calc. 431.0).

Synthesis of HEG-Ad

Adamantyl hexa(ethylene glycol), HEG-Ad, was synthesized according to a protocol adapted from literature.^[14] To a solution of hexa(ethylene glycol)

(12.5 mL, 50.9 mmol), 1-bromo adamantane (1.1 g, 5.1 mmol) and Et₃N (2.0 mL, 14.3 mmol) were added. The solution was stirred at 120 °C overnight. After cooling the reaction mixture to room temperature dichloromethane (50 mL) was added. The solution was washed with 2 M HCl (4 x 50 mL) and once with brine (50 mL). The organic layer was dried over MgSO₄ and the solvent was evaporated under reduced pressure to give HEG-Ad as a brown, transparent oil (0.87 g, 2.1 mmol, 40%). MS (ESI): *m/z* = 417.4 [M+H]⁺, (calc. 416.6 g/mol).

Synthesis of ^{ad}E

6-aminohexyl-1-adamantane carboxamide

1,6-Hexanediamine (5.81 g, 50.0 mmol) was dissolved in 180 mL CH₂Cl₂ and, subsequently, Et₃N (0.77 mL, 5.55 mmol) was added. The reaction mixture was cooled to 0 °C and a solution of adamantane carboxylic acid chloride (1.0 g, 5.04 mmol) in 20 mL CH₂Cl₂ was added dropwise during the course of 1 h. The reaction mixture was stirred for 30 min at 0 °C, left to slowly warm to room temperature (RT) and quenched with 150 mL of a saturated aqueous NaHCO₃ solution. The resulting aqueous phase was, then, washed 3 times with (50 mL) CH₂Cl₂ and the combined organic phases were washed three times with water and once with brine. The solution was dried over MgSO₄, concentrated *in vacuo* and the product, 6-aminohexyl-1-adamantane carboxamide (0.9 g, 3.22 mmol, 64%), was analyzed with NMR spectroscopy.

¹H-NMR (300 MHz, CDCl₃): δ (ppm) 3.1 (t, 2H), 2.7 (t, 2H), 2.0-1.7 (m, 15H), 1.4-1.2 (m, 8H).

Synthesis of Fmoc-glutamic acid adamantane hexane amide-carboxylic acid α-*t*Bu-ester

To a solution of Fmoc-L-glutamic acid α-*t*Bu-ester (1.56 g, 3.66 mmol) in 30 mL DMF, N,N'-diisopropylcarbodiimide (DIC) (1.54 g, 12.2 mmol) and HOBT (1.65 g, 12.2 mmol) were added under Ar atmosphere. The reaction mixture was stirred for 30 min at RT for the activation of the carboxylic acid to take place. 6-aminohexyl-1-adamantane carboxamide (0.85 g, 3.05 mmol) was dissolved in 10 mL DMF and added to the reaction mixture, which was stirred at RT for 2 h. The progress of the reaction was followed by NMR (measured by the intensity of the free amine peak) and stopped after approximately 2 h, at which time all the free amine had been consumed. The solvent was removed *in vacuo* and the resulting residue was dissolved in 150 mL EtOAc:Hexane 1:1 and washed 3 times with sat. NH₄Cl (30 mL) in order to remove DMF, any prevailing traces of DMF were, then, removed *in vacuo*. The crude product was, subsequently, dissolved in CH₂Cl₂ and washed 3 times with sat. NaHCO₃ (50 mL), twice with water (50 mL) and

once with brine. The organic phase was dried over MgSO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography (SiO_2 , chloroform:MeOH, 95:5) and resulted in 1.7 g (2.50 mmol) of pure product corresponding to 82% yield.

$^1\text{H-NMR}$ (300MHz, CDCl_3): δ (ppm) 7.8-7.3 (m, 8H), 5.6 (d, 2H), 5.5 (m, 1H), 4.4 (t, 1H), 4.4 (t, 1H), 4.2 (m, 2H), 3.1 (t, 4H), 2.0-1.7 (m, 15H), 1.43 (s, 9H), 1.4-1.2 (m, 8H). MS (ESI-TOF): m/z : 686.4 g/mol $[\text{M}+\text{H}]^+$ and 708.4 g/mol $[\text{M}+\text{Na}]^+$ (calc. 685.89 g/mol).

Synthesis of Fmoc-glutamic acid adamantanehexaneamide-carboxylic acid, (Fmoc- $\alpha\alpha\text{E}$)

1.65 g (2.40 mmol) Fmoc-glutamic acid adamantanehexaneamide-carboxylic acid α -tBu ester was dissolved in 30 mL CH_2Cl_2 , to which 30 mL TFA were added. The solution was stirred for 4 h at RT and subsequently, the reaction mixture was concentrated *in vacuo*. The resulting solution was washed 3 times with water, once with brine and dried over MgSO_4 . All residual CH_2Cl_2 was removed *in vacuo* and the obtained yellowish solid (1.3 g, 2.04 mmol, 85%) was analyzed by NMR spectroscopy and MS spectrometry and stored at -20°C until further use.

$^1\text{H-NMR}$ (300MHz, CDCl_3): δ (ppm) 7.8-7.3 (m, 8H), 5.6 (d, 2H), 5.5 (m, 1H), 4.4 (t, 1H), 4.4 (t, 1H), 4.2 (m, 2H), 3.1 (t, 4H), 2.0-1.7 (m, 15H), 1.4-1.2 (m, 8H).

MS (ESI-ToF): m/z : 630.3 $[\text{M}+\text{H}]^+$ and 652.2 $[\text{M}+\text{Na}]^+$ (calc. 629.8).

Automated solid phase peptide synthesis

Peptides of the following sequences, end-capped with maleimido caproic acid (mic), were synthesized using standard Fmoc-chemistry in solid phase peptide synthesis (SPPS) [18]: the triply adamantane substituted mic-G $\alpha\alpha$ ER $\alpha\alpha$ ED $\alpha\alpha$ EK and its reference peptide mic-GEREDEK. Rink Amide MBHA resin was used as solid phase and all amino acids carried Fmoc-protected α -amino groups. Each amino acid was dissolved in N-methyl-2-pyrrolidone (NMP, 0.3 M), stabilized with 0.3 M HOBT and all reactions were carried out at RT. Extensive washing steps with NMP were carried out after each reaction in order to prevent cross-contaminations between steps.

In short, syntheses were carried out from C- to N-terminus as follows:

A solution containing an Fmoc-protected amino acid was added to the reaction vessel containing the amine-terminated Rink amide resin. Coupling of the free carboxylic acid group of the Fmoc-protected amino acid to the free amine of the resin was done using HBTU and DIPEA as coupling reagents and reaction time was set to 90 min. This resulted in the Rink amide resin terminated with the newly coupled amino acid displaying its Fmoc-protected amines on the resin. In order

to increase yields, all couplings with natural amino acids were done twice (double couplings); all others once (single couplings). After coupling, Fmoc cleavage was carried out using first 40% piperidine, then 20% piperidine in NMP. After Fmoc-cleavage the next amino acid was added and the cycle was repeated for each amino acid until the sequence had been completed. The resin was washed with NMP, CH₂Cl₂ and MeOH each three times and dried for at least 4 h *in vacuo*. Subsequently, the peptide was cleaved from the resin as well as deprotected in a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIS) and water (TFA:TIS:H₂O, 95:2.5:2.5) overnight. The resin was rinsed three times more with pure TFA and the combined fractions were concentrated *in vacuo*. From this mixture the peptide was precipitated three times using cold diethyl ether and a centrifugation step (8000xg, 2 min) and the combined precipitated fractions were re-dissolved in water (mixed with acetonitrile containing ~1% TFA for the adamantane substituted peptide) and lyophilized. Peptides (re-dissolved in acetonitrile/water mixtures) were purified using reverse-phase HPLC (using varying mixtures of water and acetonitrile, both containing 0.1% TFA) as liquid phase, subsequently lyophilized and analyzed by mass spectrometry.

MS (ESI): mic-G^{ad}ER^{ad}ED^{ad}EK m/z 1836 g/mol [M+H]⁺ (calc. 1835 g/mol).

MS (ESI): mic-GEREDEK m/z 1056 g/mol [M+H]⁺ (calc. 1054 g/mol).

Automated peptide synthesis was carried out using a Syro1 (MultiSynTech) and peptides were purified on a Waters HPLC system with photoarray detector using a preparative reverse phase C18 column (Xterra, Waters).

Cloning of TagRFP and TFP for high-level expression

^NHis₆-¹⁴TagRFP

The following primers were used for PCR amplification of TagRFP using pTagRFP-C (Evrogen JSC) as DNA template: 5'-cgcgaatccaatgagcgagctgattaaggagaacatgca-3' containing a unique BamHI restriction site (underlined) and 5' cgcgaattcccttggtccccagtttgctag-3' containing a unique EcoRI restriction site (underlined). The PCR product was purified and digested with BamHI and EcoRI restriction enzymes (NEB) and ligated into pRSETB plasmid (Invitrogen) digested with the same restriction enzymes. pRSETB contains an N-terminal hexahistidine-tag (^NHis₆-tag) for nickel-affinity purification and an Enterokinase recognition site (DDDDK) to allow for the subsequent cleavage of the His₆-tag. The resulting plasmid, pRSETB-TagRFP, was first transformed into *E. coli* (XL10 gold, Stratagene) using standard procedures in the presence of ampicillin (100 mg/L). pRSETB-TagRFP plasmid was transformed into *E. coli* BL21 pLysS using standard procedures in the presence of ampicillin (100 mg/L) and chloramphenicol (34 mg/L). Single colony transformants were

selected and the pre-culture was grown overnight at 37 °C. This pre-culture was used to inoculate 2 L of LB medium containing ampicillin (100 mg/L) and chloramphenicol (34 mg/L) at 37 °C with shaking until an O.D.₆₀₀ = 0.6 was reached. The culture was cooled to 16 °C before protein expression was induced with IPTG to a final concentration of 1 mM and incubated overnight at 16 °C. Cells were harvested by centrifugation at 4000xg at 4 °C for 20 min. The resulting cell pellet was resuspended for 20 min in BugBuster reagent with benzonase nuclease (Novagen) according to the supplier's instruction. The lysate was cleared by centrifugation at 16000xg for 30 min at 4 °C. Ni-NTA agarose beads (QIAGEN) were added to the protein supernatant at a 10:1 v/v ratio, respectively, and incubated at 4 °C for at least an hour with slow but continuous mixing. The agarose beads were filtered and washed with wash buffer (20 mM Tris buffer, 300 mM NaCl, 20 mM imidazole, pH 8.0) and eluted with elution buffer (20 mM Tris buffer, 300 mM NaCl, 1 M imidazole, pH 8.0). The purified ^NHis₆-¹⁴TagRFP fractions (~30 μM) were subsequently desalted using PD10 columns (GE Healthcare) into 0.1xPBS (0.8 mM phosphate buffer (PB), 14.4 mM NaCl, 0.27 mM KCl, pH 7.4), aliquoted, snap-frozen in liquid nitrogen and stored at -80 °C. Proteins were characterized using SDS- and native PAGE, UV-Vis, steady state- and time-resolved fluorescence spectroscopy and MALDI-TOF mass spectrometry.

^NHis₆-^CHis₆-¹⁴TagRFP

For the insertion of a second, C-terminal His₆-tag (^CHis₆-tag), the TagRFP gene was amplified using 5'-cgcggaatccaatgagcgagctgattaaggagaacatgca-3' (BamHI restriction site is underlined) and 5'-gcggaattcttagtggtggtggtggtgcttggtgccccagtttgcta-3' (EcoRI restriction site is underlined) as forward and reverse primers, respectively, and pRSETB-TagRFP as DNA template. After PCR purification, the gene product and pRSETB-TagRFP were digested sequentially, firstly with EcoRI, then BamHI according to the manufacturer's instructions. DNA ligations were performed using T4-ligase (NEB) at 16 °C overnight and the resulting pRSETB-TagRFP-His₆ plasmid was transformed into *E. coli* XL10 gold competent cells according to standard procedures.

pRSETB-TagRFP-His₆ plasmid was transformed into *E. coli* BL21 pLysS in the presence of ampicillin (100 mg/L) and chloramphenicol (34 mg/L). Single colony transformants were selected and the pre-culture was grown overnight at 37 °C. The pre-culture was used to inoculate 2 L cultures of *E. coli* BL21 pLysS cells, which were grown at 37 °C to O.D.₆₀₀ = 0.6 before protein expression was induced upon addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration

of 1 mM (left overnight, 16 °C). The cells were harvested by centrifugation (4000xg, 20 min) and lysed using BugBuster (as described above). Protein purification and characterisation of the N- and C-terminal (doubly) His₆-tagged TagRFP (^NHis₆-^CHis₆-¹⁴TagRFP) was carried out as described for ^NHis₆-¹⁴TagRFP.

^NHis₆-¹⁰TagRFP, ^NHis₆-^CHis₆-¹⁰TagRFP, ^NHis₆-¹⁴TagRFP and ^NHis₆-^CHis₆-¹⁴TagRFP mutants

By site-directed mutagenesis (QuikChange Multi kit, Stratagene Technologies), selected mutations were introduced into pRSETB-TagRFP (both, singly and doubly His₆-tagged TagRFP) using the following primers:

S128C forward 5'-ggtgaacttcccatgcaacggccctgtga-3'

reverse 5'-tcacagggccgttgcattggaagttcacc-3'

C222S forward 5'-ggctgtggccagatactccgacctccc-3'

reverse 5'-gggagggtcggagtatctggccacagcc-3'

C114S forward 5'-gcctccaggacggctccctcatctacaac-3'

reverse 5'-gtttagatgagggagccgtcctggaggc-3'

Mutations S128C, C222S and C114S yielded mutants '¹⁰TagRFP' containing one accessible cysteine residue. Mutations C222S and C114S yielded mutants '¹⁴TagRFP' containing no accessible cysteine residues. All mutant variants were expressed and purified under the same conditions as for the wild-type protein, to yield ^NHis₆-¹⁰TagRFP, ^NHis₆-^CHis₆-¹⁰TagRFP, ^NHis₆-¹⁴TagRFP and ^NHis₆-^CHis₆-¹⁴TagRFP mutants. Characterisation by SDS- and native PAGE, UV-Vis, steady state and time-resolved fluorescence spectroscopy and MALDI-ToF mass spectrometry were carried out and the mutants showed no discernible differences in their (photophysical) properties to the wild type variants ^NHis₆-TagRFP, ^NHis₆-^CHis₆-TagRFP, thus indicating that their overall structure upon site-directed mutagenesis is retained.

TagRFP without His₆-tags

Removal of the His₆-tag from the wild-type TagRFP was accomplished by Enterokinase digestion using Enterokinase (Invitrogen) and its corresponding Enterokinase removal kit (EKaway, Invitrogen) according to the manufacturers instruction. In short, Enterokinase digestion of ^NHis₆-¹⁴TagRFP was carried out overnight at 37 °C using protein stock solution with varying amounts of enzyme where undigested protein served as negative control. Cleavage was nearly complete using 4 units of Enterokinase per 20 µg of ^NHis₆-¹⁴TagRFP. After digestion Enterokinase was removed by letting it bind to the resin with the removal kit's Enterokinase-binding resin, which was, in turn, removed by centrifugation (5000xg, 2 min). Subsequently, residual, undigested protein was removed using

NiNTA-affinity chromatography resulting in a flow-through fraction of pure, His₆-tag-free ¹⁴TagRFP.

TFP

For Teal Fluorescent Protein (TFP), the following primers were used for PCR amplification using pET15b-TFP (kindly provided by W.F. Rurup, University of Twente, The Netherlands) as DNA template: 5'-ctccacggatccatggtagcaagggcgag-3' containing a unique BamHI restriction site (underlined) and 5'-ctccacgaattcctgtacagctcgccatgc-3' containing a unique EcoRI restriction site (underlined). The PCR product was cloned into pRSETB as described above resulting in pRSETB-TFP, lacking a His₆-tag due to a frameshift just before the inserted gene. Site-directed mutagenesis was carried out to yield pRSETB-TFP (G174C) using the above protocol and the following primers: forward 5'-tgctggagggctgcggccaccac-3' and reverse 5'-gtggtggccgcagccctccagca-3'.

Purification of TFP and ^{G174C}TFP was carried out using an Äkta FPLC system with a 5 mL ResourceQ anion exchange chromatography column (GE Healthcare) according to the manufacturer's instructions using sterile filtered wash buffer (40 mM PB, pH 8.0) and elution buffer (40 mM PB, 1 M NaCl pH 8.0). Both proteins were characterized using the same techniques as mentioned above.

^NHis₆-^CHis₆-^{S128C}His₆-¹⁰TagRFP, ^NHis₆-^{S128C}His₆-¹⁰TagRFP and ^{G174C}Ad₃-TFP

^NHis₆-¹⁰TagRFP ^NHis₆-^CHis₆-¹⁰TagRFP and ^{G174C}TFP, which possess a single accessible cysteine, were conjugated with thiol-reactive maleimide-functionalized peptides. Maleimide-(His)₆ was used to give ^NHis₆-^CHis₆-^{S128C}His₆-¹⁰TagRFP and ^NHis₆-^{S128C}His₆-¹⁰TagRFP. Maleimide-GEREDEK was used to give reference TFP, while maleimide-G^{ad}ER^{ad}ED^{ad}EK was used to give ^{G174C}Ad₃-TFP, where ^{ad}E is an adamantyl ester of glutamic acid, E, see above.

As negative and positive control were used ^NHis₆-¹⁴TagRFP and ^NHis₆-¹⁴TagRFP-^CHis₆, resp., to react with maleimide-(His)₆. TFP was mixed with maleimido-Atto488 dye to serve as negative controls. Conjugations were carried out by first reducing the cysteines using 10 mM phosphate buffer (PB), 0.1 mM dithiothreitol (DTT) (pH 8.0) for 30 min and removing the DTT again (zebaspin, Thermo Scientific) and by then incubating a 10:1 mixture of maleimide:protein (~20 μM) in 10 mM PB (pH 8.0) in the dark for 24 h at RT. The reactions were subsequently quenched upon addition of a 10-fold molar excess of DTT with respect to the maleimide for 30 min at RT after which the samples were rebuffered (Zeba spin, Thermo Scientific) into 0.1xPBS at least three times to remove any residual peptide, dye and DTT. Characterisation was performed as described above and conjugation yields were typically between 30–50%.

Purification of the His₆-tagged conjugates were conducted using an Äkta FPLC system and a 1 mL HisTrap HP column (GE Healthcare) according to the manufacturer's instructions. Conjugates containing 2 and 3 His₆-tags could be separated from the native proteins containing 1 and 2 His₆-tags, respectively, and were isolated and characterized using SDS- and native PAGE and MALDI-ToF MS. The overall purity was found to be >80% after purification.

Surface plasmon resonance

Surface plasmon resonance (SPR) measurements were performed in Kretschmann configuration either using a Resonant Technologies RT2005 SPR setup as described in literature^[18] or in Chapter 4, or using an IBIS Technologies imaging SPR (iSPR) system (see below). In both cases glass substrates covered with a 50 nm gold layer were used. On the gold β -cyclodextrin (CD) or NiNTA were self-assembled into a monolayer via Au-sulfur interactions. In the case of the Resonant Technologies set-up, these substrates were attached to a 70 μ L volume microfluidic cell mounted on a prism which in turn was mounted on a double goniometer head, with which the angle of incident of the exciting laser on the prism could be controlled. Light from a 2 mW HeNe laser of 633 nm wavelength passed through the prism and onto the substrate under a tunable angle of incident. The intensity of the reflected light from the substrate was measured by a large-area photodiode. The set-up allowed determination of changes of resonance angle with an accuracy of 0.002 degrees. The gold substrate was optically matched to the prism using index matching oil. SPR experiments were performed at a continuous flow of 20 μ L/min (all TagRFP variants) or 100 μ L/min (all TFP variants). Imaging SPR measurements on the iSPR (IBIS Technologies), which is also set up in Kretschmann configuration using a laser of 800 nm wavelength, were also performed on the above substrates and various regions of interest were assigned for which SPR sensograms were each determined separately. Here too, the resonance angle is determined by continuously scanning through the surface plasmon resonance dip. Phosphate buffered saline (PBS) (with 0.01% Tween20 for TagRFPs, with 0.1 mM HEG-Ad for TFPs) was used as a running buffer (RB).

Spectroscopy and steady state anisotropy measurements

Absorption spectra of all fluorescent proteins, their mutants and conjugates were recorded using a Perkin Elmer LAMBDA850 UV/Vis spectrophotometer. Fluorescence spectra and lifetime data of all fluorescent proteins, their mutants and conjugates were recorded using a JobinYvon-Horiba Fluoromax4 fluorimeter including a TCSPC system for time-dependent measurements with pulsed LEDs for

excitation of 561 nm and 462 nm wavelengths. Steady state anisotropies, r_{ss} , of full monolayers of TagRFP were determined as an average of the anisotropies in the wavelength range of the emission peak of the sample. The anisotropy was determined from measurements with different polarizer and analyzer settings, respectively: intensity with horizontal and horizontal polarizer and analyzer (I_{HH}), intensity with horizontal polarizer and vertical analyzer (I_{HV}), with vertical-vertical polarizers (I_{VV}) and vertical-horizontal (I_{VH}). The G-factor, $G = I_{HV}/I_{HH}$, and the anisotropy, $r_{ss} = (I_{VV} - G \cdot I_{VH}) / (I_{VV} + 2 \cdot G \cdot I_{VH})$, were calculated using the appropriate intensity values averaged over the wavelength range used.

NMR spectroscopy and mass spectrometry measurements

NMR measurements were carried out on either a Varian Unity INOVA 300 MHz or a Bruker Avance 600 MHz UltraShield spectrometer and mass spectrometry of small molecules and peptides on a Waters micromass LCT mass spectrometer in ESI mode, while protein masses were determined using an Applied Biosystems Voyager-DE-RP MALDI-ToF mass spectrometer with cinnamic acid as matrix.

Infrared reflection absorption spectroscopy measurements

Infrared spectra were recorded on monolayers of proteins on gold. The gold was covered with a monolayer of thiolated-NTA using a Thermo Fisher PM-IRRAS system under an angle of incidence of about 81°.

NiNTA and β CD monolayers on gold

Preparation of monolayers of NiNTA on gold coated substrates

SPR sensors (1 inch, Ssense) were piranha cleaned (**Warning!** Piranha solution should be handled with caution. It has been reported to detonate unexpectedly) for 30 s, rinsed with water, dried in a flow of N_2 and subsequently immersed in solution of 2 mM mercapto-undecanoic acid (MUA) in 1:1 EtOH:water and left at least 16 h under ambient conditions to react. To remove any excess MUA the samples were rinsed thoroughly with water and dried in a flow of N_2 . The resulting carboxylic acid-terminated monolayers were activated using a freshly prepared aqueous solution of 300 mM N-hydroxy succinimide (NHS) and 40 mM N-(dimethyl aminopropyl)-N'-ethylcarbodiimide (EDC) for 30 min under ambient conditions. After the reaction, the samples were rinsed with water and dried in a flow of N_2 and the activated ester-terminated monolayer was immediately reacted with a 1 mM aqueous solution Na_2Na -bis(carboxymethyl)-L-lysine hydrate for 2 h under ambient conditions. Samples were then washed with water, incubated for 30 min in a 5 g/L aqueous solution

of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, washed with water, dried in a flow of N_2 and could be stored under dry N_2 for a maximum of 4 weeks before use.

Preparation of monolayers of βCD on gold coated substrates

SPR sensors (1 inch, Ssense) were piranha cleaned for 30 s, rinsed with water, dried in a stream of N_2 and subsequently immersed in solution of 5 μM CD-hepta(ϵ -amino di(dodecyl)thioether) in 2:1 chloroform:EtOH and left at least 16 h under Ar at 60 °C. Samples could be kept for a maximum of 1 week in the reaction mixture under argon atmosphere at 60 °C. Samples were only removed from the reaction mixture directly prior to a measurement, washed with chloroform, EtOH, water, EtOH, 3 times, every time in this order, dried in a flow of N_2 and immediately used for measurements.

Preparation of bifunctional βCD /NTA-, βCD /PEG- and NTA/PEG-line-patterned glass substrates using NIL

βCD /NTA-line-patterns

4 inch borofloat (Schott, Borofloat 33) wafers were immersed in a piranha solution for 10 min, rinsed with water, dried in a flow of N_2 and subsequently spin-coated (Speedline, p6700) with a 6% w/v 350 kDa poly(methyl methacrylate) (PMMA) solution in toluene for 30 s at 3000 rpm. To remove residual solvent the wafer was baked at 120 °C for 10 min. NIL was carried out using a Peltier temperature controlled (Julabo FP 50) HP Specac NIL setup at 200 °C and 1 ton for 10 min. As imprint master a 4 inch 1H,1H,2H,2H-perfluorodecyltrichlorosilane (PFDTs, AB111155, ABCR) coated Si-wafer with 24 1x1 cm large patterns of 10 to 25 μm -wide ridges separated by 5 μm -wide 0.5 μm deep trenches was used. In detail, the patterned imprint master was brought into contact with the spin-coated PMMA layer on a borofloat wafer, cushioned with Kunze heat conducting cushioning foil (KU-TXE100) on one side and 4 layers of aluminium foil on the other side and sealed with aluminium foil. This stack was heated to 200 °C, without applying pressure, then the pressure was carefully increased to the equivalent of 1 ton and these conditions were kept constant for 10 min requiring occasional re-adjustment of the pressure. Subsequently, the heating was switched off, while keeping the pressure constant. Upon reaching 100 °C the pressure was left to decrease simultaneously with the temperature. Upon reaching 80 °C the stack was removed from the NIL setup, disassembled and residual layer removal was carried out using an SPI supplies, Plasmaprep II oxygen-plasma cleaner. The progress of residual layer removal was monitored for each of the 1x1 cm patterns individually using an Olympus BH2 light microscope in transmission mode with a

halogen lamp as light source. Upon completion of removing the residual layer (with only 5 μm -wide ridges of PMMA remaining) the substrates were silanized by overnight chemical vapor deposition of TPEDA *in vacuo*. To remove the excess of silane, the samples were rinsed thoroughly with ethanol. The resulting amine-terminated line-pattern was reacted with phenyl diisothiocyanate (DITC). To this end, the samples were immersed in a 0.04 M solution of ITC in ethanol for 2 h at 50 °C under argon. After the reaction, the samples were rinsed with ethanol and dried in a flow of N_2 . The ITC-terminated monolayer was then functionalized with NTA(*t*Bu) $_3$ -NH $_2$. To this end, the samples were incubated in a 1 mM solution of NTA(*t*Bu) $_3$ -NH $_2$ in ethanol at 50 °C for 2 h under argon. All remaining PMMA was stripped from the borofloat substrates by 30 min sonication in 1 L of acetone, rinsing with acetone and drying with N_2 . Subsequently, the unfunctionalized (5 μm -wide) lines were silanized by overnight chemical vapor deposition of TPEDA and ITC as described above. Subsequently, the ITC-terminated lines were reacted with β -cyclodextrin-heptaamine^[19] by incubating the samples in a 1 mM solution of β -cyclodextrin-heptaamine in water at 50 °C for 2 h under Ar. Samples were then washed with water and dried in a flow of N_2 . To remove the *t*Bu-protecting groups the samples were immersed in (TFA) overnight, rinsed with copious amounts of water and dried in a flow of N_2 . The resulting bi-functional NTA/CD line-patterns could be stored under N_2 for a maximum of 4 weeks prior to use.

β CD/PEG- and NTA/PEG-line-patterns

In order to fabricate NTA/PEG- or β CD/PEG-line-patterns the same procedure as described above was used for functionalization of the first set of lines. In the case of NTA/PEG, however, the regular $\text{Na}_\alpha\text{Na}_\alpha$ -bis(carboxymethyl)-L-lysine hydrate instead of the *t*Bu-protected NTA was used precluding the deprotection step afterwards. For β CD/PEG patterns β -cyclodextrin-heptaamine was reacted with the DITC in the first step instead of with NTA-NH $_2$. After stripping of the remaining PMMA, the second set of lines (5 μm wide) was functionalized with a solution of 100 μL PEG-(trimethoxy)silane (AB111226, ABCR) in 60 mL dry toluene by leaving the samples to react overnight at room temperature under Ar. After washing the samples with toluene they were dried under a flow of N_2 . The resulting mono-functional NTA/PEG- and CD/PEG-line-patterns could be stored under N_2 for a maximum of 4 weeks prior to use.

Protein immobilization on (patterned) substrates

NTA-layers were incubated with a 1 mM $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ solution in water for 30 min and the samples were rinsed briefly with water. Similarly, CD-layers were incubated with a 1 mM adamantane hexa(ethylene glycol) (HEG-Ad) solution in

PBS. The solution was removed with a pipette from the surface and the sample was rinsed three times with a buffer containing 1 mM HEG-Ad. The concentration of HEG-Ad was kept constant in all buffers used in all following incubation steps. For the subsequent immobilization of proteins, samples were incubated with the appropriate protein solution (concentrations see text) for times ranging from 1 h to overnight (see text) using humidity chambers. Protein solutions were then removed with a pipette and retained for further use and samples washed on an orbital shaker (80 rpm) in the appropriate buffers for 1 to 24 h (see text). Samples were then rinsed with the appropriate buffer, subsequently with water and dried in a flow of N₂. Samples were imaged using an Olympus IX70 inverted fluorescence microscope, see below.

Fluorescence Microscopy

Fluorescence microscopy images were recorded using an Olympus inverted microscope IX71 equipped with a mercury burner U-RFL-T lamp as light source and a digital Olympus DR70 colour camera for image acquisition. Olympus filter cubes with appropriate band-pass or long-pass filters and dichroic mirrors (Semrock) were used. TFP exhibits an excitation peak at 460 nm and an emission peak at 490 nm. TagRFP exhibits an excitation peak 555 nm and an emission peak at 585 nm. Fluorescence micrographs were acquired using a 20x Fluorplan objective from Olympus and exposure times of 0.2-10 s.

3.6 Appendix

Protein	Absorption max (nm)	Emission max (nm)	Fluorescence lifetime (ns)	Mass calc. (g/mol)	MALDI TOF MS Mass meas. (m/z)
TagRFP wt	555	582	2.4	30309	30350
NHis ₆ - ¹⁴ TagRFP (1H)	555	579	2.4	30277	30000
NHis ₆ -CHis ₆ - ¹⁴ TagRFP (2H)	555	582	2.4	31100	30400
NHis ₆ - ⁵¹²⁸ CHis ₆ - ¹⁰ TagRFP (1+1H)	555	585	2.4	31116	30800
NHis ₆ -CHis ₆ - ⁵¹²⁸ CHis ₆ - ¹⁰ TagRFP(3H)	N/A	N/A	N/A	32164	31700
4-4 G ^{174C} TFP	466	495	3.3	26953	26700
4-4 G ^{174C} TFP+micGEREDEK (ref TFP)	N/A	N/A	N/A	28007	27200
4-4 G ^{174C} TFP+micGadERadEDadEK (Ad ₃ -TFP)	N/A	N/A	N/A	28788	28300

Table 3.1 – Optical properties and mass analysis data for TagRFP and TFP with mutants.

3.7 References

- [1] For examples see: a) P. L. Edmiston, J. E. Lee, S.-S. Cheng, S. S. Saavedra, *Journal of the American Chemical Society* **1997**, 119, 560. b) L. L. Wood, S.-S. Cheng, P. L. Edmiston, S. S. Saavedra, *Journal of the American Chemical Society* **1997**, 119, 571. c) P. L. Edmiston, S. S. Saavedra, *Biophysical Journal* **1998**, 74, 999. d) P. L.

- Edmiston, S. S. Saavedra, *Journal of the American Chemical Society* **1998**, 120, 1665. e) Y.-Z. Du, S. S. Saavedra, *Langmuir* **2003**, 19, 6443.
- [2] S. Hutschenreiter, A. Tinazli, K. Model, R. Tampe, *EMBO Journal* **2004**, 23, 2488.
- [3] E. M. Merzlyak, J. Goedhart, D. Shcherbo, M. E. Bulina, A. S. Shcheglov, A. F. Fradkov, A. Gaintzeva, K. A. Lukyanov, S. Lukyanov, T. W. J. Gadella, D. M. Chudakov, *Nature Methods* **2007**, 4, 555.
- [4] I. Topol, J. Collins, A. Nemukhin, *Biophysical Chemistry* **2010**, 149, 78.
- [5] K. Kato, H. Sato, H. Iwata, *Langmuir* **2005**, 21, 7071.
- [6] a) J. Huskens, H. Van Bakkum, J. A. Peters, *Computers & Chemistry* **1995**, 19, 409. b) J. Huskens, A. Mulder, T. Auletta, C. A. Nijhuis, M. J. W. Ludden, D. N. Reinhoudt, *Journal of the American Chemical Society* **2004**, 126, 6784. c) M. J. W. Ludden, A. Mulder, K. Schulze, V. Subramaniam, R. Tampé, J. Huskens, *Chemistry – A European Journal* **2008**, 14, 2044.
- [7] J. C. Love, L. A. Estroff, J. K. Kriebel, R. G. Nuzzo, G. M. Whitesides, *Chemical Reviews* **2005**, 105, 1103.
- [8] S. Lata, A. Reichel, R. Brock, R. Tampé, J. Piehler, *Journal of the American Chemical Society* **2005**, 127, 10205.
- [9] J. R. Lakowicz, *Analytical Biochemistry* **2001**, 298, 1.
- [10] G. Chirico, F. Cannone, S. Beretta, A. Diaspro, B. Campanini, S. Bettati, R. Ruotolo, A. Mozzarelli, *Protein Science* **2002**, 11, 1152.
- [11] B. L. Frey, R. M. Corn, *Analytical Chemistry* **1996**, 68, 3187.
- [12] E. Goormaghtigh, V. Cabiaux, J.-M. Ruysschaert, *European Journal of Biochemistry* **1990**, 193, 409.
- [13] M. W. J. Beulen, J. Bügler, B. Lammerink, F. A. J. Geurts, E. M. E. F. Biemond, K. G. C. van Leerdam, F. C. J. M. van Veggel, J. F. J. Engbersen, D. N. Reinhoudt, *Langmuir* **1998**, 14, 6424.
- [14] M. J. W. Ludden, A. Mulder, R. Tampé, D. N. Reinhoudt, J. Huskens, *Angewandte Chemie International Edition* **2007**, 46, 4104.
- [15] a) J. Huskens, A. Mulder, T. Auletta, C. A. Nijhuis, M. J. W. Ludden, D. N. Reinhoudt, *Journal of the American Chemical Society* **2004**, 126, 6784. b) U. Kauscher, B. J. Ravoo, *Beilstein Journal of Organic Chemistry* **2012**, 8, 1543.
- [16] D. A. Uhlenheuer, D. Wasserberg, C. Haase, H. D. Nguyen, J. H. Schenkel, J. Huskens, B. J. Ravoo, P. Jonkheijm, L. Brunsveld, *Chemistry – A European Journal* **2012**, 18, 6788.
- [17] S. Lata, J. Piehler, *Nature Protocols* **2006**, 1, 2104.
- [18] R. B. Merrifield, *Journal of the American Chemical Society* **1963**, 85, 2149.
- [19] P. R. Ashton, R. Königer, J. F. Stoddart, D. Alker, V. D. Harding, *The Journal of Organic Chemistry* **1996**, 61, 903.

Chapter 4

A Supramolecular Approach to Growth Factor Immobilization Using His₆-tagged VHH Single-domain Antibodies

In this chapter, a supramolecular strategy is presented for the assembly of growth factors employing His₆-tagged single-domain antibodies (VHH). A combination of orthogonal supramolecular interactions of β -cyclodextrin (β CD) host-guest and N-nitrilotriacetic acid (NTA)-histidine interactions was employed to generate reversibly bound and homogeneous layers of growth factors. A single-domain antibody VHH fragment was identified to bind to the human bone morphogenetic protein 6 (hBMP-6) growth factor and could be recombinantly expressed in E-coli. The VHH fragment was equipped with a C-terminal hexahistidine tether to facilitate the assembly on β CD surfaces using a heterofunctional linker that contains an adamantyl group to bind to the β CD receptors and an NTA moiety to interact with the His tag. After exploring the thermodynamic and kinetic stability of the VHH assemblies on β CD surfaces using a variety of experimental techniques including microcontact printing (μ CP), surface plasmon resonance (SPR), microscale thermophoresis (MST) and theoretical models for determining the thermodynamic behavior of the system, hBMP-6 was assembled onto the VHH-functionalized surfaces. After analyzing the structural integrity of the immobilized hBMP-6 using immunostaining, the biological activity of hBMP-6 was demonstrated in cell differentiation experiments. Early osteogenic differentiation was analyzed in terms of alkaline phosphatase (ALP) activity of KS483 mouse progenitor cells and the results indicated that the reversibly immobilized growth factors were functionally delivered to the cells.

4.1 Introduction

Many current efforts to develop therapies for regenerating a variety of tissues in the body rely on growth factor delivery. Such growth factors are of interest to apply as therapeutic tools owing to their profound effect on cell behavior including stimulation of proliferation, migration and differentiation.^[1] Crucial to the success of the therapy is the mode of growth factor delivery. Strategies relying on bolus drug delivery or systemic administration are of limited use because the inherent instability of many growth factors requires very high levels of protein for a measurable effect and the potential exists for uncontrolled activities at distant sites.^[2] In alternative strategies growth factors are locally delivered by diffusion for prolonged times from polymeric systems loaded with growth factors by simple mixing, encapsulation or layer-by-layer assembly.^[3]

Since growth factors are bound in the extracellular matrix to proteins and glycosaminoglycans under physiological conditions, in an elegant approach to mimic such a condition, Griffith-Cima and coworker demonstrated the covalent tethering of growth factors to surfaces to be a viable method for prolonged growth factor-induced cell responses.^[4] However, it remains a key challenge to optimally allow cell surface events involved in signal transduction – the binding of the growth factor to the cell receptors and possibly aggregation of the receptors in the cell membrane - to occur when growth factors are covalently tethered.^[5] Site-specific immobilization has emerged recently as a practical alternative to random immobilization strategies to generate homogeneous protein layers as well as increased accessibility to binding sites to engage in protein-protein interactions after oriented immobilization of the proteins via a site distant to the binding site.^[6] Supramolecular methods satisfy important requirements of growth factor immobilization, such as the use of mild reagents, buffered conditions, bio-orthogonality, specificity, stability, better packing and faster kinetics.^[7] Furthermore, supramolecular methods also imply reversibility and thus the possibility to tune the delivery of the growth factor. Recently, principles of supramolecular chemistry have been used for displaying a high density of binding epitopes to transforming growth factor β -1 on the surface of nanotube gels supporting the survival and promoting chondrogenic differentiation of human mesenchymal stem cells.^[8]

We here report the development of a supramolecular assembly scheme to enable the oriented immobilization of bioactive growth factors. Human bone morphogenetic protein 6 (hBMP-6) was selected to explore this supramolecular assembly. hBMP-6 belongs to the transforming growth factor-beta (TGF- β) superfamily and is known to play significant roles in cartilage and bone

morphogenesis and repair and is already used as a therapeutic protein for inducing bone growth.^[9] To orient hBMP-6 cytokines onto surfaces, we sought to use single-domain fragments derived from camelid antibodies (VHH¹ or Nanobodies®). While employing VHH constructs as a mode for growth factor delivery has not been reported, such VHH constructs have gained popularity for various therapeutic applications^[10] due to their unique characteristics such as their small size (>20 kDa) and stability to heat^[11] and changes in pH.^[12] In addition VHH constructs can be recombinantly produced and equipped with a hexahistidine (His₆)-tag. This supramolecular His₆-motif can be used for assembly onto our molecular printboard,^[13] which is based on the host-guest interaction between a supramolecular receptor monolayer of β -cyclodextrins (β CD) and adamantane-functionalized guest molecules. Due to its specificity, high immobilization efficiency and reversibility, the molecular printboard has proven to be a valuable tool for fabricating protein biochips following different supramolecular strategies^[13-14] for applications such as immunodiagnostics^[15] and biocatalysis.^[16]

To realize suitable functional assembly and delivery of growth factors, host-guest chemistry was adapted to control the binding properties of the VHH constructs to β -cyclodextrin monolayers by orthogonal linker molecules as demonstrated before.^[13] This method also offered control over the orientation of the VHH constructs to the surface by designing linkers that can bind to the protein specifically. Nonspecific protein adsorption was inhibited by using a monovalent adamantyl-oligo(ethylene glycol) as a masking molecule during the site-specific protein adsorption.^[14c] After identification of VHH binders to hBMP-6, the site-specific immobilization of VHH was first investigated using surface plasmon resonance (SPR) spectroscopy. The supramolecular VHH-based system was subsequently used for the assembly of hBMP-6 using immunostaining and SPR studies. As a demonstration of the development of a bioactive growth factor delivery from the supramolecular surfaces, the early osteogenic differentiation of KS483 mouse progenitor cells was analyzed by the alkaline phosphatase (ALP) activity.

¹ Even though widely used when referring to single-domain antibodies, VHH stands for the variable domain of the heavy chain of a heavy-chain antibody.

4.2 Results and discussion

4.2.1 Selection and production of VHH binder to hBMP-6

VHH clones that bind to hBMP-6 were selected following a phage display approach.^[10b, 17] Upon immunization of a llama with hBMP-6, the RNA encoding the VHH genetic sequence was isolated from blood lymphocytes of the animal and used after reversed-transcription for the construction of a phage library. Affinity selection (biopanning) was carried out on hBMP-6 coated plates, which were incubated with the phage VHH immune library. After the second round, the relative binding strength of selected phage VHH clones were assessed against each other using an enzyme-linked immunosorbent assay (ELISA) and resulted in the discovery of clone H1 (Figure 4.1a). Subsequently, VHH clone H1 was used for the production of VHH in *E. Coli* hosts. After purifying the VHH H1 proteins using affinity chromatography, a single band was detected as shown in Figure 4.1b by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The band of a molecular weight of ca. 16 kD is in agreement with values of related VHH binders found in literature.^[10d]

Figure 4.1 – Table a) Selection of VHH clone binding hBMP-6 via an ELISA (absorbance values at 490 nm). b) SDS-PAGE of the purified VHH shows the expected molecular weight.^[10d]

The interaction of the purified VHH construct with hBMP-6 was further analyzed using microscale thermophoresis (MST)^[18] and ELISA measurements. To determine the affinity between VHH and hBMP-6 with MST, a titration series of hBMP-6 over a range of 0.04 to 700 nM was performed while fluorescently labeled VHH was kept constant at 20 nM throughout the series. On binding of hBMP-6, a change in thermophoretic signal was observed as shown in Figure 4.2a. Subsequent fitting of the data to a 1:1 binding model yielded a dissociation constant K_d of $7.4 (\pm 0.4) \times 10^{-8}$ M and therefore $K_a = 1/K_d = 1.4 \times 10^7$ M⁻¹. These values are

comparable to the ones found in literature for VHH interactions with different binding partners.^[10b, 11, 19] The obtained affinity was compared with affinity studies carried out in an ELISA assay. For this experiment, hBMP-6 was non-specifically immobilized to the surface of a microtiter plate. To the hBMP-6 coated plates a series of VHH solutions in the range of 0-3 μM was added and assayed by ELISA (Figure 4.2b). Results revealed a K_d of $1.9 \times 10^6 \text{ M}^{-1}$, which is one order of magnitude lower than the affinity measured by MST, which is a known effect of randomly oriented proteins.^[7a]

Figure 4.2 – VHH binding to hBMP-6 analyzed a) in solution by MST and b) on microtiter plates by ELISA. In both cases a titration experiment was performed and the results were fitted to a 1:1 binding model to determine either a) the dissociation constant or b) the binding constant.

4.2.2 Immobilization of His₆-VHH onto βCD surfaces

With these VHH constructs with high affinity towards hBMP-6 in hand, we sought to employ the C-terminal His₆-tag on the VHH for the assembly onto βCD surfaces through specific interaction with NTA linkers. For the specific attachment of VHH at the βCD surfaces through host-guest interactions, the step-wise assembly process previously developed in our group was adopted using the nitrilotri(acetic acid) (NTA)-Ni(II)-His₆-tag interaction and orthogonal linkers (Scheme 4.1).

Scheme 4.1 – Chemical structures of the building blocks and scheme for a) the step-wise assembly process or b) the one-pot assembly of His₆-VHH-hBMP-6 on β CD surfaces.

Three consecutive assembly steps were followed, i.e. in the first assembly step, an ethylene glycol based mono-adamantyl linker (**HEG-Ad**) for minimizing the non-specific protein adsorption and a solution of NTA-monoadamantyl linker (**NTA-Ad**) and His₆-VHH was used in the presence of Ni(II) during the second assembly step. Onto these VHH surfaces, in the third assembly step hBMP-6 was immobilized (Scheme 4.1, route a). Alternatively, after assembling **HEG-Ad** a one-pot assembly step with all components was performed (Scheme 4.1, route b). The assembly was performed at a physiological pH of 7.4, which ensures complexation of Ni(II) ions to the majority of the NTA moieties,^[13a] while two metal coordination sites are accessible for complexation with two histidines of the VHH construct. While six histidines are present on the VHH, a range of species of different valency in adamantyl moieties (VHH·(Ni(II)·**NTA-Ad**)_x (x=1-3) can form. However, only higher valency complexes are more likely to bind to the β CD host surface with different affinities determined by the effective concentration (C_{eff}) (see Appendix 4.6.1).

Monolayers of β CD on gold were prepared as described in literature^[20] and placed in an SPR flow cell. After the baseline became stable while flowing PBS buffer, a solution of **HEG-Ad** (0.1 mM in PBS) was flown for 10 min until a stable signal was observed. Successively, a solution of 0.5 μ M VHH-Ni(II)-**NTA-Ad** (in a ratio of 1:5:5) in the presence of 0.1 mM **HEG-Ad** was injected and flown continuously (0.1 mL min⁻¹). The observed increase of the SPR signal reached thermodynamic equilibrium after 10 min (Figure 4.3a).

Figure 4.3 – a) SPR data of a titration series of **His₆-VHH-Ni(II)-NTA-Ad** (1:5:5) to β CD plates in the presence of 0.1 mM **HEG-Ad**. Symbols indicate switching of the buffer to: 0.1 mM **HEG-Ad** (▲), increasing concentrations of **His₆-VHH-Ni(II)-NTA-Ad** (1:5:5) + 0.1 mM **HEG-Ad** (□ ▢ ▣ ▤ ▥ ▦ ▧ ▨ ▩), 10 mM β CD/EDTA in PBS (●) and PBS (↓). b) Experimental change of SPR resonant angle ($\Delta\theta$ at thermodynamic equilibrium; black markers) versus VHH concentrations fitted to the theoretical model. The contribution of the assembly of the different molecules to the signal is given. c) Speciation model corresponding to the possible valences for the **His₆-VHH-Ni(II)-NTA-Ad** complex at the surface.

This observation indicates that the various **VHH-Ni(II)-NTA-Ad** species have replaced monovalent **HEG-Ad**. To demonstrate the reversibility of the assembly of **His₆-VHH**, a solution containing 10 mM of competing ligand EDTA and 10 mM of β CD in PBS were added to the flow cell while monitoring the SPR response. Although a sudden initial increase of the SPR intensity was observed due to the large change in refractive index when using a concentrated solution of β CD, the baseline was totally restored upon rinsing the system with PBS for 5 min. This result indicates the complete disassembly of the molecules from the β CD surface.

To assess the thermodynamic stability of the VHH constructs at the β CD surface, a titration series of VHH-Ni(II)-**NTA-Ad** in the range of 0.5-20 μ M was performed while recording the change in the SPR resonant angle. Between each titration step a disassembly cycle was performed to restore the β CD surface as described above. Upon addition of **HEG-Ad**, which represents the first step of the assembly of each titration step, a constant increase in SPR intensity was observed (Figure 4.3a). The second step of the assembly was marked by a gradual increase in the change of the SPR resonant angle saturating above 20 μ M of VHH (Figure 4.3a and b). The SPR data were satisfactorily fitted to a thermodynamic model that accounts for all species possibly bound to the β CD surface (see Appendix 4.6.2 for details). The model shows that at low concentrations of VHH ($< 1 \mu$ M), the major contribution to the (still low) SPR signal originates from the assembly of monovalent **HEG-Ad**, while above 2 μ M of VHH, when the coverage becomes appreciable, the major contribution to the rise in SPR signal originates from trivalent VHH $\cdot$ (Ni(II)-**NTA-Ad**)₃ species (70%) as expected, indicating that the trivalent complex is the main species contributing to the coverage at the whole range of concentrations until surface saturation (Figure 4.3b and c). Using the intrinsic binding constants $K_{i,\text{HEG-Ad}} = 2.6 \times 10^4 \text{ M}^{-1}$ and $K_{i,\text{NTA-Ad}} = 1.2 \times 10^4 \text{ M}^{-1}$ as found in literature,^[14c] an association constant K_1 of $6.3 \times 10^4 \text{ M}^{-1}$, which represents the first interaction of Ni(II)-**NTA-Ad** to the His₆-tag, and a value for C_{eff} of 0.02 M were found. From these values, the second (K_2) and third (K_3) association constants of the consecutive bonding of His₂ units to Ni(II)-**NTA-Ad** were calculated to be $1.5 \times 10^4 \text{ M}^{-1}$ and $1.95 \times 10^3 \text{ M}^{-1}$ respectively using the statistical factors as derived in Appendix 4.6.1. These values are in agreement with values found in literature,^[13a, 21] while the value for C_{eff} in this case was smaller as compared to values used in literature (0.1 M) for optimizing the binding constant of maltose binding proteins (MBP) to β CD surfaces.^[13a] This discrepancy was attributed to the difference in size and shape of VHH in comparison to MBP. While VHH ($M_w = 16 \text{ kD}$, prolate spheroid, $4.4 \times 2.8 \text{ nm}$)^[10f] occupies less than three β CD cavities, MBP ($M_w = 41 \text{ kDa}$, cubic, $3 \times 4 \times 6.5 \text{ nm}$)^[13a] exceeds three β CD cavities. Even though both proteins have a single His₆-tag, the larger protein size of MBP can sterically reduce the probing volume for the (intramolecular) binding of the second and third adamantyl moieties in the case of MBP and at the same time shield the β CD cavities available within this probing volume from interactions of neighboring His-tagged proteins, while VHH, being a smaller protein, allows other protein units to interact with neighboring hosts at the molecular printboard while simultaneously causing a larger probing volume. As a consequence, this difference in size rationalizes the observed lower value of C_{eff} in the case of VHH. The overall binding constant for the trivalent complex on the

β CD surface ($[\text{VHH}] = 2 \mu\text{M}$ and $[\text{Ni(II)-NTA-Ad}] = 10 \mu\text{M}$) was calculated to be: $K = \sim 10^6 \text{ M}^{-1}$ in agreement with literature^[22] and for the divalent complex $K = \sim 10^5 \text{ M}^{-1}$. In both cases, the multivalent interactions lead to highly stable assemblies bound to the surface while still reversible.

To visualize the VHH assemblies using fluorescence microscopy, His₆-VHH was fluorescently labeled with a Cy3-dye and used for patterning employing the well-established microcontact printing (μ CP) method with a polydimethylsiloxane (PDMS) stamp.^[23] Such stamps were inked for 2 min with a mixture of $1 \mu\text{M}$ Cy3-labeled VHH:Ni(II):**NTA-Ad** (in ratio of 1:5:5) in PBS buffer. After inking, the stamp was blown dry and put into conformal contact with a β CD-functionalized glass surface for 5 min. After removing the stamp, the slides were directly imaged. As a reference, the same experiment was performed in which the PDMS stamp was inked with a $1 \mu\text{M}$ solution of VHH without Ni(II):**NTA-Ad** added. In both cases, uniform dot patterns of $50 \mu\text{m}$ diameter and spaced by $10 \mu\text{m}$ were clearly visible with an excellent contrast with the background as imaged using fluorescence microscopy (Figure 4.4). After extensive rinsing for 30 min with PBS, the patterns disappeared in the case of the reference experiment, which lacked the supramolecular **NTA-Ad** linker, whereas clear patterns remained visible in the case when **NTA-Ad** was co-printed. These results indicate that the interactions between VHH and the surface are governed by specific NTA-Ni(II)-His₆-tag interactions as envisioned. Subsequently, after washing a slide having patterns of Cy3-His-VHH/Ni(II)/**NTA-Ad** with a solution containing 10 mM of both β CD and EDTA the patterns disappeared, which is characteristic of reversible supramolecular interactions between the protein and the surface. The observations made by fluorescence microscopy suggest that homogeneous layers of oriented proteins are fabricated as a result of specific and reversible supramolecular interactions, in agreement with the SPR studies.

Figure 4.4 – Fluorescence microscopy images of Cy3-His₆-VHH patterned by μ CP on a β CD surface in the absence (top) or presence (bottom) of Ni(II)-NTA-Ad, a,c) directly after printing, b,d) after rinsing with PBS for 30 min) and e) after rinsing with a 10 mM solution of both β CD and EDTA. Insets show fluorescence intensity profiles of the patterns. Exposure time: 500 ms.

To acquire more information about the disassembly of the VHH proteins from the β CD surface using *in vitro* conditions, a series of substrates patterned with Cy3-His₆-VHH-Ni(II)-NTA-Ad were immersed in cell culture medium and at specific time points examined using fluorescence microscopy (Figure 4.5a). When plotting the decay of the fluorescence intensity versus time (Figure 4.5b) after 7 days around 80% of the fluorescence of the Cy3-labeled VHH disappeared from the β CD surfaces. When the data were fitted with first-order kinetics a dissociation rate constant of $k_d = \sim 10^{-6} \text{ s}^{-1}$ was found, which compares favorably to examples in literature of immobilizing His₆-tagged proteins on covalently modified NTA-surfaces.^[24] The ability to extend the release of VHH beyond days provides an interesting opportunity to apply our supramolecular surfaces for the delivery of growth factors to cells.

Figure 4.5 – Fluorescence microscopy images (a-d) of Cy3-His₆-VHH patterned by μ CP on β CD glass slides recorded a) immediately after printing, and after immersion for b) 24 h, c) 48 h and d) 168 h in cell culture medium. e) Release of Cy3-VHH is plotted relative to the initial fluorescence intensity versus time. Inset shows the linear fitting of the corresponding logarithmic values. Exposure time: 200 ms.

4.2.3 Functional assembly and delivery of hBMP-6 using supramolecular interactions

To investigate whether hBMP-6 can be assembled onto β CD slides employing the interaction with VHH, SPR studies were performed following the one-pot assembly scheme. After recording a baseline by continuously flowing a solution of 0.1 mM **HEG-Ad**, the flow was switched to a solution of hBMP-6 in the presence of different concentrations of His₆-VHH (and Ni(II)·**NTA-Ad**) while monitoring the SPR intensity (Figure 4.6c). When a 50 nM solution of hBMP-6 was used in the presence of a 2 μ M solution of His₆-VHH, an increase in the SPR resonant angle was observed when compared to the case when His₆-VHH·Ni(II)·**NTA-Ad** was flown without hBMP-6. A similar observation was made when using a 210 nM solution of hBMP-6 in the presence of a 5 μ M solution of His₆-VHH. When a solution of only hBMP-6 was flown directly after the first assembly step no increase in the SPR intensity was observed indicating that no non-specific interactions occurred between hBMP-6 and the background surface. These SPR studies indicate that

hBMP-6 can be assembled onto β CD slides from solution via specific interactions with VHH.

As a next step an immunoassay was carried out to investigate whether hBMP-6 is structurally intact after immobilizing onto β CD surfaces following a step-by-step assembly. To this end, patterns of dots of VHH were fabricated on glass slides as described above and subsequently incubated with a solution containing hBMP-6 (700 nM) for 1 h. After sequential coupling for 1 h of a primary antibody specific to hBMP-6 (mouse-anti-hBMP-6-Ab) and a FITC-labeled secondary Ab (goat-anti-mouse-Ab-FITC) specific to the primary Ab, the slides were imaged using fluorescence microscopy (Figure 4.6b). The recorded images show clearly that the fluorescently labeled antibody was selectively immobilized on the printed VHH patterns. When the β CD surfaces were additionally blocked with BSA prior to incubating the slides with hBMP-6 and antibodies the background was further reduced. When the immunoassay was carried out on the VHH patterns in the absence of hBMP-6 no fluorescent patterns were observed (Figure 4.6a). These results indicate that after supramolecularly immobilizing VHH, VHH was able to specifically bind hBMP-6 in agreement with MST and ELISA studies, while in addition hBMP-6 retained its folded structure in the immobilized state.

Figure 4.6 – Fluorescence microscopy images after immunostaining β CD slides patterned with VHH-Ni(II)-NTA-Ad (dots of 100 μ m in diameter and spaced by 20 μ m) a) without and b) including incubation of hBMP-6. Insets show a scheme representing each assembly. Exposure time: 5000 ms. c) Collection of SPR sensograms of flowing VHH-Ni(II)-NTA-Ad and/or hBMP-6 as indicated in the legend over a β CD slide. The solutions were pre-mixed for at least 30 min before injection (arrow) in the presence of 0.1 mM of HEG-Ad.

To explore the bioactivity of immobilized hBMP-6 following either the step-by-step or the one-pot assembly as described above, KS483 mouse progenitor cells were cultured on hBMP-6 functionalized surfaces for 7 days. Evaluation of the fluorescence in a Live/Dead assay showed negligible cytotoxicity (Figure 4.7c). From a series of hBMP-6 functionalized surfaces and references, the alkaline phosphatase (ALP) production was measured after 7 days and normalized to the cell number and expressed as relative induction in comparison to glass substrates (Figure 4.7a and b). When hBMP-6 was assembled on the surface through VHH in a step-by-step assembly process, the ALP activity was 6-fold higher in comparison to the case when VHH functionalized surfaces were used that lack hBMP-6 (Figure 4.7a). In addition, ALP levels were enhanced at most by 2-fold when hBMP-6 was non-specifically immobilized from solution on surfaces that lack VHH-Ni(II)-NTA-Ad or β CD. No induction of ALP level was observed for VHH

functionalized surfaces. These results confirm that upon specific binding of hBMP-6 to VHH only, the cells are responsive to hBMP-6 in terms of early osteogenic differentiation, which is marked by ALP typically after 7 days. Moreover, when immobilized hBMP-6 was deliberately denatured by exposure to 95 °C temperature for 2 h, the ALP activity was significantly decreased indicating that hBMP-6 lost most of its functional structure and that hBMP-6 is responsible for producing ALP. Following an ELISA experiment to determine the amount of hBMP-6 dissociating from hBMP-6 surfaces, which were prepared following the step-by-step procedure, the released amount of hBMP-6 was determined to be 142.4 ng ($\pm$ 17.3) of hBMP-6 after 7 days of cell culture. These results explain the higher ALP activity observed for this type of hBMP-6 surfaces when compared with reference experiments in which 100 ng of hBMP-6 was added to the cell culture medium, irrespective of whether substrates were functionalized with VHH. However, when cells were seeded on hBMP-6 surfaces that were prepared following the one-pot assembly procedure using a solution of 100 ng of hBMP-6 and 0.5 μ M VHH·Ni(II)·**NTA-Ad**, a 6-fold relative induction of the ALP production was observed with respect to the case when hBMP-6 was left out of the assembly solution to give VHH-only surfaces (Figure 4.7b). Taken together, the results show that irrespective of the assembly procedure followed, sufficient amounts of hBMP-6 can be functionally assembled through VHH on the molecular printboard and successively delivered to the cells.

Figure 4.7 – ALP activity normalized by the total DNA content of KS483 cells after 7 days of culture, expressed as relative induction with respect to a) a glass control following a step-by-step or to b) a VHH-only surface following a one-pot assembly procedure of hBMP-6. shBMP-6 indicates that the growth factor was supplemented in the cellular medium in a soluble factor while bhBMP-6 was presented bound to a platform and dbhBMP-6 was denatured at high temperature upon immobilization. # $p < 0.05$ compared to VHH-Ni(III)-NTA-Ad, ## $p < 0.01$ compared to VHH-Ni(III)-NTA-Ad and ** $p < 0.01$ compared to all cases. c) Live-dead assay showing KS483 cells seeded on supramolecular surfaces presenting hBMP-6.

4.3 Conclusions

Following a phage display approach, a VHH clone binding to hBMP-6 was identified. The identified clone was used to produce the VHH binder in *E. coli*. The interaction of the purified VHH construct with hBMP-6 was analyzed using MST and ELISA measurements, yielding a K_d in the range of $10^6 - 10^7 \text{ M}^{-1}$. Using the C-terminal His₆-tag of the VHH, the assembly of VHH has been achieved on βCD surfaces adopting the nitrilotri(acetic acid) (NTA)-Ni(II)-His₆-tag interaction and orthogonal linkers. Thermodynamic analysis of the SPR titration data showed that the majority of the VHH binds in a trivalent fashion to the surface with an overall K_d of $\sim 10^6 \text{ M}^{-1}$. Homogeneous patterns of VHH assemblies were successfully fabricated using microcontact printing and visualized using fluorescence microscopy. The reversibility of the binding of the assemblies was confirmed by

SPR and fluorescence microscopy experiments, while under *in vitro* conditions a dissociation rate of $\sim 10^{-6} \text{ s}^{-1}$ of the VHH from the surface was found. Based on SPR and immunoassay studies, the surface assembly of hBMP-6 was shown to proceed via specific interactions with VHH while retaining its folded structure. The results from seeding KS483-4C3 mouse progenitor cells on hBMP-6 functionalized surfaces confirm not only that hBMP-6 is bound specifically to VHH but also that upon specific binding of hBMP-6 to VHH only, the cells are responsive to hBMP-6 in terms of early osteogenic differentiation expressed by ALP production. In addition, the combined results show that irrespective of the assembly procedure followed, either a step-by-step or a one-pot procedure of all surface components, both procedures lead to the functional assembly and delivery of sufficient amounts of hBMP-6 on the supramolecular platform.

4.4 Acknowledgements

The major part of the selection and production of VHH was performed by E.D. Rodrigues. E.B.M Landman and J. van Weerd are gratefully acknowledged for their help and fruitful discussions.

4.5 Experimental section

Materials

Commercial grade reagents were purchased from Sigma-Aldrich and were used without further purification unless otherwise stated and solvents were dried over molecular sieves. Recombinant human BMP-6 (hBMP-6) was purchased from R&D Systems as a carrier-free, 0.2 μm filtered solution (0.04 mg/mL) in acetonitrile and TFA and further diluted to the desired working concentration in PBS buffer. High-purity water (Millipore, $R = 18.2 \text{ M}\Omega\text{cm}$) was used.

^1H -NMR spectra were recorded on a Varian Unity INOVA (300 MHz) spectrometer. Spectra are reported in ppm downfield from TMS as an internal standard. Analytical TLC was performed using Merck prepared plates (silica gel 60 F-254 on aluminum). Merck silica gel (40-63 μm) was used for flash column chromatography. Electrospray Ionization (positive mode) high resolution mass spectra were recorded on a WATERS LCT mass spectrometer.

Synthesis

Hexa(ethylene glycol) mono(adamantyl ether) (HEG-Ad):

HEG-Ad was synthesized according to a protocol adapted from literature.^[14c] To a solution of hexa(ethylene glycol) (12.5 mL, 50.9 mmol) 1-bromoadamantane (1.1 g, 5.1 mmol) and triethylamine (Et₃N) (2.0 mL, 14.3 mmol) were added. The solution was stirred at 120 °C overnight. After cooling to room temperature, dichloromethane (50 mL) was added. The solution was washed with 2 M hydrochloric acid (4 x 50 mL) and once with brine (50 mL). The organic layer was dried over MgSO₄ and the solvent was evaporated under reduced pressure to give **HEG-Ad** as a yellow-brown oil (0.87 g, 2.1 mmol, 40%). MS (ESI): *m/z* = 417.4 [M+H]⁺, (calc. 416.6 for C₂₂H₄₀O₇).

Mono(adamantyl) L-lysine-nitrilotri(acetic acid) (NTA-Ad).

NTA-Ad was synthesized according to a protocol adapted from literature.^[14c, 25] H-Lys(Z)-OtBu HCl (4.0 g, 10.7 mmol) was dissolved in 100 mL dry DMF. Subsequently, *t*-butyl bromoacetate (6.36 mL, 43.1 mmol) and N,N-diisopropylethylamine (DIPEA) (9.2 mL, 52.8 mmol) were sequentially added. After purging the flask with N₂, the solution was stirred overnight at 55 °C under inert N₂ atmosphere. DMF was removed *in vacuo* at 60 °C and the partially solidified reaction mixture was resuspended in 60 mL of cyclohexane and ethylacetate (in a ratio of 3:1) and filtered over a sintered glass funnel. The precipitate was washed (3 x 40 mL) with the same solvent mixture, concentrated under reduced pressure and purified by silica gel chromatography with cyclohexane and ethylacetate (in a ratio of 3:1) as the mobile phase to quantitatively yield 6.0 g (10.5 mmol) Lys(Z)-NTA(OtBu)₃ (R_f = 0.50). Then, Lys(Z)-NTA(OtBu)₃ (6 g, 10.5 mmol) was dissolved in methanol (300 mL) and the solution was purged with vacuum-N₂ cycles. Subsequently, 10% Pd/C (0.120 g) was added under N₂. The solution was mixed vigorously and stirred for 6 h under hydrogen atmosphere at room temperature. After releasing H₂ while being replaced by N₂, the Pd/C catalyst was removed by filtration over celite and the solvents were removed from the filtrate under reduced pressure to give L-Lys-NTA(OtBu)₃ (3.68 g, 8.5 mmol; 81%). MS (ESI): *m/z* = 432.3 g/mol [M+H]⁺, (calc. 431 g/mol for C₂₂H₄₂N₂O₆). Subsequently, L-Lys-NTA(OtBu)₃ (1.95 g, 4.5 mmol) was dissolved in dichloromethane (100 mL). To this solution DIPEA (1 mL) and adamantyl acid chloride (0.8 g, 4.05 mmol) were added. After stirring this solution at room temperature for 2 h, the solution was washed with 1 N NaOH (4 x 75 mL), 1 N HCl (4 x 75 mL) and brine (75 mL). The organic phase was then dried over MgSO₄ and evaporated to dryness to give Lys-NTA(OtBu)₃-Ad (2.07 g, 3.5 mmol, 77%). MS (ESI): *m/z* = 593.8 g/mol [M+H]⁺, (calc. 592.8 g/mol for C₃₃H₅₆N₂O₇). Finally, Lys-NTA(OtBu)₃-Ad (2.1 g, 3.5 mmol)

and triisopropylsilane (4.2 mL, 20.5 mmol) were dissolved in trifluoroacetic acid (60 mL). After stirring this solution at room temperature for 4 h, diisopropylether (60 mL) was added to give a white precipitate. The precipitate was collected by filtration over a sintered glass funnel and thoroughly rinsed with diethylether to give **NTA-Ad** as a white powder (1.3 g, 3.1 mmol, 88%). ^1H NMR (300 MHz, DMSO- d_6 , 20 °C, TMS) δ : 12.42 (bs, 3H, COOH), 7.24 (t, 1H, NHCO), 3.60 (m, 4H, CH₂COO), 3.32 (t, 1H, NCH), 3.01 (m, 2H, NHCH₂), 1.95 (m, 3H, AdCH), 1.65-1.75 (m, 14H, AdCH₂ + AdCH₂ + CH₂CH), 1.26-1.38 (m, 4H, CH₂CH₂CH₂CH₂); MS (ESI): m/z = 426.6 $[\text{M}+\text{H}]^+$, (calc. 425.0 for C₂₁H₃₂N₂O₇).

Llama immunization and VHH phage library construction

A VHH phage display library was generated from the immunization of 2 llamas. Llamas were immunized with hBMP-6. hBMP-6 was mixed with the adjuvant Stimune (CEDI Diagnostics, Lelystad, The Netherlands) and injected intramuscularly at days 0, 14, 28 and 35. The VHH phage display library was generated as previously described.^[10b, 17] After immunization, total RNA was extracted from peripheral blood lymphocytes, converted into complementary DNA and cloned in a phage-display plasmid.

Selection of VHH targeting growth factors

Phage display was used to select phages binding to hBMP-6 using an immunized llama VHH phage display library. The phages were selected via the panning method by coating, overnight at 4 °C, a MaxiSorp plate (Nunc, Thermo Scientific) with decreasing concentrations of hBMP-6 (5 μg , 2 μg and 0.2 μg) in phosphate buffered saline (PBS, Gibco). Phage VHH immune library (approximately 10^{10} colony forming units (cfu)), was incubated for 2 h at room temperature to the wells blocked with 4% Marvel (dried skimmed milk, Premier International Foods) in PBS. After thorough washing, the phage VHH clones binding to the coating were eluted by incubating 100 mM Et₃N for 15 min at room temperature and immediately neutralized by the addition of 1M Tris-HCl pH 7.5. DNA information of the selected phages was rescued by infection of Escherichia coli (*E. coli*) TG1 strain and subsequent selection on agar plates for ampicillin resistance. To obtain recombinant bacteriophages expressing the VHH as fusion proteins with the bacteriophage gene III product, transformed TG1 *E. coli* were grown to logarithmic phase and then infected with helper phage VCSM13 (Stratagene, La Jolla, CA, USA). The phage particles were produced overnight in medium containing ampicillin and kanamycin. The phages were precipitated with polyethylene glycol (PEG) and used in the second panning round of selection on

wells coated with hBMP-6 as described above. Selected phages from single colonies from the second round of selection were sequenced (Macrogen).

VHH plasmids, production and purification

E. coli strain TG1 and/ or DH5a was used for the maintenance of the plasmids, infection by the phages and expression of proteins. DNA information of the individually selected and isolated VHH were sub-cloned into a plasmid containing C-terminal Myc and His tags. *E. coli* TG1 and/ or DH5a was grown in Luria Broth (LB) or Yeast extract and tryptone (YT) medium containing 2% (w/v) glucose and antibiotic, ampicillin 100 µg/ml. VHH were produced from *E. Coli* TG1 by isopropyl β-D-1-thiogalactopyranoside (IPTG) lac promoter induction at 37 °C for 4 hours under non static conditions. VHH proteins were produced in *E. coli* TG1 and purified from the periplasmic fraction via the C-terminal his-tag by cobalt affinity chromatography (TALON His-Tag Purification Resin, ClonTech). Release from the column was induced by 200 µM imidazole solution in PBS and eluents were dialyzed against PBS for two days at 4 °C to remove imidazole. Purified VHH were analyzed by means of SDS polyacrylamide gel electrophoresis (SDS-PAGE). The final VHH concentration was determined from the UV absorption at 280 nm (NanoDrop 1000 Spectrophotometer, Thermo Scientific) and the theoretical molar extinction coefficient.

His₆-VHH H1 was labeled with a fluorescent dye using the Amersham Cy3 mono-reactive dye pack (GE Healthcare Europe GmbH, Germany) according to the manufacturers protocol and purified via dialysis against PBS overnight to a ratio of 4 dyes/protein.

Phage and VHH ELISA for binder selection

Either the supernatant (as a source of phages) or the periplasmatic fractions resulting from the VHH productions were directly incubated (1h at room temperature) in ELISA plates coated with hBMP-6. Bound VHH were detected upon 1h incubation at room temperature with a rabbit anti-VHH (α-K212, self-produced) (1/5000) and a 1h incubation at room temperature with a secondary donkey anti-rabbit antibody coupled to a peroxidase (DARPO) (1/10000) second antibody. H₂O₂ and ortho-phenylenediamine (OPD) were used at a ratio 1/1000 as substrates for the peroxidase reaction. Plates were measured with a Bio-Rad model 550 plate reader. Absorbance of OPD was measured at 490 nm after the reaction was stopped by adding 1M H₂SO₄.

hBMP-6 ELISA

Cell culture medium was collected after 7 days and the amount of hBMP-6 was quantified by an ELISA assay according to the manufacturer's instructions (Duo Set ELISA development kit, R&D systems).

Preparation of β CD surfaces

On gold substrates ^[20]: Gold substrates for SPR (BK7 glass/2-4 nm Ti/50 nm Au) were obtained from Ssens B.V. (Hengelo, The Netherlands). The substrates were cleaned by immersion in piranha solution (3:1 concentrated H₂SO₄/ 30% H₂O₂) for 10 sec (**Warning!** Piranha solution should be handled with caution. It has been reported to detonate unexpectedly) and rinsed thoroughly with water. Subsequently, the gold plates were placed in absolute EtOH for 10 min in order to remove the oxide layer. Afterwards, the substrates were immersed in a 0.1 mM β CD heptamine solution in EtOH/CHCl₃ (1:2 v/v) overnight at 60 °C. Finally, the substrates were rinsed in three series of CHCl₃, EtOH and water and immediately used for SPR or otherwise stored in the β CD heptamine solution for up to three days.

On glass substrates ^[26]: Microscope glass slides were cleaned and activated by immersion in piranha solution for 25 min and rinsed thoroughly with water. After drying under a flow of dry N₂, the substrates were immediately placed at the bottom of a desiccator with a vial containing 100 μ L of (trimethoxysilyl)propyl]ethylenediamine (TPEDA) and the desiccator was then evacuated with a diaphragm vacuum pump for 10 minutes and left overnight. Subsequently, the substrates were thoroughly rinsed with ethanol and dichloromethane and dried under a flow of dry N₂. Subsequently, the substrates were immersed in a solution of 10 mM 1,4-phenylene diisothiocyanate in dry toluene for 2 h at 60 °C. Samples were rinsed with toluene and dried under a flow of dry N₂. Finally, samples were immersed in an aqueous solution of 0.1 mM β CD-heptamine and allowed to react for 2 h at 60 °C. After rinsing with water, the β CD-functionalized surfaces were immediately used or stored in a N₂ atmosphere up to a week.

PDMS preparation and μ CP experiments

Silicon wafer-based masters with etched structures were prepared by UV photolithography. The master's surface was fluorinated using 1H,1H,2H,2H-perfluorododecyltrichlorosilane (PFDTs). Poly(dimethylsiloxane) (PDMS) (Dow Corning) stamps were fabricated by curing the degassed mixture (10:1 elastomer, curing agent) of the components of SYLGARD® 184 silicone elastomer

kit on the surface of the master at 60 °C overnight. PDMS stamps were first oxidized in an oxygen-plasma reactor (Plasma-Prep II plasma etcher, SPI supplies, USA) at an oxygen pressure of 1.0 bar, a vacuum pressure of 160 mbar and a current of 40 mA for 20 sec. The stamps were then stored in water. Prior to use, the stamps were dried with a flow of dry N₂ and then incubated with 100 µL of a 1 µM solution of Cy3-His₆-VHH:Ni(II):**NTA-Ad** (1:5:5) for 2 min. After removing the excess of the inking solution, the dried stamps were brought into conformal contact with the βCD functionalized surfaces for 5 min. Afterwards the substrates were visualized with fluorescence microscopy or washed as described in the main text.

For all experiments in this chapter, NiCl₂ was used as Ni(II) source and Cy3-His₆-VHH:Ni(II):**NTA-Ad** were pre-incubated together for 30 min to maximize complexation and in addition, ratio of 1:5:5 was used, which offers two extra Ni(II) ions and **NTA-Ad** than the three required to saturate the His₆-tag.

The quantification of protein bound to the substrates at different time points was performed via the fluorescence intensity quantification of protein arrays by using the particle counting routine of the free software ImageJ.

For the immobilization of the N-(lissamine-rhodamine B)-labeled divalent adamantyl guest, the same concentrations and printing times were used.

For the immobilization of hBMP-6, His₆-VHH(H1):Ni(II):**NTA-Ad** (1:5:5) was printed as described above. Subsequently, the background was blocked with a 0.1 mM BSA solution in PBS buffer via a 1 h incubation at room temperature. The substrates were then rinsed 3 times with PBS buffer including 1 mM Tween-20 during 10 min on the orbital shaker and finally rinsed with PBS buffer. The substrates were incubated with a solution of 700 nM hBMP-6 in PBS buffer containing 1% BSA for 1h at room temperature and rinsed as previously described.

hBMP-6 immunostaining

Following protein immobilization via µCP in the desired patterns, substrates were incubated with PBS containing 1% BSA for 1 h and washed again three times as described above to block the background against non-specific protein interactions. The hBMP-6 protein patterns were then incubated with 100 µL of a solution of 5 µg/mL of monoclonal anti-BMP-6 antibody produced in mouse (mouse IgG2B isotype, clone 74219.11) in PBS buffer including 1% BSA. After 1 h, the substrates were washed three times 10 min with PBS buffer including 1 mM Tween-20, rinsed with water and dried with a flow of dry N₂. Finally, 100 µL of a solution (20 µg/mL) of anti-mouse IgG (whole molecule, subclasses G1, Gg2a, G2b, and G3)-FITC produced in goat in PBS buffer including 1% BSA was used to

incubate the samples for 1 h. The substrates were then washed again three times 10 min with PBS buffer including 1 mM Tween 20, rinsed with water and dried with a flow of dry N₂ and used for fluorescence microscopy imaging.

Fluorescence Microscopy

Fluorescence microscopy images were recorded using an Olympus inverted microscope IX71 equipped with a mercury burner U-RFL-T as light source and a digital Olympus DR70 camera for image acquisition using the following Olympus filter cubes: 510 nm $\leq \lambda_{\text{ex}} \leq 550$ nm for red emission ($\lambda_{\text{em}} \geq 580$ nm) and 460 nm $\leq \lambda_{\text{ex}} \leq 490$ nm for green emission ($\lambda_{\text{em}} \geq 520$ nm). All fluorescence microscopy images were acquired in air.

SPR

SPR experiments were performed using an RT2005 surface plasmon spectrometer (Resonant Technologies GmbH, Germany) in Kretschmann configuration using a mechanically modulated HeNe-laser ($\lambda = 632.8$ nm). Refractive index matching oil (Cargille; series B; $n_D^{25\text{ }^\circ\text{C}} = 1.7000 \pm 0.0002$) between glass substrate and prism (Schott, LaSFN9) was used and a teflon flow cell (100 μL) of 0.9 cm in diameter was mounted on top of the gold layer on the surface of the glass substrate. The prism, sample and flow cell were mounted on a double goniometer with an electronic control unit to precisely set the angle position, the light was detected using a photodiode. A syringe pump (Aladdin WPI Ltd, US) was used to flow the different solutions into the cell via Tygon R3607 tubes (0.76 mm diameter) at a flow rate of 0.1 mL/min. All measurements were performed by tracking the SPR resonance angle while using a temperature controller, which was set to 21 $^\circ\text{C}$.

MST

Microscale Thermophoresis (MST) measurements were performed using a Monolith NT115 (NanoTemper Technologies GmbH, Germany). A solution of 60 μM of VHH was labeled with a fluorescent dye (NT-647, NT-532 or NT-488) using Monolith NTTM Protein Labeling Kit (amine reactive) with the subsequent removal of excess dye within 45 min. The non-labeled titrant (hBMP-6) was diluted with PBS buffer in a dilution series with the highest concentration of 700 nM. 10 μL of the different dilutions were mixed with 10 μL of the fluorescent molecule. Mixed samples were loaded into glass capillaries and the MST analysis was performed.

Cell culture

Prior to cell seeding, the substrates were sterilized with 70% ethanol and subsequently washed twice with PBS to remove any excess of ethanol. The mouse pre-osteoblast cell line KS483-4C3 was used as a model for osteogenic differentiation. Cells were plated at a density of 5000 cells/cm² and cultured in Minimal Essential Medium (MEM) alpha supplemented with 10% fetal bovine serum (FBS) and 100 U penicillin/streptomycin (Gibco) and 1% (v/v) Penicillin-Streptomycin solution (with 10,000 units penicillin and 10 mg streptomycin/mL, Sigma-Aldrich). Live/Dead assay (LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells, Molecular Probes®) was performed according to the protocol from the provider.

ALP quantification

For osteogenic differentiation, 0.2 mM ascorbic acid was added to the medium together with 100 ng/mL hBMP-6 delivered in a soluble form or a comparable concentration delivered via the substrate. After seven days of culturing KS483-4C3 cells, the cells were lysed in 200 µL lysis buffer (0.1 M K₂HPO₄ + 0.1 M KH₂PO₄ + 0.1% Triton X100, pH 7.8). ALP was detected and quantified using the CDP-star® Chemiluminescent Substrate (Sigma Aldrich). ALP production was corrected for DNA content, which was measured using the CyQuant Cell Proliferation assay (Invitrogen). Experiments were repeated at least three times. Error bars represent standard errors, and statistical analysis was performed using the one-way analysis of variance test (ANOVA) to evaluate the statistical differences and a Tukey's Post Hoc test (# $p < 0.05$ compared to VHH·Ni(II)·NTA-Ad, ## $p < 0.01$ compared to VHH·Ni(II)·NTA-Ad and ** $p < 0.01$ compared to all cases * $p < 0.05$ or ** $p < 0.005$).

4.6 Appendix

4.6.1 Thermodynamic model for fitting SPR titration data

The orthogonal interactions of **NTA-Ad** with both the βCD surface, in a monovalent fashion, as well as with His₆-tagged VHH in the presence of Ni(II), in a multivalent fashion, leads to the coexistence of multiple species with different valencies. In addition, the use of **HEG-Ad** to suppress unspecific protein adsorption yields competitive binding to βCD. Scheme 4.2 represents all possible equilibria between all species in our system.

Scheme 4.2 - Equilibria for all species participating in the immobilization of His₆-VHH at a βCD surface (above), for the binding of Ni(II)·NTA-Ad to a βCD surface (middle) and for the competitive binding of HEG-Ad to βCD (below). Complexation steps given in bold of Ni(II)·NTA-Ad to His₆-VHH take place in solution. K_1 is the equilibrium constant for the interaction between a His₂ unit of the His₆-VHH to Ni(II)·NTA, $K_{i,NTA}$ and $K_{i,HEG}$ are the intrinsic binding constants for the interaction between βCD and NTA-Ad or HEG-Ad respectively. For subsequent bindings to the βCD surface the binding will be affected by the effective concentration parameter (C_{eff}).

To fit our SPR data the following assumptions were made for the current system under study using prior knowledge of Ludden et al. [13a]

- i) At the working pH of 7.4 all **NTA-Ad** is complexed to Ni(II). [13a]
- ii) The complexation of His₆-VHH to 1-3 Ni(II)·NTA-Ad units occurs exclusively in solution, which determines the valence of the complex (solution steps are given in bold in Scheme 4.2).
- iii) Statistical factors relate the binding constants K_2 and K_3 with K_1 following the binding scheme of **NTA-Ad** with histidines in the presence of Ni(II) taking into account that the initial binding of Ni(II)·NTA-Ad to His₆-tag is 5 times as likely than to a His₂ unit (Scheme 4.3a). Therefore,

$$K_1 = 5K_{i,His_2} \cdot \text{Ni(II)} \quad (1)$$

From five possible cases in which VHH is bound to a single Ni(II)·NTA-Ad, two of them offer three His₂ units free for interacting and three of them only two. Moreover, the dissociation of His₂ from

$\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2$ is twice as likely when compared to the dissociation of His from $\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})$ (Scheme 4.3b). Therefore,

$$K_2 = \frac{(3 \cdot \frac{2}{5} + 2 \cdot \frac{3}{5})}{2} K_{i, \text{His}_2 \cdot \text{Ni(II)}} = \frac{6}{25} K_1 \quad (2)$$

From two $\text{VHH} \cdot \text{Ni(II)} \cdot \text{NTA-Ad}$ cases offering three His₂ free for interacting with a second Ni(II)·NTA-Ad unit, only two of the His₂ allow binding to a third His₂ unit (Scheme 4.4b), whereas from three $\text{VHH} \cdot \text{Ni(II)} \cdot \text{NTA-Ad}$ cases offering two His₂ free for interacting with a second Ni(II)·NTA-Ad unit, only in one case, both of the His₂ units allow binding to a third His₂ unit, whereas in the other two cases, binding to a third His₂ unit is not possible (Scheme 4.4b). In addition, upon binding VHH to three His₂, the dissociation is three times as likely when compared to case of the dissociation of His₂ from $\text{VHH} \cdot \text{Ni(II)} \cdot \text{NTA-Ad}$. Therefore,

$$K_3 = \frac{\frac{2 \cdot 2 + 1}{5 \cdot 3 \cdot 5}}{3} K_{i, \text{His}_2 \cdot \text{Ni(II)}} = \frac{7}{225} K_1 \quad (3)$$

Scheme 4. 3 - Equilibria for the complexation of pairs of neighboring histidines from the His₆-VHH to Ni(II)·NTA-Ad in solution (a) and origin of the statistical factors associated to subsequent bindings (b). Dots represent Ni(II)·NTA-Ad units for the first (solid gray) and second (faint gray) bindings. Capital letters in the dots indicate positions of equal statistics. Green check marks and red crosses indicate whether a third His₂ unit is available or not, resp., for the binding of an additional Ni(II)·NTA-Ad unit.

With these assumptions in mind, the following mass balances were constructed:

$$\begin{aligned}
 [\text{VHH}]_{\text{tot}} &= [\text{VHH}]_{\text{free}} + [\text{VHH} \cdot \text{Ni(II)} \cdot \text{NTA-Ad}] + \\
 &[\text{VHH} \cdot \text{Ni(II)} \cdot \text{NTA-Ad} \cdot \beta\text{CD}] + [\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2] + \\
 &[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2 \cdot \beta\text{CD}] + [\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2 \cdot (\beta\text{CD})_2] + \\
 &[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3] + [\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3 \cdot \beta\text{CD}] + \\
 &[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3 \cdot (\beta\text{CD})_2] + [\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3 \cdot (\beta\text{CD})_3]
 \end{aligned} \quad (4)$$

$$\begin{aligned}
 [\text{Ni(II)} \cdot \text{NTA-Ad}]_{\text{tot}} &= [\text{Ni(II)} \cdot \text{NTA-Ad}]_{\text{free}} + [\text{Ni(II)} \cdot \text{NTA-Ad} \cdot \beta\text{CD}] + \\
 &[\text{VHH} \cdot \text{Ni(II)} \cdot \text{NTA-Ad} \cdot \beta\text{CD}] + 2([\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2] + [\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2 \cdot \beta\text{CD}] + \\
 &[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2 \cdot (\beta\text{CD})_2]) + 3([\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3] + [\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3 \cdot \beta\text{CD}] + \\
 &[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3 \cdot (\beta\text{CD})_2] + [\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3 \cdot (\beta\text{CD})_3])
 \end{aligned} \quad (5)$$

$$[\text{HEG-Ad}]_{\text{tot}} = [\text{HEG-Ad}]_{\text{free}} + [\text{HEG-Ad} \cdot \beta\text{CD}] \quad (6)$$

$$\begin{aligned}
 [\beta\text{CD}]_{\text{tot}} &= [\beta\text{CD}]_{\text{free}} + [\text{VHH} \cdot \text{Ni(II)} \cdot \text{NTA-Ad} \cdot \beta\text{CD}] + \\
 &[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2 \cdot \beta\text{CD}] + [\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3 \cdot \beta\text{CD}] + \\
 &2([\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2 \cdot (\beta\text{CD})_2] + [\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3 \cdot (\beta\text{CD})_2]) + \\
 &3[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3 \cdot (\beta\text{CD})_3] + [\text{Ni(II)} \cdot \text{NTA-Ad} \cdot \beta\text{CD}] + \\
 &[\text{HEG-Ad} \cdot \beta\text{CD}]
 \end{aligned} \quad (7)$$

The binding constant for the complexation of the first His₂ unit of the VHH to the Ni(II)·NTA-Ad can be described as:

$$K_1 = \frac{[\text{VHH} \cdot \text{Ni(II)} \cdot \text{NTA-Ad}]}{[\text{VHH}] \cdot [\text{Ni(II)} \cdot \text{NTA-Ad}]} = 5K_{i, \text{His}_2 \cdot \text{Ni(II)}} \quad (8)$$

Relating the binding of subsequent histidine pairs according to the statistical factors presented in Scheme 4.3, the binding constants for divalent and trivalent NTA-Ad species with the surface are:

$$K = \frac{[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2 \cdot \beta\text{CD}]}{[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_2] \cdot [\beta\text{CD}]} = 2K_{i, \text{NTA}} \quad (9)$$

$$K = \frac{[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3 \cdot \beta\text{CD}]}{[\text{VHH} \cdot (\text{Ni(II)} \cdot \text{NTA-Ad})_3] \cdot [\beta\text{CD}]} = 3K_{i, \text{NTA}} \quad (10)$$

Subsequent bindings are regulated by the effective concentration (C_{eff}), which incorporates characteristics such as the linker length and lattice parameter in the probing volume, giving the following binding constants:

$$K = \frac{[VHH \cdot (Ni(II) \cdot NTA-Ad)_2 \cdot (\beta CD)_2]}{[VHH \cdot (Ni(II) \cdot NTA-Ad)_2 \cdot \beta CD] \cdot [\beta CD]} = \frac{1}{2} C_{eff} K_{i,NTA} \quad (11)$$

$$K = \frac{[VHH \cdot (Ni(II) \cdot NTA-Ad)_3 \cdot (\beta CD)_2]}{[VHH \cdot (Ni(II) \cdot NTA-Ad)_3 \cdot \beta CD] \cdot [\beta CD]} = C_{eff} K_{i,NTA} \quad (12)$$

$$K = \frac{[VHH \cdot (Ni(II) \cdot NTA-Ad)_3 \cdot (\beta CD)_3]}{[VHH \cdot (Ni(II) \cdot NTA-Ad)_3 \cdot (\beta CD)_2] \cdot [\beta CD]} = \frac{1}{3} C_{eff} K_{i,NTA} \quad (13)$$

Substituting the equilibrium constant equations (8-13) into the mass balances (equations 4-7) (not shown) yields a set of numerically solvable equations with $[VHH]$, $[\beta CD]$, $[Ni(II) \cdot NTA-Ad]$ and $[HEG-Ad]$ as variables. These equations, were solved by using a Simplex algorithm in a spreadsheet approach.^[27] By fixing the values of the intrinsic binding constants for the monovalent interactions with the βCD surface, which are reported in literature for our system components,^[14c] while K_1 and C_{eff} were iteratively optimized to fit the theoretical SPR resonant angle change $\Delta\theta_{max}$ with the experimentally observed SPR data, assuming a contribution of all surface binding units (i.e. $VHH \cdot (Ni(II) \cdot NTA-Ad)_x$ ($x=1-3$) and **HEG-Ad**) to the SPR signal. Finally, the overall stability constant for $VHH \cdot (Ni(II) \cdot NTA-Ad)_3 \cdot (\beta CD)_3$ was calculated using:

$$K = (K_{i,NTA})^3 K_1 K_2 K_3 C_{eff}^2 \cdot [Ni(II) \cdot NTA-Ad]^3 \quad (14)$$

Where, $K_{i,NTA} = 1.2 \cdot 10^4 \text{ M}^{-1}$,^[14c] $K_1 = 6.3 \cdot 10^4 \text{ M}^{-1}$ ($K_2 = 1.50 \cdot 10^4 \text{ M}^{-1}$ and $K_3 = 1.95 \cdot 10^3 \text{ M}^{-1}$), $C_{eff} = 0.02 \text{ M}$ and $[Ni(II) \cdot NTA-Ad] = 10 \text{ } \mu\text{M}$

4.6.2 Thermodynamic model for fitting ELISA titration data

In a similar manner as described above, a simplified model to describe the interaction between non-immobilized VHH and hBMP-6 was used assuming a 1:1 interaction between the two components. The signal was related directly to the complex formation without accounting for possible interferences from the multiple steps of the ELISA procedure. Scheme 4.4 represents the equilibrium between all species, their binding constant, the simplified mass balances and the theoretical relationship between the absorbance at 490 nm and the total

concentration of VHH which was assumed to be exponential. In this case, optimized K_{VHH} and $A_{490,max}$ in a least-squares routine for the best fitting.

$$K_{VHH}$$

$$K_{VHH} = \frac{[VHH \cdot hBMP-6]}{[VHH] \cdot [hBMP-6]} \quad (15)$$

$$[VHH]_{tot} = [VHH] + [VHH \cdot hBMP-6] = [VHH] \cdot (1 + K_{VHH}[hBMP-6]) \quad (16)$$

$$[hBMP-6]_{tot} = [hBMP-6] + [VHH \cdot hBMP-6] = [hBMP-6] \cdot (1 + K_{VHH}[VHH]) \quad (17)$$

$$A_{490} = A_{490,max} \cdot (1 - e^{(-K_{VHH}[VHH]_{tot})}) \quad (18)$$

Scheme 4.4 - Equilibrium of the binding of the VHH to hBMP, simplified mass balances and exponential theoretical relationship between the absorbance at 490 nm and the total VHH concentration.

4.7 References

- [1] For examples see: a) S. D. Boden, J. Kang, H. Sandhu, J. G. Heller, *Spine* **2002**, 27, 2662. b) S. Takeshita, L. P. Zheng, E. Brogi, M. Kearney, L. Q. Pu, S. Bunting, N. Ferrara, J. F. Symes, J. M. Isner, *The Journal of Clinical Investigation* **1994**, 93, 662. c) S. Takeshita, L. Q. Pu, L. A. Stein, A. D. Sniderman, S. Bunting, N. Ferrara, J. M. Isner, M. Jeffrey, J. F. Symes, *Circulation* **1994**, 90, 228. d) I. Baumgartner, A. Pieczek, O. Manor, R. Blair, M. Kearney, K. Walsh, J. M. Isner, *Circulation* **1998**, 97, 1114. e) R. C. Hendel, T. D. Henry, K. Rocha-Singh, J. M. Isner, D. J. Kereiakes, F. J. Giordano, M. Simons, R. O. Bonow, *Circulation* **2000**, 101, 118. f) C. H. Lee, J. L. Cook, A. Mendelson, E. K. Moiola, H. Yao, J. J. Mao, *The Lancet* **2010**, 376, 440.
- [2] G. D. Yancopoulos, S. Davis, N. W. Gale, J. S. Rudge, S. J. Wiegand, J. Holash, *Nature* **2000**, 407, 242.
- [3] For examples see: a) J. Murray, L. Brown, R. Langer, *Cancer Drug Delivery* **1984**, 1, 119. b) E. R. Edelman, E. Mathiowitz, R. Langer, M. Klagsbrun, *Biomaterials* **1991**, 12, 619. c) W. R. Gombotz, D. K. Pettit, *Bioconjugate Chemistry* **1995**, 6, 332. d) M. J. Mahoney, W. M. Saltzman, *Proceedings of the National Academy of Sciences* **1999**, 96, 4536. e) K. Y. Lee, M. C. Peters, K. W. Anderson, D. J. Mooney, *Nature* **2000**, 408, 998. f) T. P. Richardson, M. C. Peters, A. B. Ennett, D. J. Mooney, *Nature Biotechnology* **2001**, 19, 1029. g) T. Crouzier, L. Fourel, T. Boudou, C. Albigès-Rizo, C. Picart, *Advanced Materials* **2011**, 23, H111. h) T. Crouzier, K. Ren, C. Nicolas, C. Roy, C. Picart, *Small* **2009**, 5, 598.
- [4] P. R. Kuhl, L. G. Griffith-Cima, *Nature Medicine* **1996**, 2, 1022.
- [5] S. R. Meyers, M. W. Grinstaff, *Chemical Reviews* **2011**, 112, 1615.
- [6] For reviews see for example: a) T. L. Foley, M. D. Burkart, *Current Opinion in Chemical Biology* **2007**, 11, 12. b) Y.-W. Wu, R. S. Goody, *Journal of Peptide*

- Science **2010**, 16, 514. c) Y.-X. Chen, G. Triola, H. Waldmann, *Accounts of Chemical Research* **2011**, 44, 762.
- [7] For reviews see for example: a) P. Jonkhøj, D. Weinrich, H. Schröder, C. M. Niemeyer, H. Waldmann, *Angewandte Chemie International Edition* **2008**, 47, 9618. b) C. M. Niemeyer, *Angewandte Chemie International Edition* **2001**, 40, 4128.
- [8] R. N. Shah, N. A. Shah, M. M. Del Rosario Lim, C. Hsieh, G. Nuber, S. I. Stupp, *Proceedings of the National Academy of Sciences* **2010**, 107, 3293.
- [9] a) S. Vukicevic, L. Grgurevic, *Cytokine & Growth Factor Reviews* **2009**, 20, 441. b) R. H. Li, J. M. Wozney, *Trends in Biotechnology* **2001**, 19, 255. c) C. M. Kemmis, A. Vahdati, H. E. Weiss, D. R. Wagner, *Biochemical and Biophysical Research Communications* **2010**, 401, 20.
- [10] For examples see: a) C. Hamers-Casterman, T. Atarhouch, S. Muyldermans, G. Robinson, C. Hammers, E. B. Songa, N. Bendahman, R. Hammers, *Nature* **1993**, 363, 446. b) S. Muyldermans, *Reviews in Molecular Biotechnology* **2001**, 74, 277. c) M. Harmsen, H. De Haard, *Applied Microbiology and Biotechnology* **2007**, 77, 13. d) J. Wesolowski, V. Alzogaray, J. Reyelt, M. Unger, K. Juarez, M. Urrutia, A. Cauerhff, W. Danquah, B. Rissiek, F. Scheuplein, N. Schwarz, S. Adriouch, O. Boyer, M. Seman, A. Licea, D. Serreze, F. Goldbaum, F. Haag, F. Koch-Nolte, *Medical Microbiology and Immunology* **2009**, 198, 157. e) P. Vanlandschoot, C. Stortelers, E. Beirnaert, L. Itatí Ibañez, B. Schepens, E. Depla, X. Saelens, *Antiviral Research* **2011**, 92, 389. f) V. Cortez-Retamozo, N. Backmann, P. D. Senter, U. Wernery, P. De Baetselier, S. Muyldermans, H. Revets, *Cancer Research* **2004**, 64, 2853. g) K. De Groeve, N. Deschacht, C. De Koninck, V. Cavelliers, T. Lahoutte, N. Devoogdt, S. Muyldermans, P. De Baetselier, G. Raes, *Journal of Nuclear Medicine* **2010**, 51, 782.
- [11] R. H. J. van der Linden, L. G. J. Frenken, B. de Geus, M. M. Harmsen, R. C. Ruuls, W. Stok, L. de Ron, S. Wilson, P. Davis, C. T. Verrips, *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **1999**, 1431, 37.
- [12] M. Harmsen, C. van Solt, A. van Zijderveld-van Bommel, T. Niewold, F. van Zijderveld, *Applied Microbiology and Biotechnology* **2006**, 72, 544.
- [13] a) M. J. W. Ludden, A. Mulder, K. Schulze, V. Subramaniam, R. Tampé, J. Huskens, *Chemistry – A European Journal* **2008**, 14, 2044. b) P. Maury, M. Escalante, M. Péter, D. N. Reinhoudt, V. Subramaniam, J. Huskens, *Small* **2007**, 3, 1584.
- [14] For examples see: a) M. Escalante, Y. Zhao, M. J. W. Ludden, R. Vermeij, J. D. Olsen, E. Berenschot, C. N. Hunter, J. Huskens, V. Subramaniam, C. Otto, *Journal of the American Chemical Society* **2008**, 130, 8892. b) D. A. Uhlenheuer, D. Wasserberg, C. Haase, H. D. Nguyen, J. H. Schenkel, J. Huskens, B. J. Ravoo, P. Jonkhøj, L. Brunsveld, *Chemistry – A European Journal* **2012**, 18, 6788. c) M. J. W. Ludden, A. Mulder, R. Tampé, D. N. Reinhoudt, J. Huskens, *Angewandte Chemie International Edition* **2007**, 46, 4104. d) M. J. W. Ludden, M. Péter, D. N. Reinhoudt, J. Huskens, *Small* **2006**, 2, 1192.
- [15] M. J. W. Ludden, X. Li, J. Greve, A. van Amerongen, M. Escalante, V. Subramaniam, D. N. Reinhoudt, J. Huskens, *Journal of the American Chemical Society* **2008**, 130, 6964.
- [16] A. González-Campo, B. Eker, H. J. G. E. Gardeniers, J. Huskens, P. Jonkhøj, *Small* **2012**, 8, 3531.
- [17] G. P. Smith, V. A. Petrenko, *Chemical Reviews* **1997**, 97, 391.
- [18] C. J. Wienken, P. Baaske, U. Rothbauer, D. Braun, S. Duhr, *Nature Communications* **2010**, 1, 100.
- [19] For examples see: a) S. Spinelli, L. G. J. Frenken, P. Hermans, T. Verrips, K. Brown, M. Tegoni, C. Cambillau, *Biochemistry* **2000**, 39, 1217. b) E. De Genst, K. Silence, K. Decanniere, K. Conrath, R. Loris, J. r. Kinne, S. Muyldermans, L. Wyns, *Proceedings of the National Academy of Sciences of the United States of America* **2006**, 103, 4586.
- [20] M. W. J. Beulen, J. Bügler, B. Lammerink, F. A. J. Geurts, E. M. E. F. Biemond, K. G. C. van Leerdam, F. C. J. M. van Veggel, J. F. J. Engbersen, D. N. Reinhoudt, *Langmuir* **1998**, 14, 6424.

- [21] S. Lata, A. Reichel, R. Brock, R. Tampé, J. Piehler, *Journal of the American Chemical Society* **2005**, 127, 10205.
- [22] M. De, S. Rana, V. M. Rotello, *Macromolecular Bioscience* **2009**, 9, 174.
- [23] C. Wendeln, B. J. Ravoo, *Langmuir* **2012**, 28, 5527.
- [24] I. Nakamura, A. Makino, M. Ohmae, S. Kimura, *Macromolecular Bioscience* **2010**, 10, 1265.
- [25] S. Lata, J. Piehler, *Nature Protocols* **2006**, 1, 2104.
- [26] S. Onclin, A. Mulder, J. Huskens, B. J. Ravoo, D. N. Reinhoudt, *Langmuir* **2004**, 20, 5460.
- [27] J. Huskens, H. van Bakkum, J. A. Peters, *Computers & Chemistry* **1995**, 19, 409.

A Fluorogenic Monolayer to Detect the Immobilization of Peptides that Combine Cartilage Targeting and Regeneration*

Strategies to generate platforms combining tissue targeting and regeneration properties are in great demand in the regenerative medicine field. Here we present the detected immobilization of cysteine-terminated peptides with relevant biological properties by using a novel fluorogenic surface.

First, the peptide CGRGDS was synthesized to function as a cell adhesion signal. The selective immobilization of this adhesive peptide was directly detected using a fluorogenic surface. Adhered cells were confined to patterns of these peptides matching with the fluorogenic areas. These results show that the fluorogenic signal can be used to detect the chemo-selective immobilization of non-fluorescent biomolecules and to correlate the cell response to the patterned peptides. Second, the peptides CLPLGNSH and CLRGRYW were synthesized to function as peptide binders to transforming growth factor (TGF)- β 1 and collagen type II (CII), respectively, and immobilized as detected by the fluorogenic platform. After analyzing the sequence specificity and cross-reactivity of the binding of TGF- β 1 and CII to the respective peptide regions employing immunofluorescence assays, both peptides were co-immobilized in a step-wise process as detected by the fluorogenic surface. TGF- β 1 and CII could self-sort from a mixture in a regio-selective manner resulting in a bifunctional protein platform. Surfaces of CLPLGNSH reported excellent bioactivity when pre-loaded with TGF- β 1 in terms of early chondrogenic processes of human articular chondrocytes (HACs).

* Part of this chapter has been published in: C. Nicosia, J. Cabanas-Danés, P. Jonkheijm, J. Huskens, *ChemBioChem* **2012**, 13, 778.

5.1 Introduction

Designing functional materials that combine both targeting and regenerating properties is enormously desired to improve the properties of biomaterials commonly used in the regenerative medicine field. In particular, articular cartilage regeneration will greatly benefit from such dual material platforms because damaged cartilage shows restricted abilities to effectively form new tissue maintaining the native structural and mechanical properties.^[1]

Employing peptides with specific targeting and regenerative properties is an effective strategy to induce specifically designed biological function. Such peptides are often detected employing phage display technology, which allows for rapidly screening thousands of peptide sequences to interact with specific target proteins (e.g., see in Chapter 4).^[2] Taking advantage of this approach, Stupp et al. functionalized the periphery of peptide amphiphiles^[3] with specific peptide sequences having regenerative properties for different clinical situations.^[4] For example, when such functionalized peptide amphiphiles were able to capture transforming growth factors (TGF)- β 1, successfully regenerated articular cartilage was found in a full thickness chondral defect in a rabbit model.^[4a] In another report by Hubbel et al. cartilage targeting was elegantly demonstrated using polymeric nanoparticles that were functionalized with a specific peptide sequence.^[5]

Here, we present a method to prepare a bifunctional peptide platform for targeting of collagen type II (CII), which is a major component of the collagen fibrillar network of cartilage,^[6] and delivery of TGF- β 1, which maintains articular cartilage cells in its differentiated phenotype^[7] while inducing chondrogenic differentiation in bone marrow mesenchymal progenitor cells.^[8] For this purpose, we used a self-reporting fluorogenic reactive monolayer, recently described by Nicosia et al.,^[9] that allows detecting the immobilization of cysteine-terminated peptides by a Michael addition reaction.^[10] Using these fluorogenic surfaces yields a fast, simple, sensitive and non-destructive detection of the immobilization products while direct visualization of the modified area is feasible using fluorescence microscopy. Fluorogenic reporting methods overcome the use of expensive and time-consuming labeling of compounds. First, the fluorescently detected immobilization of non-fluorescently labeled RGD peptide^[11] is used for identifying the site-specific adhesion of C2C12 cells (mouse myoblast cell line).^[9] Second, a TGF- β 1 binding peptide CLPLGNSH^[4a] and a CII binding peptide CLRGRYW^[5] were co-immobilized as detected by the fluorogenic platform. These dual platforms were used to bind TGF- β 1 and collagen type II yielding a platform with regenerative and targeting properties respectively. Finally, the

activity of TGF- β 1 tethered on a layer of CLPLGNSH was tested on human articular chondrocytes (HACs), reporting an increased glycosaminoglycans (GAG) production.

5.2 Results and discussion

5.2.1 Immobilization strategy of peptides

For the fabrication of the fluorogenic platform, glass slides were first modified with an azide-terminated self-assembled monolayer (SAM) (Scheme 5.1a).^[12] Then, alkyne-functionalized fluorogenic coumarin **1** was immobilized via a Huisgen 1,3-dipolar cycloaddition reaction^[13] by performing a 1 h reactive microcontact printing (μ CP) step employing an oxidized polydimethylsiloxane (PDMS) stamp inked with a solution of coumarin **1**, Cu(I)(CH₃CN)₄PF₆ and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA). The presence of a methyl-4-oxo-2-butenate group adjacent to the coumarin allows reaction with thiols and quenches the coumarin emission prior to reaction with such thiols.^[9, 14] Owing to this quenching, upon printing of **1**, the fluorescence of the pattern was almost indistinguishable from the background.^[9] Subsequently, onto this self-reporting platform the covalent immobilization of cysteine-terminated peptides was performed by means of the fluorogenic Michael addition to the methyl-4-oxo-2-butenate moiety of **1** (Scheme 5.1a).

To establish the method for the immobilization of cysteine-modified peptides on the self-reporting surfaces, a collection of peptides was synthesized via solid phase peptide synthesis (SPPS). The synthesized peptides carry an N-terminal cysteine and have proven biological properties such as integrin binding properties (CGRGDS, **3**)^[11], binding of TGF- β 1 (CLPLGNSH, **4**)^[4a, 15] and binding to CII (CLRGRYW, **5**).^[5] As control for the specificity of the immobilization reaction, a peptide was synthesized in which the cysteine was replaced by methionine (MGRGDS, **3'**). As control for the specific binding of proteins to the immobilized peptides, two scrambled peptides were synthesized (CHNLGLPS, **4'** and CWRGLRY, **5'**).

a)

b)

Scheme 5.1 – a) Signaled peptide immobilization strategy consisting of the reactive μCP of coumarin **1** on an azide-terminated SAM, followed by the covalent immobilization and detection of cysteine-terminated peptides by means of the fluorogenic Michael addition reaction to the methyl-4-oxo-2-butenate moiety. b) Chemical structures of the compounds employed in this chapter.

5.2.2 Immobilization of CGRGDS for localized cell adhesion

To investigate the applicability of the fluorogenic platform to detect the peptide immobilization, patterned surfaces of **1** (Figure 5.1a) were fabricated and incubated with a 10 mM solution of **3** or **3'** in PBS for 1 h following Scheme 5.1a. While the sample treated with **3** showed a 10-fold enhancement of the fluorescence intensity (Figure 5.1c),^[9] the intensity of the sample incubated in a solution containing the corresponding methionine derivative (**3'**) remained almost indistinguishable from the background (Figure 5.1d) and unchanged with respect to **1**. When the fluorogenic platform was incubated with cysteine ethyl ester an 8-fold enhancement of the coumarin fluorescence was observed relative to **1** (Figure 5.1b). These results indicate that, the peptide immobilization proceeds exclusively via the thiol of the cysteine-containing peptides. In addition, when assuming full coverage for a small thiol-terminated molecule such as in the case of cysteine ethyl ester, a close-packed surface was found in the case of the RGD peptide.

Figure 5.1 – Fluorescence microscopy images corresponding to substrates patterned with a) coumarin **1** and after incubation with b) 10 mM L-cysteine ethyl ester hydrochloride, c) 10 mM of **3** and d) 10 mM of **3'** following Scheme 5.1. Insets show fluorescence intensity profiles. C2C12 cells were subsequently incubated for 6 h onto the CGRGDS (**3**)-modified substrates either without (e, g) or with (f, h) backfilling with **2** as shown in the insets. Images were recorded after staining the C2C12 cells with phalloidin against actin fibers.

As RGD is known to induce integrin mediated cell adhesion, as a next step, C2C12 cells from a mouse myoblast cell line were seeded on RGD-patterned surfaces for 6 h. The remaining non-functionalized azide-terminated areas on the surface were backfilled via a Huisgen 1,3-dipolar cycloaddition reaction with propargylhexa(ethylene glycol) (**2**) prior to cell seeding. Fluorescence micrographs showed actin fibers in the cells, which are co-localized on the blue fluorescent coumarin patterns. When samples were not backfilled, cells partially

spread to areas that were not functionalized with RGD (Figure 5.1e and g) while backfilling with **2** led to a significant increase in spatial selectivity yielding clearly localized cells on the RGD patterns (Figure 5.1f and h).

5.2.3 Detected co-immobilization of CLPLGNSH and CLRGRYW and binding of TGF- β 1 and collagen type II

Incubating fluorogenic platforms with a solution of **4** or **4'** in PBS yielded an 8-fold enhancement of the coumarin fluorescence while the intensity enhancement upon incubation with a solution of **5** or **5'** in PBS:DMSO (1:3) was ca. 6-fold (Figure 5.2). Differences in surface coverage could be related to differences in solubility and peptide structure. After incubation of platforms functionalized with the CLPLGNSH (**4**) peptide in a solution containing TGF- β 1 (1 μ g/mL) in PBS for 1 h, an immunofluorescence assay was performed to visualize the binding of TGF- β 1 to the immobilized peptide. First a primary antibody was incubated for 1 h followed by an incubation of a secondary antibody labeled with for 1 h. After rinsing, the substrates were analyzed using fluorescence microscopy showing clearly red fluorescent patterns that coincide with the coumarin patterns (Figure 5.2a, b). When this assay was performed on substrates functionalized with a scrambled peptide sequence (**4'**) (Figure 5.2d, e) or with an unrelated peptide sequence (**5**) (Figure 5.2f, g) no red fluorescent patterns could be discerned while the blue fluorescence was retained.

Another series of experiments was performed on platforms of peptide CLRGRYW (**5**). After incubating these platforms with bovine collagen type II (BCII) (50 μ g/mL) an immunofluorescence assay was performed. After sequential incubation with primary and secondary antibodies, green fluorescent lines were observed using fluorescence microscopy indicative of successful binding of BCII to the immobilized peptide **5** (Figure 5.2f, h). Whereas the green fluorescent lines were excellently confined onto patterns of **5**, minimal binding of BCII was detected on patterns of scrambled peptide sequence **5'** (Figure 5.2i, j). In addition, binding of BCII was absent on patterns of the TGF- β 1 peptide **4** indicating negligible cross-reactivity among sequences (Figure 5.2c).

These results show that i) the binding of TGF- β 1 and BCII is sequence specific and selective, and ii) bound TGF- β 1 and BCII retain their structural integrity.

Figure 5.2 – Fluorescence images corresponding to substrates patterned with coumarin **1** after 1h incubation with a) **4**, d) **4'**, f) **5** and i) **5'** and b, e, g) after 1 h incubation with 1 $\mu\text{g}/\text{mL}$ TGF- β 1 and immunofluorescence staining (red) or c, h, j) after 1h incubation with collagen type II and immunofluorescence staining (green). Insets provide both intensity profiles and images captured using the blue channel when the main picture was recorded using either the red or the green channel.

Having these results in hand, we sought to explore the possibility of detecting the immobilization of multiple peptides on the same platform and to study the selectivity of their binding properties. To achieve the co-immobilization of peptides CLPLGNSH (**4**) and CLRGRYW (**5**) the procedure as depicted in Scheme 5.2 was followed. First, fluorogenic coumarin **1** was immobilized by μCP in patterns consisting of 100 μm wide lines separated by 100 μm followed by the printing of peptide **4** in a 1 h step. To this end, a PDMS stamp with dots of 100 μm in diameter and separated by 100 μm was inked with a 10 mM solution of **4** in PBS

for 30 min and placed in conformal contact with the functionalized substrates for 1 h. Subsequently, the substrates were incubated in a solution containing 10 mM of peptide **5** in order to backfill the unreacted regions of fluorogenic **1**. Finally, the bifunctional peptide surfaces were exposed to a solution containing both TGF- β 1 (1 μ g/mL) and BCII (50 μ g/mL) and inspected using fluorescence microscopy subsequent to immunofluorescence staining. The results are presented in Figure 5.3. Initially, patterns of **1** showed quenched coumarin fluorescence in agreement with our previous results (*vide supra*) and with literature.^[9] The coumarin fluorescence intensity is then regio-selectively restored upon printing the first peptide **4** as signaled by the fluorogenic platform (Figure 5.3b). In this case, the intensity of **1** was restored only in the printed dots that matched with patterned lines of **1** indicating the success of the Michael addition reaction. An incubation step with the second peptide **5** led to a recovery of the coumarin intensity in the remaining areas resulting in observing a continuous blue fluorescent line pattern of coumarin **1** (Figure 5.3c). Upon close inspection, the 100 μ m dots of **4** were still distinguishable in agreement with the larger enhancement of the coumarin intensity when compared to immobilizing **5** as observed above. Subsequently, the platform co-functionalized with the two peptides was incubated with a solution containing both TGF- β 1 (1 μ g/mL) and BCII (50 μ g/mL) for 1 h. After rinsing, an immunostaining assay was performed against TGF- β 1 (red) (Figure 5.3d) yielding a clear match with the patterned regions of peptide **4** while the remaining areas were undistinguishable from the background. Then, an immunostaining assay was performed against BCII. From the recorded fluorescence images, green areas were observed that coincide with the remaining areas of the initial blue line pattern (Figure 5.3e,g,h).

Scheme 5.2 – Surface functionalization by a) reactive μ CP of **1** on an azide-terminated SAM followed by immobilization of **4** by reactive μ CP as reported by the fluorogenic reaction, and subsequent immobilization of **5** by incubation. The bifunctional platforms were then incubated in a solution containing both TGF- β 1 and BCII for 1 h and immunostained accordingly.

Figure 5.3 – a-h) Fluorescence images of a) a pattern of **1**, b) the subsequent printing of **4** and c) after incubation with **5**. d) shows the immunostaining of TGF- β 1 (red) after the platforms were incubated with a mixture containing both TGF- β 1 and BCII and e-f) show the platforms after immunostaining of BCII (green) using e) the green or f) the red channels. g) is the overlay image of both e) and f) and h) is the 3D surface plot corresponding to g). Insets show the image taken using the blue channel when the main image was taken using either the red or the green channels.

When overlaying both fluorescence images the TGF- β 1 and BCII regions could be clearly observed while the intensity of the coumarin pattern was fully restored. Therefore, the self-reporting surface resulted in an excellent tool to visualize the local step-wise peptide immobilization with the subsequent protein self-sorting of a mixture. In addition, this novel finding opens the possibility to combine both targeting and regenerative properties in a unique layer.

In another experiment the approach presented in Scheme 5.2 was followed using the same stamp for all the surface functionalization steps (Figure 5.4). In this manner, after cross-printing peptide **4** on patterns of coumarin **1**, the functionalized regions are equal in size and shape as compared to the reactive background. Upon incubation with the second peptide (**5**) a rise in fluorescence was observed in the initially dark regions, however two fluorescent regions were distinguished from the fluorescent intensity profiles along the lines of coumarin **1** (Figure 5.4c). This observation can be used to evaluate the specific binding of TGF- β 1 and BCII to the correct peptide areas. After immunostaining the bifunctional platforms for TGF- β 1 and BCII, a clear confinement of TGF- β 1 was observed in regions functionalized with **4**, while BCII was bound to regions functionalized with **5** (Figure 5.4f).

Figure 5.4 – Fluorescence images of a) a pattern of **1**, b) the subsequent cross-printing of **4** (arrows indicate printing direction) and c) after incubation with **5**. d) and e) show the immunostaining of BCII (green) and TGF-β1 (red) respectively after the platforms were incubated with a mixture containing both proteins. f) is the overlay image of both d) and e) and g) is the 3D surface plot corresponding to f). Inset in f) shows the image recorded using the blue channel.

5.2.4 Regenerative potential of platforms functionalized with CLPLGNSH

To demonstrate the biological activity of bound TGF-β1, a platform was fabricated that consisted of a full layer of peptide **4**. After printing **1** on an azide-terminated SAM using a flat featureless PDMS stamp, the fluorogenic surface was incubated with either a solution containing **4** or **4'**. The increase in the fluorescence intensity (Figure 5.5a) indicates that the peptides were successfully immobilized. These platforms were then incubated with TGF-β1 (100 ng) and used for culturing human articular chondrocytes (HACs) for one week and the cellular morphology was analyzed (Figure 5.5b). After this period of time, cells cultured in absence of TGF-β1 exhibited an elongated morphology, which resembles a fibroblast-like phenotype.^[16] In contrast, those cultured in the presence of TGF-β1, either bound or supplemented in the culture medium, showed a more round to polygonal morphology, which resembles chondrocyte-like phenotypes. In addition, the presence of pre-chondrogenic cell condensations was observed as an early skeletogenesis event, common in cultures supplemented with TGF-β1.^[17] These cellular condensations ranged in size and number being maximized for cells cultured on platforms of **4** with bound TGF-β1, followed by cells seeded on a glass slide with TGF-β1 (100 ng) supplemented in the medium, while cells seeded on a platform of **4'** pre-loaded with 100 ng of growth factor

showed the fewest and smallest cell condensations of them all. These results not only show again that platforms of surface-bound **4** are useful for the binding with TGF- β 1 but also that the growth factor remains active and is readily accessible for interacting with specific cellular receptors. When comparing the case when the same amount of TGF- β 1 was delivered in solution, the observed stimulation in the case of TGF- β 1 bound to **4** was enhanced, which could be related to a higher local concentration of growth factor at the surface. The enhanced stimulation led to larger sizes and higher quantities of cell condensations. These observations were in agreement with the observations made on platforms with immobilized **4'**. In this case reduced sizes and quantities of pre-chondrogenic cell condensations were observed when compared to those with bound **4**. This indicates that the presence of TGF- β 1 was important and confirms the specific binding between peptide **4** and TGF- β 1.

Figure 5.5 – a) Fluorescence images corresponding to the functionalization of an azide-terminated SAM with **1** using a flat PDMS stamp and after incubation with **4** and **4'**. b) HACs morphology after 7 days on the different functionalized substrates by bright field microscopy. sTGF- β 1 refers to soluble (s) growth factor while bTGF- β 1 indicates bound (b) protein. c) Live-dead assay showing HACs seeded on different functionalized substrates.

After performing a Live/Dead assay after 7 days of culture (Figure 5.5c), the same cell morphologies were observed for the different cases while negligible cytotoxicity effects were observed. Despite cells were observed to have similar spreading behaviors independently of the surface nature, when no TGF- β 1 was present, we observed higher numbers of adhered cells on either surfaces functionalized with **1** or peptide when compared to the bare N₃ SAM, which is in agreement with literature.^[18]

From the analysis of the glycosaminoglycan (GAG) levels in HAC lysates after 7 days (Figure 5.6a), a significantly higher production of GAGs by HACs was observed in the case where HACs were seeded on platforms of **4** with bound TGF- β 1 when compared to the growth factor delivered in solution to cells seeded on glass or platforms with peptide only. Interestingly, the presence of growth factor in the medium did not result in an enhanced GAG production by cells seeded on glass when compared with a negative control lacking TGF- β 1. Taking into account that the relative induction of the GAG content for the highest value is only 1.4, this effect could be attributed to the short duration of the experiment. In addition, HACs were stained for GAGs after 7 days with Alcian blue. Whereas the staining of cells seeded on platforms functionalized with peptide **4** was of low intensity in the absence of TGF- β 1, cells cultured on platforms of peptide **4** that were incubated with TGF- β 1 presented an intense color after staining indicating a higher production of GAGs.

Figure 5.6 – a) GAG levels normalized by the total DNA content of HACs after 7 days of culture, expressed as relative induction with respect to an azide-terminated SAM. b) Alcian blue staining of cells seeded on **4** without b) or with c) TGF- β 1 immobilized. Error bars represent standard deviation (SD) of the mean of triplicates. * $p < 0.05$ compared to all cases after the ANOVA analysis of the data accompanied by a Tukey post-hoc test.

5.3 Conclusions

In conclusion, a fluorogenic platform was successfully used to detect the chemoselective immobilization of different cysteine-terminated peptides with relevant properties in terms of cell adhesion, regeneration and targeting. We initially detected the immobilization of a cellular adhesive peptide which maintained its cell adhesive properties as confirmed by the confinement of cells within the patterned regions.

In addition, after analyzing specificity and cross-reactivity in terms of protein binding, biological functionalities could be combined by co-immobilizing two different peptide sequences at different parts on a single substrate. The co-immobilized peptides were able to selectively capture their envisioned protein binding partners TGF- β 1 and BCII from a mixture. The resulting bifunctional platform contains both regeneration and targeting properties and represents a novel strategy for the functionalization of existing biomaterials.

5.4 Acknowledgments

C. Nicosia is gratefully acknowledged for the synthesis of **1** and **2**, for his help and fruitful discussions.

5.5 Experimental section

Materials

Commercial grade reagents were purchased from Sigma-Aldrich and were used without further purification unless stated otherwise and solvents were dried over molecular sieves. Recombinant human TGF- β 1 was purchased from R&D Systems as a 2 μ g carrier-free lyophilized powder from a 0.2 μ m filtered solution in acetonitrile and TFA. TGF- β 1 was reconstituted according to the protocol of the provider at a concentration of 20 μ g/mL and further diluted to the desired working concentration in PBS buffer. Collagen type II from bovine nasal septum was purchased as a powder and reconstituted as indicated by the provider to a stock concentration of 5 mg/mL and further diluted to the desired working concentration in PBS buffer. High-purity water (Millipore, R = 18.2 M Ω cm) was used. Electrospray Ionization (positive mode) high resolution mass spectra were recorded on a WATERS LCT mass spectrometer.

For information on the synthesis and characterization of the compounds **1** and **2** the reader is referred to the work of Nicosia et al. [9]

Automated solid-phase peptide synthesis

Fmoc-Rink amide MBHA resin (Multisynthetech GmbH, 50 mg, substitution 0.64 mmol/g) was placed in the reaction vessel of the fully-automated peptide synthesizer (Syro), and swollen for 30 min in 3 mL N-methyl-2-pyrrolidone (NMP) (Biosolve BV). After emptying the vessels the resin was washed with 1 mL piperidine (40%v)/NMP for 3 min and with 1 mL piperidine (20%v)/NMP for 12 min. The resin was washed with NMP (6 x 1 mL) prior to adding the following solutions to the vessel: Fmoc-amino acid (Multisynthetech GmbH, 4.5 equivalents with respect to the resin) dissolved in a solution of HOBt (Biosolve BV, 0.3 M) in NMP, HBTU (Multisynthetech, 0.29 M) in NMP and DIPEA (0.58 M) in NMP. The solid phase reaction was carried out for 80 min at room temperature and the vessel was emptied under reduced pressure. The deprotection and coupling cycles were repeated until the synthesis of the Fmoc protected peptide sequence was complete. The cleavage of the side chain protecting groups and the release of the sequence from the resin was achieved by manually incubating the resin with a mixture of 94% trifluoroacetic acid (TFA) (Acros), 2.5% H₂O, 2.5% 1,2-ethanedithiol (EDT) and 1% triisopropylsilane (TIS) overnight. The peptide was purified by several cycles of precipitation using cold diethyl ether (Acros), redissolved in water and lyophilized overnight. MS (ESI): m/z = 593.2 [M+H]⁺, (calcd. 592.6 for C₂₀H₃₆N₁₀O₉S) for CGRGDS. MS (ESI): m/z = 621.3 [M+H]⁺, (calcd. 620.7 for C₂₂H₄₀N₁₀O₉S) for MGRGDS. MS (ESI): m/z = 839.8 [M+H]⁺, (calcd. 839.0 for C₃₅H₅₈N₁₂O₁₀S) for CLPLGNSH. MS (ESI): m/z = 840.2 [M+H]⁺, (calcd. 839.0 for C₃₅H₅₈N₁₂O₁₀S) for CHNLGLPS. MS (ESI): m/z = 952.8 [M+H]⁺, (calcd. 952.1 for C₄₃H₆₅N₁₅O₈S) for CLRGRYW. MS (ESI): m/z = 952.4 [M+H]⁺, (calcd. 952.1 for C₄₃H₆₅N₁₅O₈S) for CWRGLRY.

Substrate and monolayer preparation

Microscope glass slides were used for monolayer preparation.^[12-13] The substrates were oxidized with piranha solution for 30 min (concentrated H₂SO₄ and 33% aqueous H₂O₂ in a 3:1 ratio; **(Warning! Piranha solution should be handled carefully: it has been reported to detonate unexpectedly)** and rinsed with water (MilliQ). After drying them in a flow of N₂, the substrates were immediately immersed in 0.1 vol.% 11-bromoundecyltrichlorosilane (ABCR) in dry toluene for 45 min at room temperature. Following monolayer formation, the substrates were rinsed with toluene to remove any excess of silanes, with ethanol and subsequently dried in a N₂ flow. Next, the bromo-functionalized substrates were immersed into a saturated solution of sodium azide (NaN₃) (Acros) in DMF for 48h at 70 °C. The substrates were thoroughly rinsed with MilliQ water and ethanol and dried in a N₂ flow.

PDMS preparation and μ CP experiments

Stamps were prepared by casting a 10:1 (v/v) mixture of poly(dimethylsiloxane) and curing agent (Sylgard 184, Dow Corning) against a silicon master. After overnight curing at 60 °C, the stamps were oxidized by oxygen plasma for 10 sec (power tuned at 50 mA) and subsequently inked by dropping the inking solution onto the stamp (1.5 mM **1** (in CH₃CN), 0.5 mM Cu(I)(CH₃CN)₄PF₆ and 0.5 mM TBTA (CH₃CN/EtOH = 2/1) (catalyst mixture). The inking solution was prepared by mixing 75 μ L of 2 mM solution of **1** in CH₃CN and 25 μ L of 2 mM of catalyst mixture). After inking the stamps for 4 min, the stamps were blown dry in a flow of N₂ and subsequently brought into conformal contact with the azide-functionalized substrate for 60 min. Fresh stamps were used for each printing following the same inking procedure. After removal of the stamp, the printed substrates were rinsed with ethanol, sonicated in acetonitrile for 2 min, rinsed again with ethanol, blown dry with N₂ and imaged using fluorescence microscopy. Printing of **4** was performed by inking PDMS for 30 min with a 10 mM solution of **4** in PBS. After removing the excess of the inking solution and drying the stamps in a flow of N₂, the stamps were brought into conformal contact with the substrate for 60 min.

Interfacial reaction with thiols

After immobilizing **1**, the platform was used for reacting with cysteine-terminated peptides from solution. Solutions of 10 mM of L-cysteine ethyl ester hydrochloride and peptides **3**, **3'**, **4** and **4'** were prepared in PBS at pH 7.4 while peptide **5** and **5'** were prepared in DMSO:PBS (3:1) and used for incubating the patterned substrates for 1 h. Substrates were then rinsed and briefly sonicated in PBS and in MilliQ water. For cell adhesion experiments a backfilling with a hydrophilic compound was necessary to improve the selectivity of the cell patterning. Therefore, after reaction with **3** some samples were incubated in a water solution containing 30 mM propargylhexa(ethylene glycol),^[9] 50 mM NaCl, 1 mM CuSO₄ and 40 mM ascorbic acid over night at room temperature.

TGF- β 1 and BCII binding and immunofluorescent staining

Upon functionalization of the surfaces with peptides, the substrates were incubated with solutions containing either 1 μ g/mL TGF- β 1 in PBS, 50 μ g/mL BCII in PBS or both together for 1 h. The substrates were then rinsed three times for 10 min with PBS buffer including 1 mM Tween-20 on the orbital shaker and finally rinsed with PBS buffer. Afterwards, substrates were incubated with PBS containing 1% BSA for 1 h and washed again three times as described above to block the

background against non-specific protein interactions. Protein patterns were then incubated with 200 μL of a solution of 5 $\mu\text{g}/\text{mL}$ of monoclonal anti-TGF- $\beta 1$ antibody (R&D systems) produced in mouse (mouse IgG_{2b} isotype, clone 141322) in PBS buffer including 1% BSA. After 1 h, the substrates were washed three times for 10 min with PBS buffer including 1 mM Tween-20, rinsed with water and dried with a flow of dry N₂. Consecutively, 200 μL of a solution (4 $\mu\text{g}/\text{mL}$) of anti-mouse IgG_{2b} labeled with Alexa Fluor® 546 produced in goat (Invitrogen) in PBS buffer (including 1% BSA) was used to incubate the samples for 1 h. The substrates were then washed again three times 10 min with PBS buffer including 1 mM Tween-20, rinsed with water and dried with a flow of dry N₂ and used for fluorescence microscopy imaging. For staining collagen II, polyclonal anti-collagen II $\alpha 1$ produced in rabbit (Abnova) was diluted 100 times in PBS buffer (including 1% BSA) and 200 μL of this solution were used to incubate each patterned sample for 1 h. Samples were washed as described above. Subsequently, a solution of 20 $\mu\text{L}/\text{mL}$ of Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Life technologies™) in PBS (including 1% BSA) was used to incubate the substrates for 1 h and they were washed and dried prior to inspection by fluorescence microscopy.

Cell adhesion studies

All substrates were sterilized prior to cell seeding with 70% ethanol and subsequently washed twice with PBS to remove any excess of ethanol. C2C12 (Mouse myoblast) cells were plated at a density of 50000 cells/cm² in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich), supplemented with 4.5 g/L glucose, L-glutamine and NaHCO₃; 1% (v/v) penicillin-streptomycin solution (with 10,000 units penicillin and 10 mg streptomycin/mL, Sigma-Aldrich) and cultured at 37 °C in a humidified 5% CO₂ atmosphere for 6 h. Subsequent to incubation, adhered cells were carefully washed twice with sterile PBS to remove loosely attached cells. Cells were fixed with a 4% formaldehyde solution in PBS for 10 min at room temperature and stained for F-actin with Alexa Fluor® 568 phalloidin (Invitrogen) following the protocol from the provider. Afterwards, the substrates were washed two times with PBS and water and then dried in a flow of N₂.

Cell differentiation studies

Macroscopically intact articular cartilage from femoral condyles was obtained from patients undergoing total knee replacement. Chondrocytes were isolated after overnight digestion with Collagenase type II and used at passage two. Chondrocytes were plated at a density of 5000 cells/cm² in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich), supplemented with 4.5 g/L glucose, 1% (v/v) penicillin-streptomycin solution (with 10,000 units penicillin and 10 mg

streptomycin/mL, Sigma-Aldrich), 50 µg/mL L-ascorbic acid-2-phosphate (AsAP), 100 µg/mL sodium pyruvate, 40 µg/mL proline, 1% of a mixture of 1.0 mg/mL recombinant human insulin, 0.55 mg/mL human transferrin (substantially iron-free), and 0.5 µg/mL sodium selenite (ITS supplement) and 10^{-7} M dexamethasone. 100 ng of TGF- β 1 was either supplemented to the medium or bound to the surfaces as indicated in the results and discussion section. Cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere for 7 days.

Live/Dead assay (LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells, Molecular Probes®) was performed according to the protocol from the provider.

GAG quantification and staining

For the quantification of GAGs in cell lysates, HACs were digested in a proteinase K (1 mg/mL) digestion buffer at 56 °C overnight. Briefly, the digestion buffer was freshly prepared by mixing 100 mL of Tris/EDTA buffer (6.055 g Tris and 0.372 g EDTA in 1 L water adjusting pH to 7.6 with HCl) with 18.5 g iodoacetamide. After warming this solution to 56 °C for 1 h, 1 mg pepstatin A and proteinase K were added to complete the digestion buffer. After digestion, GAG levels were quantified using 25 µL of each digested sample and adding 5 µL of 2.3 NaCl and 150 µL of dimethylmethylene blue (DMMB) solution (95 mL 0.1 M HCl, 2.04 g glycine, 2.37 g NaCl and 16 mg DMMB in 1 L water, adjusting pH to 3.0 using HCl). Immediately after adding the DMMB solution, the absorbance was recorded both at 525 and 590 nm and their quotient was used to determine the GAG concentration according to a standard curve ranging from 0 to 100 µg/mL of GAGs. GAG levels were corrected for the DNA content, which were measured using the CyQuant Cell Proliferation assay (Invitrogen).

For GAG staining, cells were fixed in 10% buffered formalin and stained using 0.5% Alcian Blue (Sigma Aldrich) in H₂O (pH set to 1 using HCl) for 30 minutes.

Fluorescence microscopy

Fluorescence microscopy images were recorded using an Olympus inverted research microscope IX71 equipped with a mercury burner U-RFL-T as light source and a digital Olympus DR70 camera for image acquisition. UV excitation ($300 \text{ nm} \leq \lambda_{\text{ex}} \leq 400 \text{ nm}$) and blue emission ($410 \text{ nm} \leq \lambda_{\text{em}} \leq 510 \text{ nm}$) was filtered using a Dapi Olympus filter cube. Green excitation ($510 \text{ nm} \leq \lambda_{\text{ex}} \leq 550 \text{ nm}$) and red emission ($\lambda_{\text{em}} \geq 580$) was filtered using a Olympus filter cube. All fluorescence microscopy images were acquired in air.

5.6 References

- [1] For reviews see for example: a) S. W. O'Driscoll, *The Journal of Bone & Joint Surgery* **1998**, 80, 1795. b) J. S. Temenoff, A. G. Mikos, *Biomaterials* **2000**, 21, 431.
- [2] For reviews see for example: a) G. P. Smith, V. A. Petrenko, *Chemical Reviews* **1997**, 97, 391. b) J. Scott, G. Smith, *Science* **1990**, 249, 386.
- [3] For reviews see for example: a) T. Aida, E. W. Meijer, S. I. Stupp, *Science* **2012**, 335, 813. b) H. Cui, M. J. Webber, S. I. Stupp, *Peptide Science* **2010**, 94, 1.
- [4] For examples see: a) R. N. Shah, N. A. Shah, M. M. Del Rosario Lim, C. Hsieh, G. Nuber, S. I. Stupp, *Proceedings of the National Academy of Sciences* **2010**, 107, 3293. b) K. Rajangam, H. A. Behanna, M. J. Hui, X. Han, J. F. Hulvat, J. W. Lomasney, S. I. Stupp, *Nano Letters* **2006**, 6, 2086. c) J. C. Stendahl, L. J. Wang, L. W. Chow, D. B. Kaufman, S. I. Stupp, *Transplantation* **2008**, 86, 478. d) A. Mata, Y. Geng, K. J. Henrikson, C. Aparicio, S. R. Stock, R. L. Satcher, S. I. Stupp, *Biomaterials* **2010**, 31, 6004. e) L. W. Chow, R. Bitton, M. J. Webber, D. Carvajal, K. R. Shull, A. K. Sharma, S. I. Stupp, *Biomaterials* **2011**, 32, 1574. f) M. J. Webber, J. Tongers, C. J. Newcomb, K. T. Marquardt, J. Bauersachs, D. W. Losordo, S. I. Stupp, *Proceedings of the National Academy of Sciences* **2011**, 108, 13438.
- [5] D. A. Rothenfluh, H. Bermudez, C. P. O'Neil, J. A. Hubbell, *Nature Materials* **2008**, 7, 248.
- [6] A. P. Hollander, I. Pidoux, A. Reiner, C. Rorabeck, R. Bourne, A. R. Poole, *The Journal of Clinical Investigation* **1995**, 96, 2859.
- [7] J. S. Dounchis, R. S. Goomer, F. L. Harwood, M. Khatod, R. D. Coutts, D. Amiel, *Journal of Orthopaedic Research* **1997**, 15, 803.
- [8] B. Johnstone, T. M. Hering, A. I. Caplan, V. M. Goldberg, J. U. Yoo, *Experimental Cell Research* **1998**, 238, 265.
- [9] C. Nicosia, J. Cabanas-Danés, P. Jonkheijm, J. Huskens, *ChemBioChem* **2012**, 13, 778.
- [10] M. Heggli, N. Tirelli, A. Zisch, J. A. Hubbell, *Bioconjugate Chemistry* **2003**, 14, 967.
- [11] a) D. Liu, Y. Xie, H. Shao, X. Jiang, *Angewandte Chemie International Edition* **2009**, 48, 4406. b) W.-S. Yeo, M. Mrksich, *Langmuir* **2006**, 22, 10816.
- [12] N. Balachander, C. N. Sukenik, *Langmuir* **1990**, 6, 1621.
- [13] D. I. Rozkiewicz, D. Jańczewski, W. Verboom, B. J. Ravoo, D. N. Reinhoudt, *Angewandte Chemie International Edition* **2006**, 45, 5292.
- [14] L. Yi, H. Li, L. Sun, L. Liu, C. Zhang, Z. Xi, *Angewandte Chemie International Edition* **2009**, 48, 4034.
- [15] H. A. Behanna, J. J. J. M. Donners, A. C. Gordon, S. I. Stupp, *Journal of the American Chemical Society* **2005**, 127, 1193.
- [16] M. Schnabel, S. Marlovičs, G. Eckhoff, I. Fichtel, L. Gotzen, V. Vécsei, J. Schlegel, *Osteoarthritis and Cartilage* **2002**, 10, 62.
- [17] a) A. M. DeLise, L. Fischer, R. S. Tuan, *Osteoarthritis and Cartilage* **2000**, 8, 309. b) H. J. Kwon, *Biochemical and Biophysical Research Communications* **2012**, 424, 793.
- [18] G. A. Hudalla, W. L. Murphy, *Langmuir* **2009**, 25, 5737.

Chapter 6

Spatiotemporal Regulation of hBMP-6 Growth Factor by Reversible Covalent Surface Tethering

Many growth factor delivery systems rely only on material degradation or growth factor diffusion through the material matrix to control the spatiotemporal availability of the growth factor in the vicinity of specific cell membrane receptors. In addition, for many cases the relationship between the tethering strategy and the biological activity of the growth factor remains unclear. Here we present a tethering strategy that allows the delivery of human bone morphogenetic protein 6 (hBMP-6), thereby controlling its spatial and temporal availability to induce cell signaling. The delivery is mediated by the hydrolysis of the siloxane linker. In addition, we demonstrate that this system benefits from the advantages of both the delivery of mobile growth factor and tethered growth factor at the same time, in terms of orientational freedom and presentation of confined high local concentrations of bioactive signaling proteins for the interaction with cells. Finally, we extrapolate these fundamental findings on Polyactive™ films with the aim of developing a system with a delivery strategy independent from the material bulk mechanical properties.

6.1 Introduction

Growth factors are endogenously present either bound to the extracellular matrix (ECM)^[1] or as soluble molecules secreted by cells. Bound growth factors, as opposed to their soluble analogs, provide control over the local concentration and protection from enzymatic degradation.^[2] Such bound growth factors are inactive to induce signaling and, characteristically, become active upon release from the ECM by the proteolytic removal of the signal peptide from the growth factor chain. In such a way, active, mature human bone morphogenetic protein 6 (hBMP-6), a member of the transforming growth factor- β (TGF- β) superfamily, is generated, yielding a disulfide-linked homodimeric protein.^[3] BMP-6 is known to regulate differentiation of various cells implicated in cartilage and bone formation during skeletal development and fracture repair.^[4] For example, BMP-6 plays a role in stimulating chondrocyte differentiation^[5] and has been involved in the development of embryonic kidney and urinary systems,^[6] as well as in liver growth and differentiation.^[7] In the case of keratinocyte differentiation, variable responses to BMP-6 doses have been observed, alternating between stimulation and repression of the epidermal cells to control cell proliferation and epidermal thickness.^[8] BMP-6 also strongly induces alkaline phosphatase activity in osteoblasts and myoblasts.

Throughout the last decades many efforts have been devoted to develop methods to immobilize growth factors aiming to mimic the endogenous growth factor delivery system and thereby improving the performance of biomaterials in tissue engineering and therapeutic applications.^[9] For example, hBMP-2 has been mixed with biomaterials to be released by diffusion and thereby activating cell differentiation.^[10] Other approaches have been focusing on linking ECM elements, such as heparins,^[11] or mimics thereof,^[12] to biomaterials in order to position growth factors on their surfaces. Alternatively, direct coupling of growth factors has also been pursued using non-covalent His-tag technology^[13] or covalent EDC or imine chemistry.^[14] Despite this progress, it remains a challenge to develop an immobilization method that allows spatiotemporal regulation of growth factor concentration and relate it to cell response.

In this chapter we investigate the *in vitro* potential of the hydrolytic removal and subsequent signal induction by growth factors that were initially immobilized onto biomaterials. We exploit the reversible properties of imine and siloxane bonds as a linkage between the surface and the growth factor. Such reversible chemistry has been successfully applied for the controlled formation of for example gels, polymers and functional surfaces.^[15] Among reversible covalent bonds, the imine and siloxane bonds are especially attractive as these bonds can be hydrolyzed

under physiological conditions.^[16] For example, Sreenivasachary et al. have shown the release of biologically active molecules that were initially entrapped into a hydrogel formed by reversible covalent bonds.^[17] Differences in the percentage of released molecules as well as the duration of that release were observed to be mainly dependent on the percentage of the occluded compound in the solvent of the gel. Reinhoudt et al. and Giuseppone et al. have used reversible imine bonds to fabricate various mixed surface patterns and gradients to control the interfacial composition.^[18] Given the great potential of adopting reversible covalent chemistry for constructing and functionalizing adaptive materials and surfaces, to date this type of chemistry has not been explored for the spatiotemporal delivery of growth factors. Herein we demonstrate the effect on cell response when adopting a reversibly covalently tethered growth factor on a commercially available biomaterial.^[19] We demonstrate that a confined reservoir of active hBMP-6 is created upon hydrolysis and that such high local concentration of GF allows for the efficient interaction with specific cell membrane receptors.

6.2 Results and discussion

6.2.1 hBMP-6 immobilization on siloxane surfaces

To establish the method of releasing immobilized hBMP-6 upon hydrolysis, model studies on glass slides were performed. To this end, reactive epoxide-terminated monolayers were prepared on glass substrates by vacuum deposition of 3-glycidylpropyltrimethoxysilane (GPTMS).^[20] An advancing contact angle value of $55^\circ \pm 2^\circ$ was recorded while a film thickness of $0.9 \text{ nm} \pm 0.2 \text{ nm}$ was estimated using ellipsometry, which is indicative of successful monolayer formation and in agreement with literature (Table 6.1).^[20a]

Monolayer	$\Theta_a (^\circ)$	$\Theta_r (^\circ)$	Ell. Thickness (nm)	N (XPS)
GPTMS	52 ± 2	40 ± 5	0.9 ± 0.2	Not detected
GPTMS + hBMP6	64 ± 5	47 ± 2	1.9 ± 0.2	1.0 ± 0.3
PGMA	60 ± 1	52 ± 4	80 ± 3.1	Not detected
PGMA + hBMP6	65 ± 1	41 ± 4	87 ± 2.1	0.7 ± 0.2

Table 6.1 – Table summarizing the advancing (Θ_a) and receding (Θ_r) water contact angle values, ellipsometric thicknesses and selected XPS data of the two epoxide-terminated linkers before and after the immobilization of hBMP-6.

Subsequently, hBMP-6 was immobilized on the epoxide-functionalized substrates by reactive microcontact printing (μ CP) (Figure 6.1) via the nucleophilic ring-opening reaction by the primary amine groups of the 12 lysine residues localized at the outer part of the growth factor. Consequently, the protein was covalently tethered to the modified substrates in a random fashion. Depending on the features of the PDMS stamp (i.e. either flat or patterned) either a full layer or a patterned microarray of tethered growth factors was fabricated.

Figure 6.1 – Schematic overview of the preparation of epoxide-terminated glass substrates with two linkers of different length: GPTMS (top) and PGMA (bottom) and subsequent immobilization of hBMP-6 (accessible lysine residues are indicated in dark grey) by reactive μ CP.

Following protein immobilization an increase in the advancing contact angle value was observed. The immobilization of hBMP-6 was further confirmed by the appearance of a N1s peak in the XPS spectrum indicating the presence of ca. 1% N while a 1 nm increase in film thickness was estimated using ellipsometry. Inspection of the monolayers using atomic force microscopy (AFM) (Figure 6.2b) showed clear patterns of hBMP-6, which were however not completely filled with the growth factor. In the AFM phase image, individual and clustered dots of light contrast were clearly observed in a background of dark contrast and similar to

the intensity of the background of the patterns (Figure 6.9c). The dots occupied 70% of the total area of the pattern and a height difference of 0.5 nm was measured between the protein patterns and the background. These results indicate that the growth factor is tethered in a lightly (ca. 70%) packed, single layer configuration. As a next step an immunoassay was performed to analyze the structural integrity of the immobilized hBMP-6. The recorded fluorescence micrograph (Figure 6.2a) shows clearly that the fluorescently labeled secondary antibody was selectively immobilized on the hBMP-6, indicating that the immobilization occurred under retention of the secondary structure.

Figure 6.2 – a) Fluorescence microscopy image (inset shows the intensity profile) of immunostained μ CP patterns of hBMP-6 (100 μ m diameter dots and 100 μ m spacing) on an epoxide-terminated surface using a passive background of NH_2 -PEG. b) Atomic force microscopy (AFM) phase image of hBMP-6 micro-patterned (10 μ m diameter dots and 5 μ m spacing) on the same surface without using a passive background and c) zoom in of the phase image.

To study the activity of hBMP-6, cell culture experiments were performed using murine preosteoblastic KS483 cells as a working model. These mesenchymal precursor cells constitute a well-established *in vitro* model system to study the ability of BMP-6 to initiate differentiation into mature mineralizing osteoblasts.^[22] KS483 cells were seeded for 24h on an epoxide-terminated surface with and without tethered hBMP-6 and on a control surface (glass) with and without soluble hBMP-6. Cell morphology was observed using fluorescence microscopy

after staining the actin cytoskeleton. All surfaces showed similar numbers of adhered cells that were similarly spreading (see Figure 6.3).

Figure 6.3 – Effect of the linker nature and the tethered hBMP-6 on cell adhesion and spreading observed 24 h after seeding, studied via actin and nucleus staining of KS483 cells on a, d) a glass control, b, e) a GPTMS SAM and c, f) a PGMA layer both without hBMP-6 (a, b, c) and with hBMP-6 (d, e, f). The growth factor added for the glass control is presented in suspension contrary to the one bound to the GPTMS or PGMA layers. The scale bar is 200 μm. Insets show fluorescence microscopy image of cells after performing a Live-Dead assay. d) The cell surface area was quantified and normalized by the cell number after 24 h for the different cases. # $p < 0.05$, ## $p < 0.01$ when compared to all other platforms and * $p < 0.05$, ** $p < 0.01$ when comparing cases with or without hBMP-6 for a given platform.

As cells spread already very fast on glass in the absence of hBMP-6, it was not surprising to find that soluble or tethered hBMP-6 did not lead to significant changes in spreading kinetics. After 7 days of culture, cells had formed a confluent layer and a negligible cytotoxicity of the surfaces was observed (Inset in Figure 6.3e).

6.2.2 Biological activity of hBMP-6 using siloxane surfaces

hBMP-6 treatment of KS483 cells induces osteoblastic differentiation as characterized by alkaline phosphatase activity. If the siloxane bond, which connects the hBMP-6 to the surface, is hydrolyzed to release hBMP-6 into the cell medium, alkaline phosphatase (ALP) activity would be observed. Therefore two complementary assays were performed to verify the activity of hBMP-6. KS483 cells were cultured for 4 days on a glass substrate with tethered hBMP-6 on the left half of the substrate while the other half was passivated using

polyethyleneglycol. After immunohistochemically staining for ALP, a clear contrast was observed between the two parts of the surface coinciding with the contrast observed after immunostaining the same substrate for hBMP-6 (Figure 6.4). These results indicate that only in the areas where functional growth factors are available ALP production is induced.

Figure 6.4 – Study of early osteogenesis in KS483 cells by tethered hBMP-6. a) Fluorescence microscopy image of hBMP-6 immobilized on half of the substrate after immunofluorescence staining. b) Immunohistochemical staining of ALP as an osteogenic early marker, which is produced after 4 days by KS483 cells (insets shows intensity profiles). c) ALP activity normalized by the total DNA content of KS483 cells after 7 days of culture, expressed as relative induction to a glass control in the presence of either soluble (s), bound (b) or denatured bound (db) hBMP-6. d) Dose response of KS483 cells cultured for 7 days in the presence of increasing concentrations of hBMP-6 in solution and two different amounts of bound hBMP-6, i.e. a full layer (100%) of hBMP-6 and a μ CP patterned layer of hBMP-6 (23% of the coverage in comparison to the full layer). * $p < 0.05$ and ** $p < 0.01$.

To compare the biological activity of hBMP-6 tethered to the siloxane surface with hBMP-6 supplemented in solution, the ALP activity in KS483 cells after 7 days of culture was normalized to the DNA content of the total number of cells in contact with the substrate and expressed as relative induction values compared to a glass control (Figure 6.4c). Interestingly, cells seeded on a layer of tethered hBMP-6 produced significantly more ALP when compared to all other cases (Figure 6.4c). Controls where tethered growth factors were partly denatured prior to cell seeding showed a significant loss of ALP activity indicating that hBMP-6 is involved in the observed ALP activity. While cells cultured on a glass substrate in the presence of a comparable amount of soluble hBMP-6 showed a 2-fold induction in ALP activity in comparison with the negative glass control, cells seeded on a layer of tethered hBMP-6 showed a 4-fold induction. Next, cells were seeded on a reactive epoxide-terminated surface in the presence of soluble hBMP-6. While in this case a lower ALP activity was observed when compared to surfaces with tethered hBMP-6, a higher ALP activity was found when compared to either glass surfaces in the presence of hBMP-6 or when epoxide surfaces were used without addition of hBMP-6. Taken together, these results indicate that tethering hBMP-6 is a more effective strategy to achieve ALP production than addition of soluble hBMP-6. We tentatively attribute this result to the possibility of releasing hBMP-6 upon hydrolysis which will yield locally mobile hBMP-6. Hence cells can sense a *higher local concentration* of active hBMP-6 facilitating the interaction with the BMP receptors at the cell membrane.^[9a]

In an additional experiment, the concentration effect of tethered and soluble hBMP-6 on the ALP activity was studied (Figure 6.4d). A large increase in ALP activity was observed when increasing the concentration of soluble hBMP-6 from 100 ng/mL to 200 ng/mL. Further increasing the concentration of soluble hBMP-6 to 400 ng/mL however did not lead to a further increase in ALP activity indicating that 200 ng/mL is sufficient to saturate the cellular receptors. This value is in the same range as found in the case of related BMPs.^[10b, 23] Interestingly, the ALP activity of cells seeded on surfaces completely covered with tethered hBMP-6 compares favorably to the case when soluble hBMP-6 at 200 ng/mL or higher was used. This indicates that through the tethering strategy a sufficient amount of active hBMP-6 is available to achieve the maximum ALP activity. The hBMP-6 dose can be easily tuned by reducing the surface concentration to 23% by immobilizing hBMP-6 using a featured μ CP stamp resulting in a decreased ALP activity.

6.2.3 Release and local confinement of hBMP-6

To address the key question whether cell signaling occurs through tethered and immobile hBMP-6 or released and mobile hBMP-6, several experiments were performed, which are described in this section.

The rate of the hydrolysis of the siloxane bond is expected to decrease when using a long brush-type linker. Such a linker is less prone to hydrolysis because the accessibility of the water molecules to the hydrolyzable bond is restricted.^[24]

To this end, layers of poly(glycidyl methacrylate) (PGMA) brushes on glass were prepared.^[24a, 25] Briefly, PGMA brushes were produced through the reaction of an atomic transfer radical polymerization (ATRP) initiator (2-bromo-2-methylpropionyl bromide) with the terminal amine moieties of a (3-aminopropyl)triethoxysilane (APTES) monolayer. Subsequently, the immobilized initiator was immersed in an oxygen-free solution of glycidyl methacrylate as the ATRP monomer for 2 h to yield a PGMA layer of 80 nm thickness, which was measured using ellipsometry (Figure 6.1a). In order to tether the growth factor, the same approach was followed as in the case of the short epoxide GPTMS linker (Figure 6.1b). Successful tethering of hBMP-6 onto the PGMA layers was confirmed using contact angle, ellipsometry and XPS measurements (Figure 6.1a). Fluorescence micrographs in Figure 6.4b show μ CP patterns of immunostained hBMP-6 that were of similar quality as compared to the hBMP-6 patterns on GPTMS surfaces (Figure 6.2a and 6.5b). However, after quantifying the immunofluorescence intensity of the images at an initial time point ($t = 0$ h), the intensity of the microarrays of hBMP-6 immobilized onto the long linker was slightly higher when compared to immobilization of hBMP-6 on the short linker. This observation is in agreement with ellipsometry measurements showing a higher increase in film thickness upon hBMP-6 immobilization onto the long linker. We next studied the temporal release of hBMP-6 during 15 days using an enzyme-linked immunosorbent assay (ELISA) to quantify hBMP-6 present in the culture medium (Figure 6.5a) while the remaining tethered hBMP-6 was quantified using immunostaining (Figure 6.5b and c). Within the first 24 h a burst release was observed in the case of the short (GPTMS) linker reaching already a therapeutic level of 100 ng/mL.^[23, 26] After this initial period, a linear release in time was observed until a plateau was reached after 7 days. In the case of the long (PGMA) linker within the first 4 days only a small amount of hBMP-6 was released, which could be attributed to hBMP-6 physisorbed on the surface of the polymeric brush that was slowly released in the culture medium via diffusion. However, after this initial period, an increase in detected hBMP-6 was found. After 5 days the released hBMP-6 reached a therapeutic value of 100 ng/mL.

Figure 6.5 – Quantification of hBMP-6 released via hydrolysis of the siloxane bond in culture medium at 37 °C and 5% CO₂ over a period of 15 days using a) an ELISA assay both without (solid lines) and with (dashed lines) 5000 cells/cm² and b) fluorescence microscopy images of immunostained micro-patterned hBMP-6 (100 x 100 μm dots) on a passive background on a GPTMS (left) or PGMA layer (right) at different incubation time points. Scale bars indicate 200 μm. c) The intensity was quantified with the software ImageJ on all the full dots of a microarray of at least three images corresponding to a same time point. d) Effect of the local confinement of hBMP-6 on the production of ALP by KS483 relative to a glass control. ** $p < 0.01$.

When the ELISA assay was performed when cells were seeded on the surfaces, remarkable differences were observed. For hBMP-6 immobilized onto the short (GPTMS) linker the formation of a confluent cell layer (reached at day 4) reduced the amount of released hBMP-6 protein close to the detection limit. This indicates that a locally confined reservoir of hBMP-6 was formed underneath the cell layer. However, when cells were seeded on surfaces of hBMP-6 immobilized onto the long (PGMA) linker, the detected release profile was found similar to the case without cells at early time points. Reduced cell adhesion and spreading is observed on PGMA layers (Figure 6.12a and e, *vide infra*). Only after day 7, upon forming a confluent cell layer, lower quantities are detected in the medium in the presence of cells. These results indicate that on PGMA a poorer confinement of the released hBMP-6 was achieved.

Quantification of the fluorescence intensity (Figure 6.5c) of the recorded micrographs of the immunostained hBMP-6 microarrays (Figure 6.5b) revealed the global loss of protein from the surface. These results confirmed our hypothesis that the hydrolysis-driven release of hBMP-6 from the long (PGMA) linker is slower when compared to the short (GPTMS) linker. Cells seeded on the long linker will be exposed to a high local concentration of surface bound, immobile hBMP-6 while cells seeded on the short linker will be exposed to a high local concentration of confined but mobile hBMP-6, as hypothesized in Figure 6.6.

Figure 6.6 – hBMP-6 presented a) in soluble form will freely interact with its cognate receptors, activate down-stream responses and be partially recycled for further interactions with yet non-metabolized growth factor. Tethered hBMP-6 via b) a hydrolyzable siloxane bond can be released, thereby creating a high local concentration of hBMP-6 in a reservoir underneath the cell layer. Though slightly delayed with respect to soluble growth factor, stimulation will be initiated upon receptor interaction with the mobile hBMP-6. Finally, tethered hBMP-6 via c) a buried hydrolyzable siloxane linker covered by PGMA brushes, can initiate cell signaling upon release of a therapeutic dose.

In order to verify the importance of the mobility of hBMP-6 for effective interaction with the BMP receptors, the ALP activity of KS483 cells was determined after 7 days. KS483 cells were seeded either in contact or physically separated from the surface using a transwell assay (Figure 6.4d). The ALP activity was higher in the case of soluble hBMP-6 as compared to the negative controls using glass while no effect on the ALP activity was observed in the presence of the transwell system. Small osteoinductive properties of cells seeded on control GPTMS surfaces were confirmed. Interestingly, in the case of the transwell assay the ALP activity was significantly lower in comparison with the case when cells were seeded in direct contact with the surface. In addition, when cells were seeded in contact to hBMP-6 tethered to the PGMA surface, a lower ALP activity was observed in comparison with hBMP-6 tethered to the GPTMS surface. These results demonstrate that cell receptors can also interact with soluble growth factor and that an increased ALP activity at an early stage requires hBMP-6 in a mobile state and locally confined underneath the cell layer.

6.2.4 Spatiotemporal bioactivity of released hBMP-6

To support the finding that the biological activity of the hBMP-6 is strongly dependent on the mobility of hBMP-6 in close proximity to the cells, it is important to note that our findings are not specific to KS483 cells. C2C12 cells, an acknowledged cellular model to study differentiation into bone cells and which also possesses BMP receptors, responded similarly. To further study the spatiotemporal biological activity of released hBMP-6, the luciferase activity was measured of C2C12 cells stably transfected with a BMP-responsive element (BRE) fused to the firefly luciferase reporter gene (BRE-Luc cells) (Figure 6.7a).^[27]

Figure 6.7 – a) Luminescence of C2C12 cells transfected with BRE-Luc, measured at different time points relative to a background using the same surface without hBMP-6. Inset shows the luminescence results at day 7 from an independent experiment. Gene expression analysis at days 1, 3 and 7 of b) RUNX2 and c) OCN was determined via the $2^{-\Delta\Delta C_t}$ method^[28] using the expression on day 1 on glass as a reference. * $p < 0.05$ and ** $p < 0.01$.

To avoid effects related to material stiffness, differences in luciferase expression relative to the background using the same surface in the absence of hBMP-6 were compared (Figure 6.7a). From these results, it was concluded that luciferase

activity was delayed to day 2 when cells were seeded on surfaces with tethered hBMP-6 using the short (GPTMS) linker in comparison to soluble hBMP-6, whereas in the case of the surfaces onto which hBMP-6 was tethered using the long (PGMA) linker a significant rise in luciferase activity could be observed only at day 7.

In addition, the mRNA expression levels of the osteoblast-specific runt-related transcription factor 2 (*RUNX2*) and osteocalcin (*OCN*) genes were analyzed (Figure 6.7b and c).^[29] Upregulation of *RUNX2* was clearly observed already one day after cell seeding, particularly in the cases of soluble hBMP-6 and of tethered hBMP-6 using the short linker. In contrast, *OCN* expression occurred only after day 3 in those cases. The observation that *RUNX2* expression precedes *OCN* expression is in agreement with literature and indicates that hBMP-6 is biologically active as expected.^[29-30] When comparing the gene expression behavior resulting from surfaces using the short and long linkers used for tethering hBMP-6, genes were typically expressed earlier on surfaces using the short linker. These results confirm that hBMP-6 tethered via the short linker activates the BMP pathway earlier in comparison to the long linker but later in comparison to soluble hBMP-6. Taken together, these results confirm that interaction with the cell receptors is only possible after releasing hBMP-6. The results are in agreement with the ELISA studies (Figure 6.5a) showing that in the case of the long linker, a substantial therapeutic amount of released hBMP-6 is achieved only after 5 days in culture, whereas for the short linker already after 1 day the therapeutic range was reached. Therefore, we believe that the biological activity of hBMP-6 growth factors is strongly related to their release from the surface.

6.2.5 hBMP-6 on Polyactive™ films

To demonstrate that our tethering strategy is applicable to biomaterials Polyactive™ (Figure 6.8a) was selected as a representative biomaterial.^[19] We hypothesize that if we are able to tune the release and biological activity of growth factors by functionalizing the surface of the biomaterial, the delivery will be independent from the degradation properties of the bulk material. To test the hypothesis, polymer films of ca. 100 µm thickness (Figure 6.8b) were used for surface activation using O₂-plasma (Figure 6.8c) to generate various oxygen-containing species (e.g. hydroxyl, carboxyl, peroxy, carbonyl or ether).^[31] Two routes have been followed that take advantage of reversible chemistry. First, a siloxane-bound GPTMS linker was installed using the hydroxyl groups on the oxidized polymer surface. However, hBMP-6 can potentially be immobilized via the epoxides but also via remaining aldehyde groups on the surface (Figure 6.8c, II). Therefore, in a second set of films hBMP-6 was immobilized directly onto the

aldehyde groups to yield a reversible imine bond between the growth factor and the polymer surface (Figure 6.8c, I). A control surface with irreversibly bound growth factor was obtained by reducing the imine bond to generate a stable, irreversible, secondary amine linkage under physiological conditions (Figure 6.8c, III). Reducing agent NaBH_3CN is sufficiently mild in the presence of biomolecules as previously reported.^[32]

Figure 6.8 – a) Molecular structure of the block-copolymer Polyactive™ (PA), consisting of a hydrophilic block of poly(ethylene glycol terephthalate) (PEGT) and a hydrophobic block of poly(butylene terephthalate) (PBT). b) 100 μm thick PA films were prepared by casting a solution of 3.4 wt% PA in chloroform overnight. c) Schematic procedure of the surface functionalization of PA films after pre-activation using O_2 plasma, with GPTMS. hBMP-6 is subsequently immobilized via reactive μCP on the activated film via the reaction between the primary amine groups (accessible lysine residues in dark grey) and aldehyde groups forming an imine bond (I), which is further reduced in order to increase its stability towards hydrolysis (II) or on the GPTMS (III) with unreacted aldehyde groups also available to tether the growth factor as in I.

The functionalization of the activated PA with GPTMS was characterized with contact angle measurements and XPS (Table 6.2).

Sample	Θ_A (°)	Θ_R (°)	C (XPS)	O (XPS)	Si (XPS)
PA	52 ± 1	15 ± 3	N/A	N/A	N/A
PAOx	38 ± 1	15 ± 1	70.3 ± 1.4	29.2 ± 0.7	0.5 ± 1.0
PAOx-GPTMS	53 ± 1	15 ± 1	60.3 ± 1.9	33.3 ± 0.2	6.4 ± 1.8

Table 6.2 – Advancing (θ_a) and receding (θ_r) water contact angle values and selected XPS data of PA films at different stages of functionalization.

Upon oxidizing the PA surface the advancing contact angle decreased with respect to the native PA films while upon installation of GPTMS the contact angle increased. Upon reaction with GPTMS, the presence of elemental Si was detected in the XPS spectra, while deconvoluted XPS spectra of the C1s region (Figure 6.9) showed an increase of the peak at 286.35 eV, which can be attributed to ether bonds (C-O).^[33]

Figure 6.9 – X-ray photoelectron (XPS) spectra of the C1s region for a PA film after the O₂-plasma treatment (left) and after introducing the epoxide-terminated silane (right). 1 was assigned to aromatic and aliphatic carbon bonds (C-C), 2 to ether bonds (C-O) and 3 due to its broadness could be assigned to ester (O-C=O), aldehyde (H-C=O) and ketones (C=O).

In addition, the peak centered at 288.52 eV, mainly attributed to ester bonds (O-C=O) that are present in native PA films, was broadened during plasma treatment indicating other carbonyl-containing species such as

aldehyde/ketone ($\text{C}=\text{O}$) groups, which typically have bands in the range of 287.1-288.1 eV. In the FT-IR spectrum an O-H stretching band appeared after plasma treatment of the native PA films (inset in Figure 6.10a).

Figure 6.10 – a) FT-IRRAS spectra of PA films at different stages of functionalization. The inset corresponds to the region associated to the O-H stretching. b) Ratio between the intensity of the asymmetric C-O-C and the C=O stretching bands.

This band was significantly reduced upon reaction with GPTMS indicating the conversion of O-H bonds into O-Si bonds. Also, the ratio of the intensities of the asymmetric C-O-C and the C=O stretching bands (Figure 6.10b) was inverted after the plasma treatment with a higher intensity for the carbonyl peak. Upon reaction with GPTMS, the ratio was inverted back with a slightly higher intensity for the C-O-C stretching band corresponding to the epoxide ring of the linker. Taken together, these results are indicative of successful activation of the PA films with groups that allow the tethering of hBMP-6 as envisioned.

hBMP-6 microarrays on the activated PA films were prepared via reactive μCP as described before. Visualization of the microarrays was possible after immunostaining the films using fluorescence microscopy (Figure 6.11b).

Figure 6.11 – a) Quantification of hBMP-6 released from the Polyactive™ films, via hydrolysis of different bonds (i.e. the siloxane of GPTMS (PAOx-GPTMS-bhBMP-6), imine (PAOx-bhBMP-6) and the amine resulting of the reduction of the imine (PAOx-rbhBMP-6)) in culture medium at 37 °C and 5% CO₂ during a period of 7 days of b) the fluorescence intensity images of immunostained microarrays of hBMP-6 (100 x 100 μ m dots) with passive background. The intensity was quantified using ImageJ on all the full dots of the microarray of at least three images corresponding to a same time point. Scale bars indicate 200 μ m. c) Luminescence of C2C12 BRE-Luc at different time points relative to the background. * $p < 0.05$ and ** $p < 0.01$.

These results show that hBMP-6 was successfully immobilized via the different tethering strategies. Quantifying the fluorescence intensity of hBMP-6 bound by each linking strategy upon exposure to cell culture medium confirmed the possibility to release hBMP-6 (Figure 6.12a). For the GPTMS linker a significant decay of fluorescence intensity was observed within 2 days which is slower in comparison when applying this linker on glass substrates. Although a decrease in the fluorescence intensity was also observed in the case in which hBMP-6 was immobilized via an imine bond, the release was slower than for the GPTMS linker. When the imine bond was reduced using NaBH₃CN, the fluorescence intensity remained nearly constant over 7 days indicating that hBMP-6 was irreversibly linked to the surface.

Prior to the analysis of the biological activity of released hBMP-6 from PA films, cell adhesion and spreading properties after 1 day on these films were assessed with and without hBMP-6. Plasma treated PA films exhibited improved cell

adhesion and spreading in comparison to native PA films as visualized after actin staining (Figure 6.12a, e, b and f), which is in agreement with literature.^[34]

Figure 6.12 – Effect of the linker nature and the tethered hBMP-6 on cell adhesion and spreading observed 24 hours after seeding, studied via actin and nucleus staining of KS483 cells on a, e) a PA control, b, f) oxidized PA, c, g) oxidized and reduced PA and d, h) PA functionalized with GPTMS both without hBMP-6 (a, b, c, d) and with hBMP-6 (e, f, g, h). The growth factor added for the PA control is presented in suspension while for the rest of cases it is bound to the platform. The scale bar is 200 μm. e) The cell surface area was quantified and normalized by the cell number after 24 hours for the different cases. # $p < 0.05$, ## $p < 0.01$ when compared to all other platforms and * $p < 0.05$, ** $p < 0.01$ when comparing cases with or without hBMP-6 for a given platform.

No differences in cell adhesion behavior were found for plasma treated PA films with imine-tethered hBMP-6. Similar cell adhesion behavior was found on these films after reduction of the imine bond (Figure 6.6c and g). However, a significant enhancement of cell adhesion was observed after the formation of a GPTMS linker on the PA films, irrespective of tethered hBMP-6 (Figure 6.6d and h). On PA films, the presence of tethered hBMP-6 did not show any effect on enhancing cell adhesion and spreading.

In a next set of cell experiments, the biological activity of the released hBMP-6 from PA films using the different linkers was verified using C2C12 BRE-Luc cells (Figure 6.11c). The luciferase activity was increased when substantial quantities of hBMP-6 were released and available to the cells. For example, in the case of the GPTMS linker, a significant released amount of hBMP-6 after 2 days coincides with the observed increase in luciferase activity. Since the release of hBMP-6 using imine-tethering is delayed to 5 days of culture, significant luciferase activity on those films is only observed after 5 days, which was suppressed by reducing the

imine bond. These results confirm that early cell differentiation is initiated by releasing hBMP-6 from the biomaterial surfaces, in agreement with the model studies on glass.

6.3 Conclusions

We have demonstrated the use of several reversible linkers for tethering hBMP-6 to model surfaces as well as biomaterials. Tethered hBMP-6 retained its functional structure and upon release from the surface its biological activity was shown. We established a relation between the hydrolytic susceptibility of the bonds, such as siloxane and imine bonds, and early cell differentiation. Important consequence of using reversible linkers for tethering growth factors is the advantage to deliver mobile growth factors in a confined space at the cell-material interface. We anticipate that this concept can be used to improve the design of a new generation of delivery systems.

6.4 Acknowledgements

E.B.M Landman is gratefully acknowledged for her help and fruitful discussions.

6.5 Experimental section

Materials

Commercial grade reagents were purchased from Sigma-Aldrich unless stated otherwise and were used without further purification. Solvents were dried over molecular sieves. Recombinant human BMP-6 (hBMP-6) was purchased from R&D Systems as a carrier-free, 0.2 μm filtered solution (0.04 mg/mL) in acetonitrile and trifluoroacetic acid (TFA) and further diluted to the desired working concentration in PBS buffer. Water used was of high-purity (Millipore, R = 18.2 M Ω cm).

Preparation of (3-glycidyloxypropyl)trimethoxysilane (GPTMS)

Either single side polished p-type Si(100) wafers (Okmetic) or microscope glass slides (76 x 26 mm, Menzel-Gläzer) were cut into pieces of approximately 1 cm². The substrates were immersed in piranha solution (3:1 concentrated H₂SO₄ / 30% H₂O₂) for 30 min (**Warning!** Piranha solution should be handled with caution. It has been reported to detonate unexpectedly) to install silanol groups. After rinsing several times with water, the substrates were dried under a flow of dry N₂ and immediately taken into the N₂ filled glove box and immersed in a 1 v/v% GPTMS toluene solution for 16 h. Subsequently, the substrates were removed from

solution and rinsed several times with toluene and ethanol. To remove unbound deposited materials, the substrates were placed in a beaker with ethanol in an ultrasonic bath for 5 min, rinsed with ethanol and dried under a flow of dry N₂. The samples were immediately used for further experiments.

Preparation of poly(glycidyl methacrylate) (PGMA) layers

These substrates were prepared following a modified literature procedures.^[24a, 25] Either single side polished p-type Si(100) wafers (Okmetic) or microscope glass slides (76 x 26 mm, Menzel-Gläser) were cut into pieces of approximately 1 cm². The substrates were activated in piranha solution (3:1 concentrated H₂SO₄/ 30% H₂O₂) for 30 min (**Warning!** Piranha solution should be handled with caution. It has been reported to detonate unexpectedly) and then rinsed several times with water. The substrates were dried under a flow of dry N₂ and immediately placed at the bottom of a dessicator with a vial containing 100 µL of (3-aminopropyl)-triethoxysilane (APTES). After evacuating the desiccator with a diaphragm vacuum pump for 10 minutes, the substrates were left in an atmosphere of APTES vapor overnight. Subsequently, the substrates were thoroughly rinsed with ethanol and dried under a flow of dry N₂.

The cleaned substrates were then immersed into a solution of triethylamine in 40 mL of dry toluene. At 0 °C, 300 µL of ATRP initiator 2-bromo-2-methylpropionyl bromide were added dropwise and left to stir for 1.5 h under a N₂ atmosphere. After rinsing the substrates with toluene, ethanol and water, the substrates were dried under a flow of dry N₂.

Then a solution of glycidyl methacrylate (5 mL, 36.7 mmol), 2,2'-dipyridyl (bpy) (141 mg, 0.904 mmol) in methanol (4 mL) and water (1 mL) was prepared and purged with argon for 30 min in a round-bottom flask sealed with a septum. In another flask, CuCl (36.4 mg, 0.368 mmol) and CuBr₂ (3.9 mg, 0.017 mmol) were added together and also purged with argon for 30 min. Monomer, ligand, and catalyst were then combined, stirred and degassed for another 30 min to facilitate the formation of the organometallic complex. Subsequently, this solution was transferred with a degassed syringe into an argon-degassed flask containing the functionalized substrates with the initiator. Upon addition, the ATRP reaction was allowed to proceed for 2 h at room temperature after which the substrates were removed from the flask. After thorough rinsing with water, ethanol, a gentle sonication in dichloromethane, rinsing with dichloromethane, the substrates were dried under a flow of dry N₂. The samples were immediately used of further experiments.

Preparation and functionalization of Polyactive™ films

Polyactive™ (PA) 1000PEG70PBT30 (M_w of PEG is 1000 g/mol and a 70/30 ratio of PEG/PBS) was a gift from PolyVation (The Netherlands). Thick PA-films were made by casting a clear 3.4 wt% PA solution in chloroform (40 mL) onto a glass petri dish ($\varnothing = 14$ cm). After standing overnight at room temperature, the as-dried cast film further dried in a vacuum oven at 60 °C. An average thickness of 100 μm was found. The film was then cut into pieces of approximately 1 cm^2 and used for immunostaining, cell adhesion and activity experiments.

Thin PA-films were made by spin coating a clear 1 wt% PA solution in chloroform (500 rpm during 3s and 3000 rpm during 30 s) on either gold substrates ($\varnothing = 2.5$ cm, 2-5 nm Ti on glass and 200 nm gold, SSENS, The Netherlands) or 1 cm^2 cut single side polished p-type Si(100) wafers (Okmetic). An average thickness of 100 nm was found using an ellipsometer. A Plasma-Prep II plasma etcher (SPI supplies, USA) was used at an oxygen pressure of 1.0 bar, a vacuum pressure of 160 mbar and a current of 40 mA for 15 sec to activate the surface of the PA-films. The films were subsequently modified with GPTMS using the same procedure as described above. In the case of PA-films, dry hexane was used in all steps instead of dry toluene to minimize swelling of the polymer.

Characterization of substrates

Modified surfaces were examined by dynamic contact angle (sessile droplet) measurements using a Krüss G10 contact angle measuring instrument, equipped with a CCD camera. Droplets (1.5-2 μL) of water were placed randomly over the surface and advancing and receding (Θ_a and Θ_r) contact angles were automatically determined during growth and shrinkage of the droplet by a drop shape analysis routine. All reported values were an average of at least three measurements.

Ellipsometric layer thickness measurements were performed on a Plasmos ellipsometer (Plasmos SD 2002 ellipsometer, $\lambda = 632.8$ nm) with a fixed angle of incidence of 70°. The indexes of refraction were considered to be constant and equal to the "bulk" values of 1.500 and 1.457 for organic layers and the underlying native oxide, respectively. The average thickness of the SiO_2 layer was measured separately on an unmodified Si substrate and subtracted from the total layer thickness. All reported thickness values were averaged from at least five different measurements on the same substrate.

XPS spectra were measured using a Physical Electronics Quantera Scanning X-ray Multiprobe instrument, equipped with a monochromatic Al K α X-ray source operated at 1486.6 eV and 25 W. Spectra were referenced to the main aliphatic C 1s peak set at 284.8 eV. The X-ray beam size was 100 μm and the data were

collected from surface areas of $100\ \mu\text{m} \times 300\ \mu\text{m}$ with a pass energy of 224 eV and a step energy of 0.8 eV for survey scans at a detector input angle of 45° . The measurement was collected after 3 cycles scanning. Charge neutralization was achieved by low-energy electrons and low-energy Argon ions.

Atomic force microscopy (AFM) experiments were carried out using a digital NanoScope III multimode AFM (Digital Instruments, Veeco Metrology Group, USA) in tapping mode, equipped with a Si_3N_4 tip and with a J-Scanner at a scan rate of 0.6 Hz. AFM imaging was performed at ambient conditions.

Polarization modulation (PM) FT-IRRAS spectra were recorded using a Tabletop Optical Module (TOM) connected with a Nicolette 6700 spectrometer (Thermo Scientific) and equipped with a PEM-100 controller (HINDS instruments), a synchronous sampling Demodulator (GWC Technologies) and with a liquid nitrogen-cooled MCT-A detector at a reflection angle of 81° . Data of a dual-channel polarization modulation experiment were collected for 250 scans at a resolution of $8\ \text{cm}^{-1}$.

Reactive μCP

Silicon wafer-based masters with etched structures were prepared by UV photolithography. The master's surface was fluorinated using 1H,1H,2H,2H-perfluorododecyltrichlorosilane (PFDTs). Poly(dimethylsiloxane) (PDMS) (Dow Corning) stamps were fabricated by curing the degased mixture (10:1 elastomer, curing agent) of the components of SYLGARD® 184 silicone elastomer kit on the surface of the master at $60\ ^\circ\text{C}$ overnight. PDMS stamps were first oxidized in an O_2 -plasma reactor (Plasma-Prep II plasma etcher, SPI supplies, USA) at an oxygen pressure of 1.0 bar, a vacuum pressure of 160 mbar and a current of 40 mA for 20 sec. The stamps were then stored in water. Prior to use, the stamps were dried with a flow of dry N_2 and then incubated with 50 μL of a hBMP-6 solution (20 $\mu\text{g}/\text{mL}$) in PBS for 5 min at room temperature. The excess of inking solution was then removed and the stamps were dried with a flow of dry N_2 and brought into conformal contact with the epoxide-terminated layers for 2 h with $15\ \text{g}/\text{cm}^2$ of pressure. After printing, the substrates were washed three times 10 min with PBS buffer including 0.005% Tween-20, then washed with PBS and dried with a flow of dry N_2 . For fluorescence microscopy imaging, a final washing with water was performed to reduce the amount of salts present on the surface.

Prior to the washing procedure upon printing on the activated PA films, a solution of 100 mg of NaBH_3CN in 40 mL of PBS with 10 mL of EtOH for 5 minutes was applied in the case when reduction of the imine bond was required.

hBMP-6 immunostaining

Following protein immobilization via reactive μ CP, the background was blocked by incubating the samples for 2 h in a solution of 0.1 wt% O-(2-aminoethyl)polyethyleneglycol 3,000 (NH_2 -PEG) in PBS buffer. Subsequently, the substrates were washed with PBS buffer, rinsed with water and dried with a flow of dry N_2 . Then, the substrates were incubated with 100 μL of a solution of 5 $\mu\text{g}/\text{mL}$ of monoclonal anti-BMP-6 antibody produced in mouse (mouse IgG2B isotype, clone 74219.11, Invitrogen) in PBS buffer including 1 mM Tween-20. After 1 h, the substrates were washed three times 10 min with PBS buffer including 1 mM Tween-20, rinsed with water and dried with a flow of dry N_2 . Subsequently, the substrates were incubated with 100 μL of a solution of 40 $\mu\text{g}/\text{mL}$ of Alexa Fluor® 546 labeled goat anti-mouse IgG2b (γ 2b) (Invitrogen) produced in goat in PBS buffer including 1% BSA. After 1 h, the substrates were washed three times 10 min with PBS buffer including 1 mM Tween-20 during, rinsed with water and dried with a flow of dry N_2 .

Cell culture and staining

All substrates were sterilized prior to cell seeding with 70% ethanol and subsequently washed twice with PBS to remove any excess of ethanol. Mouse pre-osteoblast cell line KS483-4C3 was used as a model for osteogenic differentiation. Cells were plated at a density of 5000 cells/ cm^2 and cultured in Minimal Essential Medium (MEM) alpha supplemented with 10% fetal bovine serum (FBS) and 100 U penicillin/streptomycin (Gibco) and 1% (v/v) penicillin-streptomycin solution (with 10,000 units penicillin and 10 mg streptomycin/mL). After 24 h, adhered cells were carefully washed twice with sterile PBS to remove loosely attached cells. Cells were then fixed in 10% buffered formalin for 1 h at room temperature and stained for F-actin with Alexa Fluor® 568 phalloidin or Alexa Fluor® 488 phalloidin (Invitrogen) or cell nuclei with DAPI dilactate (Invitrogen) following the protocol from the provider. After washing the substrates two times with PBS and water, the substrates were carefully blown-dry with N_2 . Cell numbers were assessed after 24 h of adhesion using a particle counting routine of the free software ImageJ, using the fluorescence pictures corresponding to the DAPI staining. Cell spreading was assessed in a similar way by quantifying the total cell area of at least 5 different fluorescence pictures corresponding to the F-actin staining samples and normalized by the corresponding cell number.

ALP staining and quantification

For osteogenic differentiation, 0.2 mM ascorbic acid was added to the medium either together with 100 ng/mL hBMP-6 delivered in soluble form or hBMP-6 was bound to the substrate. Cells were harvested at different time points for detection and quantification of alkaline phosphatase (ALP). For ALP staining, cells were fixed using 10% buffered formalin for 1 h. ALP staining was performed using the Leukocyte Alkaline Phosphatase kit according to manufacturer's protocol. For ALP quantification, KS483-4C3 cells were lysed in 200 μ L lysis buffer (0.1 M K_2HPO_4 + 0.1 M KH_2PO_4 + 0.1% Triton X100, pH 7.8), and ALP was quantified using the CDP-star® Chemiluminescent Substrate. ALP production was corrected for DNA content, measured using the CyQuant Cell Proliferation assay (Invitrogen). To avoid contact between cells and substrates, Transwell® inserts (Corning Life Sciences) were used according to the manufacturer's protocol.

Luciferase reporter activity

C2C12 cells were stably transfected with an expression construct (BRE-Luc) containing a BMP responsive element (BRE) fused to the firefly luciferase reporter (Logeart-Avramoglou, 2006).^[27] Cells were cultured at a density of 5000 cells/cm² in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 100 U penicillin/streptomycin (Gibco). At different time points, cells were lysed in Glo lysis buffer (Promega) and luciferase activity was measured using Steady-Glo luciferase reagent (Promega). Luciferase activity was normalized for DNA content, as measured by CyQuant Cell Proliferation assay (Invitrogen).

Gene expression analysis

Cells were lysed in Trizol for RNA isolation, RNA was extracted using the Nucleospin RNA II kit (Bioke) according to manufacturer's protocol. Subsequently, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Quantitative polymerase chain reaction (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad) on MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad). Gene expression was normalized using GAPDH and expressed as fold induction compared to controls. Primer sequences are listed in the following table.

Gene	Primer sequence	Product size	Annealing temperature
GAPDH	For 5' CGCTCTCTGCTCCTCCTGTT 3' Rev 5' CCATGGTGTCTGAGCGATGT 3'	82 bp	60 °C
RUNX2	For 5' GGAGTGGACGAGGCAAGAGTTT 3' Rev 5' AGCTTCTGTCTGTGCCCTCTGG 3'	133 bp	60 °C
OCN	For 5' ACTGGCTCAAGAACGTCCTG 3' Rev 5' GAGAGAATCCGGTACTGTGG 3'	438 bp	60 °C

Quantification of released hBMP-6

Cell culture medium was collected at different time points and the amount of hBMP-6 was quantified using an ELISA assay according to the manufacturer's instructions (Duo Set ELISA development kit, R&D systems) and expressed as a cumulative amount throughout the period of time under study. The quantification of hBMP-6 bound to the substrates at different time points was performed via the fluorescence intensity quantification of protein arrays by using the particle counting routine of the free software ImageJ. Prior to imaging, the substrates were washed twice with PBS and rinsed with water and after imaging they were placed back in cell culture media until further measurements.

Fluorescence microscopy

Fluorescence microscopy images were recorded using an Olympus inverted research microscope IX71 equipped with a mercury burner U-RFL-T as light source and a digital Olympus DR70 camera for image acquisition using the following Olympus filter cubes: $300 \text{ nm} \leq \lambda_{\text{ex}} \leq 400 \text{ nm}$ for blue emission ($410 \text{ nm} \leq \lambda_{\text{em}} \leq 510 \text{ nm}$), $510 \text{ nm} \leq \lambda_{\text{ex}} \leq 550 \text{ nm}$ for red emission ($\lambda_{\text{ex}} \geq 580 \text{ nm}$) and $460 \text{ nm} \leq \lambda_{\text{ex}} \leq 490 \text{ nm}$ for green emission ($\lambda_{\text{em}} \geq 520$). All fluorescence microscopy images were acquired in air.

Statistical Analysis

All experiments were repeated at least three times. Error bars represent standard errors, and statistical analysis was performed using the one-way analysis of variance test (ANOVA) to evaluate the statistical differences and a Tukey's Post-hoc test (*p < 0.05 or **p < 0.005) among all samples or between samples and controls. In the case where only differences between sample and control were to be assessed a t-test was used to determine statistical differences (*p < 0.05 or **p < 0.005)

6.6 References

- [1] R. O. Hynes, *Science* **2009**, 326, 1216.
- [2] P. Tayalia, D. J. Mooney, *Advanced Materials* **2009**, 21, 3269.
- [3] K. Yuasa, G. Futamatsu, T. Kawano, M. Muroshita, Y. Kageyama, H. Taichi, H. Ishikawa, M. Nagahama, Y. Matsuda, A. Tsuji, *FEBS Journal* **2012**, 279, 3997.
- [4] For examples see: a) R. H. Li, J. M. Wozney, *Trends in Biotechnology* **2001**, 19, 255. b) P. Ducey, G. Karsenty, *Kidney Int* **2000**, 57, 2207. c) E. Groeneveld, E. Burger, *European Journal of Endocrinology* **2000**, 142, 9.
- [5] C. D. Grimsrud, P. R. Romano, M. D'Souza, J. E. Puzas, P. R. Reynolds, R. N. Rosier, R. J. O'Keefe, *Journal of Bone and Mineral Research* **1999**, 14, 475.
- [6] M. J. Bitgood, L. Shen, A. P. McMahon, *Current Biology* **1996**, 6, 298.
- [7] T. Knittel, P. Fellmer, L. Müller, G. Ramadori, *Experimental Cell Research* **1997**, 232, 263.
- [8] For examples see: a) V. Drozdoff, N. A. Wall, W. J. Pledger, *Proceedings of the National Academy of Sciences* **1994**, 91, 5528. b) M. Blessing, P. Schirmacher, S. Kaiser, *The Journal of Cell Biology* **1996**, 135, 227. c) S. Kaiser, P. Schirmacher, A. Philipp, M. Protschka, I. Moll, K. Nicol, M. Blessing, *Journal of Investigative Dermatology* **1998**, 111, 1145.
- [9] For reviews see for example: a) Y. Ito, *Soft Matter* **2008**, 4, 46. b) F.-M. Chen, M. Zhang, Z.-F. Wu, *Biomaterials* **2010**, 31, 6279. c) G. A. Hudalla, W. L. Murphy, *Advanced Functional Materials* **2011**, 21, 1754. d) K. Lee, E. A. Silva, D. J. Mooney, *Journal of The Royal Society Interface* **2011**, 8, 153. e) V. Gribova, R. Auzely-Velty, C. Picart, *Chemistry of Materials* **2011**, 24, 854.
- [10] a) T. Crouzier, K. Ren, C. Nicolas, C. Roy, C. Picart, *Small* **2009**, 5, 598. b) T. Crouzier, L. Fourel, T. Boudou, C. Albige's-Rizo, C. Picart, *Advanced Materials* **2011**, 23, H111.
- [11] For examples see: a) S. E. Sakiyama-Elbert, J. A. Hubbell, *Journal of Controlled Release* **2000**, 69, 149. b) S. E. Sakiyama-Elbert, J. A. Hubbell, *Journal of Controlled Release* **2000**, 65, 389. c) A. Herland, K. M. Persson, V. Lundin, M. Fahlman, M. Berggren, E. W. H. Jager, A. I. Teixeira, *Angewandte Chemie International Edition* **2011**, 50, 12529. d) K. Rajangam, H. A. Behanna, M. J. Hui, X. Han, J. F. Hulvat, J. W. Lomasney, S. I. Stupp, *Nano Letters* **2006**, 6, 2086. e) K. Rajangam, M. S. Arnold, M. A. Rocco, S. I. Stupp, *Biomaterials* **2008**, 29, 3298. f) J. C. Stendahl, L. J. Wang, L. W. Chow, D. B. Kaufman, S. I. Stupp, *Transplantation* **2008**, 86, 478. g) L. W. Chow, L.-J. Wang, D. B. Kaufman, S. I. Stupp, *Biomaterials* **2010**, 31, 6154. h) L. W. Chow, R. Bifton, M. J. Webber, D. Carvajal, K. R. Shull, A. K. Sharma, S. I. Stupp, *Biomaterials* **2011**, 32, 1574.
- [12] For examples see: a) R. Mammadov, B. Mammadov, S. Toksoz, B. Aydin, R. Yagci, A. B. Tekinay, M. O. Guler, *Biomacromolecules* **2011**, 12, 3508. b) R. Mammadov, B. Mammadov, M. O. Guler, A. B. Tekinay, *Biomacromolecules* **2012**, 13, 3311. c) B. Mammadov, R. Mammadov, M. O. Guler, A. B. Tekinay, *Acta Biomaterialia* **2012**, 8, 2077. d) K. L. Christman, V. Vázquez-Dorbatt, E. Schopf, C. M. Kolodziej, R. C. Li, R. M. Broyer, Y. Chen, H. D. Maynard, *Journal of the American Chemical Society* **2008**, 130, 16585. e) S. M. Anderson, T. T. Chen, M. L. Iruela-Arispe, T. Segura, *Biomaterials* **2009**, 30, 4618.
- [13] a) K. Kato, H. Sato, H. Iwata, *Langmuir* **2005**, 21, 7071. b) T. Nakaji-Hirabayashi, K. Kato, Y. Arima, H. Iwata, *Biomaterials* **2007**, 28, 3517. c) T. Nakaji-Hirabayashi, K. Kato, H. Iwata, *Biomaterials* **2008**, 29, 4403.
- [14] For examples see: a) P. R. Kuhl, L. G. Griffith-Cima, *Nature Medicine* **1996**, 2, 1022. b) K. Alberti, R. E. Davey, K. Onishi, S. George, K. Salchert, F. P. Seib, M. Bornhauser, T. Pompe, A. Nagy, C. Werner, P. W. Zandstra, *Nature Methods* **2008**, 5, 645. c) T. Pompe, K. Salchert, K. Alberti, P. W. Zandstra, C. Werner, *Nature Protocols* **2010**, 5, 1042.
- [15] For examples see: a) P. T. Corbett, J. Leclaire, L. Vial, K. R. West, J.-L. Wietor, J. K. M. Sanders, S. Otto, *Chemical Reviews* **2006**, 106, 3652. b) J.-M. Lehn, *Chemical Society Reviews* **2007**, 36, 151. c) Y. Ura, J. M. Beierle, L. J. Leman, L. E. Orgel, M. R.

- Ghadiri, *Science* **2009**, 325, 73. d) J.-M. Lehn, *Science* **2002**, 295, 2400. e) S. J. Rowan, S. J. Cantrill, G. R. L. Cousins, J. K. M. Sanders, J. F. Stoddart, *Angewandte Chemie International Edition* **2002**, 41, 898. f) E. Moulin, G. Cormos, N. Giuseppone, *Chemical Society Reviews* **2012**, 41, 1031. g) M. von Delius, E. M. Geertsema, D. A. Leigh, *Nature Chemistry* **2010**, 2, 96. h) M. von Delius, E. M. Geertsema, D. A. Leigh, D.-T. D. Tang, *Journal of the American Chemical Society* **2010**, 132, 16134. i) B. Shi, M. F. Greaney, *Chemical Communications* **2005**, 886.
- [16] a) L. E. Gerweck, K. Seetharaman, *Cancer Research* **1996**, 56, 1194. b) M. Calistri-Yeh, E. J. Kramer, R. Sharma, W. Zhao, M. H. Rafailovich, J. Sokolov, J. D. Brock, *Langmuir* **1996**, 12, 2747. c) S. R. Wasserman, Y. T. Tao, G. M. Whitesides, *Langmuir* **1989**, 5, 1074.
- [17] a) N. Sreenivasachary, J.-M. Lehn, *Proceedings of the National Academy of Sciences* **2005**, 102, 5938. b) N. Sreenivasachary, J.-M. Lehn, *Chemistry – An Asian Journal* **2008**, 3, 134.
- [18] For examples see: a) N. Giuseppone, J.-M. Lehn, *Angewandte Chemie International Edition* **2006**, 45, 4619. b) A. Herrmann, N. Giuseppone, J.-M. Lehn, *Chemistry – A European Journal* **2009**, 15, 117. c) D. I. Rozkiewicz, Y. Kraan, M. W. T. Werten, F. A. de Wolf, V. Subramaniam, B. J. Ravoo, D. N. Reinhoudt, *Chemistry – A European Journal* **2006**, 12, 6290. d) L. Tauk, A. P. Schröder, G. Decher, N. Giuseppone, *Nature Chemistry* **2009**, 1, 649.
- [19] a) M. Kellomäki, H. Niiranen, K. Puumanen, N. Ashammakhi, T. Waris, P. Törmälä, *Biomaterials* **2000**, 21, 2495. b) M. Kellomäki, S. Paasimaa, D. W. Grijpma, K. Kolppo, P. Törmälä, *Biomaterials* **2002**, 23, 283.
- [20] a) V. V. Tsukruk, I. Luzinov, D. Julthongpiput, *Langmuir* **1999**, 15, 3029. b) D. C. Trimbach, B. Keller, R. Bhat, S. Zankovych, R. Pöhlmann, S. Schröter, J. Bossert, K. D. Jandt, *Advanced Functional Materials* **2008**, 18, 1723.
- [21] C. Wendeln, B. J. Ravoo, *Langmuir* **2012**, 28, 5527.
- [22] G. van der Horst, R. L. van Bezooijen, M. M. L. Deckers, J. Hoogendam, A. Visser, C. W. G. M. Lwik, M. Karperien, *Bone* **2002**, 31, 661.
- [23] A. Wutzl, W. Brozek, I. Lernbass, M. Rauner, G. Hofbauer, C. Schopper, F. Watzinger, M. Peterlik, P. Pietschmann, *Journal of Biomedical Materials Research Part A* **2006**, 77A, 75.
- [24] a) X. Sui, A. Di Luca, M. K. Gunnewiek, E. S. Kooij, C. A. van Blitterswijk, L. Moroni, M. A. Hempenius, G. J. Vancso, *Australian Journal of Chemistry* **2011**, 64, 1261. b) R. Zimmerman, L. Basabe-Desmonts, F. van der Baan, D. N. Reinhoudt, M. Crego-Calama, *Journal of Materials Chemistry* **2005**, 15, 2772.
- [25] S. Edmondson, W. T. S. Huck, *Journal of Materials Chemistry* **2004**, 14, 730.
- [26] P. Giannoudis, N. Kanakaris, T. Einhorn, *Osteoporosis International* **2007**, 18, 1565.
- [27] D. Logeart-Avramoglou, M. Bourguignon, K. Oudina, P. Ten Dijke, H. Petite, *Analytical Biochemistry* **2006**, 349, 78.
- [28] K. J. Livak, T. D. Schmittgen, *Methods* **2001**, 25, 402.
- [29] P. Ducey, T. Schinke, G. Karsenty, *Science* **2000**, 289, 1501.
- [30] R. T. Franceschi, G. Xiao, *Journal of Cellular Biochemistry* **2003**, 88, 446.
- [31] H. Shen, X. Hu, F. Yang, J. Bei, S. Wang, *Biomaterials* **2007**, 28, 4219.
- [32] Q. Hong, C. Rogero, J. H. Lakey, B. A. Connolly, A. Houlton, B. R. Horrocks, *Analyst* **2009**, 134.
- [33] M. B. Olde Riekerink, M. B. Claase, G. H. M. Engbers, D. W. Grijpma, J. Feijen, *Journal of Biomedical Materials Research Part A* **2003**, 65A, 417.
- [34] a) M. B. Claase, M. B. Olde Riekerink, J. D. de Bruijn, D. W. Grijpma, G. H. M. Engbers, J. Feijen, *Biomacromolecules* **2002**, 4, 57. b) P. K. Chu, J. Y. Chen, L. P. Wang, N. Huang, *Materials Science and Engineering: R: Reports* **2002**, 36, 143. c) A. H. C. Poulsson, S. A. Mitchell, M. R. Davidson, A. J. Johnstone, N. Emmison, R. H. Bradley, *Langmuir* **2009**, 25, 3718.

Cell Studies with Arrays of Multiple Proteins Fabricated Using a Hydrogel-Filled Silicon Stamp

A novel hydrogel-filled silicon stamp has been used for the fabrication of protein microarrays. A stamp comprising an array of dots (with a diameter of 5 μm) has been used for the immobilization of various amounts of proteins with a constant geometry. Another stamp including 24 different and 1 duplicate geometry layout, has been used for the fabrication of surfaces with different effective protein areas. After exploring different printing strategies and evaluating the shape fidelity of the printed features, fibronectin (FN) arrays on epoxide-terminated poly(trimethylene carbonate) (PTMC₃₇₃) films have been used for analyzing the geometrical effects of the FN adhesive cues on the adhesion and spreading of C2C12 cells (mouse myoblast cell line). Another array comprising of different concentrations of growth factor human bone morphogenetic protein 6 (hBMP-6) and FN has been successfully printed on polydimethylsiloxane (PDMS) blocks with poly(glycidyl methacrylate) (PGMA) brushes. Using this array, early osteogenic differentiation could be modulated.

The results show that using the novel silicon stamp provides a new entry to fabricate functional arrays of multiple proteins on various types of surfaces in an easily accessible manner following standard printing protocols.

7.1 Introduction

Arrays of proteins play an important role in various areas of biomedical and biotechnological research that range from biomarker detection, proteomic screens to drug discovery.^[1] Defined arrays of proteins also facilitate systemic understanding of culturing and studying cells on surfaces.^[2] For example, cellular adhesion is a process mediated by the interaction of cell-surface receptors with ligands from the extracellular matrix which results in the clustering of receptors into focal adhesion complexes. This elaborate and highly regulated process is believed to play a significant role in many essential cellular functions. Most studies involving the signaling of focal adhesion complexes with surface-immobilized proteins have employed defined structures conveniently made by conventional microcontact printing (μ CP).^[3] Adopting microcontact printing is attractive to rapidly achieve functional arrays of proteins as the stamp,^[4] central to μ CP, can consist of multiple features and is easy to make and apply.^[5]

Although promising progress has been reported on developing printing strategies for printing multiple proteins, it remains a key challenge to develop a printing strategy to achieve arrays of multiple proteins.^[6] For example, Delamarche and coworkers locally inked a stamp involving multiple types of affinity proteins through local exposure of a stamp using microcontainers.^[6a] Such fabricated stamps including sites with different affinities were used to capture target proteins that were then transfer-printed to other substrates in parallel.^[6a] In a related approach, these authors placed a microfluidic network having an ensemble of independent flowing zones onto a stamp. Sealing of the microchannels resulted in transfer of the proteins upon conformal contact with a substrate.^[6b-e] Another elegant way was introduced by Chen and coworkers who used a 3D stamp. When inking the different layers of the 3D stamp with different types of proteins, through applying increasing pressure to the stamp each layer of the stamp was successively brought in contact with the substrate to yield a protein microarray.^[6f]

In this chapter, an alternative strategy to print microarrays of multiple proteins on biomaterials is presented. The printed arrays are successfully used in cell adhesion and differentiation studies. To this end, novel silicon (Si) stamps were used that were recently developed by dr. E. Bat in our group.^[7] These Si stamps comprise a multitude of reservoirs for solutions comprising proteins, wherein each of the reservoirs has a bottom side. Each of the bottom sides comprises a multitude of microchannels connecting the reservoir with the stamping surface. The reservoirs and microchannels are filled with a hydrogel that is crosslinked and linked to the walls of the Si stamp. This design was motivated by previous work by Martin et al.

who used a hydrogel stamper consisting of an array of fragile glass microcapillaries filled with hydrogel and connected to a reservoir holding the proteins.^[8] In contrast, our design allows for multiple reservoirs, each providing an array of spots fabricated in a robust silicon wafer. In addition, our macroporous cross-linked hydrogel swells less extensively, providing sufficient mechanical strength to allow reusability of the stamps, repeated printing (>75 times) and access to a wide range of proteins that can be printed (up to 1 MDa).

Two applications of the printed protein microarrays using the Si stamps are demonstrated. First, the impact of the geometrical properties of an important external cell membrane (ECM) adhesive protein (fibronectin, FN) on C2C12 cells (mouse myoblast cell line) was studied. In this case, the design of the bottom of the reservoirs included different geometries yielding a micropatterned FN array on a soft surface. These micropatterns could be transferred to another biomaterial surface via common transfer printing.^[9] Second, since the growth factor human bone morphogenetic protein 6 (hBMP-6) is reported to convert the differentiation pathway of myoblastic cell lines into that of osteoblast lineage and induces osteoblast differentiation and maturation in osteoprogenitor and osteoblastic cell lines,^[10] a microarray with different amounts of hBMP-6 was printed on the surface of biomaterials. Such a printed hBMP-6 microarray was used to estimate the required hBMP-6 dosage to produce alkaline phosphatase (ALP), which is an early osteogenic marker.

7.2 Results and discussion

7.2.1 Preparation, design and printing procedure of hydrogel-filled Si stamps

Hydrogel-filled Si stamps (Figure 7.1a) were fabricated following a sequence of photolithography and deep reactive ion-etching (DRIE) steps according to a procedure previously developed in our group.^[7] Upon fabrication, the stamp surface was modified with 1H,1H,2H,2H-perfluorodecyltrichlorosilane (PFDTs) to ease peeling off the stamp after printing, while the remaining surfaces were modified with 3-(trichlorosilyl)propylmethacrylate (TPM) to facilitate the covalent anchoring of the hydrogel, which was required for optimal printing.^[7] After these two surface modification steps, the reservoirs and microchannels were filled with a covalently cross-linked (hydroxyethyl)methacrylate (HEMA)-based hydrogel (see Experimental Section for details) and visualized using SEM (Figure 7.1b and c). This type of hydrogel serves as a suitable environment for storing protein solutions and facilitates the release properties of the protein solution upon

conformal contact of the stamp surface with a substrate. Homogeneous layers were repeatedly printed (>75 times) on various types of polymeric substrates.^[7] The hydrogel-filled reservoirs were loaded with protein solution using a standard laboratory micropipette addressing either all reservoirs of the stamp or groups of 4 reservoirs (Figure 7.2, steps a1 and b1).

Two different stamp designs were used for different experiments. The first design (Figure 7.1d) comprising an array of dots (with a diameter of 5 μm) was used for the immobilization of various amounts of proteins with a constant geometry. The second design, while having the same amount and size of reservoirs, includes 24 different and 1 duplicate geometry lay-out and was used for fabricating surfaces with different effective protein areas. The geometry lay-out of an array is given in Figure 7.1e and consists of differently spaced features, which are squares, rectangles, circles and ellipsoids having either a surface area of 25 or 100 μm^2 .

Figure 7.1 – Designs of the hydrogel-filled Si stamps consisting of 25 reservoirs of 320 x 320 μm , spaced by 320 μm : a) shows a photograph of the Si stamp viewed from the reservoir side. SEM images of the b) reservoir and c) printing sides of the hydrogel-filled Si stamps. Scale bars are 200 and 20 μm , respectively, while the images given in the insets were recorded at 2x and 10x higher magnification, respectively. Schematic representation of stamp design d): one of the reservoirs on the stamp is expanded to show the design of the printing side: each reservoir consists of an array of 12 x 12 dots of 5 μm in diameter, 20 μm apart. Schematic representation of stamp design e): each reservoir on the stamp consists of a unique array of shapes (as drawn) varying in size, aspect ratio (top-right corner) and spacing as specified by a , which denotes the area of the shape in μm^2 and by d_x and d_y , which denote the center to center distance along the x and y axis of the stamp, respectively. The maximum possible protein coverage is given for each type of array as a percentage of the total area of an array marked with a dashed line in d).

Figure 7.2 – Printing procedures using the hydrogel-filled Si stamp. In route a (above), the full array of reservoirs is used for arraying proteins on a hard substrate (e.g. a SAM or a thin polymeric film on a glass substrate) following five steps: a1) all the reservoirs of the stamp are filled with a protein solution; a2) printing on a non-functionalized PDMS soft substrate yields a protein array on PDMS (a3) and (a4) is subsequently used as a stamp in transfer printing onto a "hard" substrate to yield a5) the protein array on the substrate of interest. In route b (bottom) different protein solutions are applied to a selection of reservoirs (separated by an empty row of reservoirs for clarity) (b1) and used for printing on a "soft" substrate (b2) yielding a protein array with different properties (b3).

Figure 7.2 depicts two printing procedures that were followed in the present study using a loaded stamp. For the first series of experiments, arrays of fibronectin (FN) were fabricated on poly (trimethylene carbonate) (PTMC₃₇₃) using the design with a plurality of geometries following a two-step printing procedure. In the first printing step, an array of FN was printed on flat non-functionalized PDMS blocks (Figure 7.2, step a2). This array on PDMS was transfer-printed onto hard surfaces of spin coated thin films of (PTMC₃₇₃) on glass in good conformal contact (Figure 7.2, step a3). Prior to printing, PTMC₃₇₃ was functionalized with an epoxide-silane to allow the formation of covalent bonds between the printed proteins and the PTMC₃₇₃ films to achieve stable protein arrays (*vide infra*). The printed FN arrays were then used to study the effect of the geometrical parameters in terms of initial cell adhesion and spreading. In a second series of experiments, arrays of hBMP-6 were fabricated on soft PDMS blocks using the design with uniform geometry lay-out following a one-step printing procedure (Figure 7.2, step b2). Prior to printing, PDMS blocks were functionalized with PGMA brushes with epoxide end-groups to achieve stable protein arrays (*vide infra*). These hBMP6 arrays were then used to gain information on the growth factor dosage in terms of early cellular differentiation.

7.2.2 Printing fibronectin arrays with different geometries on PTMC₃₇₃

After spin coating thin films of PTMC₃₇₃ on glass, the surfaces were functionalized with 3-glycidylpropyltrimethoxysilane (GPTMS) as depicted in Scheme 7.1, following the same procedure as described in Chapter 6 for the surface functionalization of Polyactive™ films with epoxide moieties upon activation using an oxygen plasma for 15 sec. After functionalizing the PTMC₃₇₃ films with GPTMS, the recorded advancing contact angle value dropped from $80.4^\circ \pm 0.4^{[10]}$ to $61.8^\circ \pm 3.2$, which is only slightly higher when compared to the wettability of GPTMS SAMs on glass ($55.1^\circ \pm 1.5$) presumably due to the formation of more loosely packed monolayers on polymer surfaces.

After loading a Si stamp with different geometry lay-outs with a solution of 2 μM of FN, which contained 10% rhodamine-labeled FN (Rh-FN) for localization purposes using fluorescence microscopy, the stamp was used to print an FN array on PDMS. Subsequent to imaging the array using a fluorescence microscope, this FN array was transfer-printed to the epoxide-functionalized PTMC₃₇₃ films forming a covalent bond upon nucleophilic ring opening of the epoxide groups by the free amines of the fibronectin (Scheme 7.1).

Scheme 7.1 – Schematic procedure of the functionalization of PTMC₃₇₃ films by pre-activation with O₂ plasma followed by reaction with GPTMS. Fibronectin (ellipse) was covalently immobilized via transfer printing onto the GPTMS activated films.

Arrays of FN were successfully printed on the PTMC₃₇₃-GPTMS films for all the geometries present in the stamp as visualized using fluorescence microscopy (Figure 7.3a). A good replica of the stamp design with fully printed individual

features of the geometries was observed except in some case when conformal contact was poor. When comparing the average fluorescence intensity of the FN arrays before transfer printing on the flat PDMS (30 ± 1 a.u.) with the fluorescence intensity of the FN arrays after transfer printing on PTMC₃₇₃ (35 ± 8 a.u.), comparable intensity values were found, whereas on the PDMS surface no remainders of the FN arrays could be detected (Figure 7.3c).

Figure 7.3 – a) Fluorescence microscopy images of FN arrays consisting of different geometries. b) Bright field image of the printing side of the Si stamp. c) Fluorescence images of the feature-less flat PDMS stamp that was used for transfer printing before (left) and after (right) contact with the receiver PTMC₃₇₃ substrate. Error (%) of the printed features (determined using fluorescence microscopy) in terms of area (d) and aspect ratio (e) in comparison to the original values of the design. The layout matches with the layout displayed in Figure 7.1e.

Upon close inspection of the bright field image of the Si stamp and the fluorescent microscopy image of the printed FN arrays, the shape fidelity (area and aspect ratio) of the fabricated geometries in the reservoirs and the printed geometries were compared with the originally desired shapes of the stamp.

Based on images such as in Figure 7.3b, the openings in the bottom of the reservoirs of the stamp were found to be on average 10% larger when compared to the original design. While circular features were indeed found to have a circularity ratio of close to 1, in the images made of the printing side of the stamp, the ellipsoidal and rectangular features were found to deviate more significantly having smaller aspect ratios of 1:1.85 and 1:3.4 in comparison with the desired ones of 1:2 and 1:4, respectively. Comparing the individual areas of each of the printed features using fluorescence microscopy images such as in Figure 7.3a with those from the original stamp design, the error in the area and aspect ratios of the printed features was calculated with respect to the original design (Figure 7.3d and e). For the majority of the shapes the error in the area was in the same range as the observed discrepancy between the originally desired stamp and the fabricated stamp. This indicates that most of the shapes, for all aspect ratios, in the printed protein arrays are in good agreement with the geometry of the used stamps. Printed shapes that were smaller and had a smaller aspect ratio than expected from their parent design mostly corresponded to the largest features of the stamp design. We tentatively attribute the observed incomplete replication of the features by printing to incomplete filling of the microchannels of the stamp, which may result from insufficient crosslinking time or to the inhomogeneous porosity of the hydrogel within those openings. A reason for printed features larger than the designed ones could be related to the applied pressure during printing, which leads to leaking.

Taken together, the Si stamps can be used to reliably print protein arrays with various feature geometries, on different substrates and multiple times without the need of reloading with fresh protein solution.^[7]

7.2.3 Cell adhesion and spreading on printed fibronectin arrays on PTMC₃₇₃

Beyond printing 25 distinct arrays of FN of $60\text{--}70 \times 10^3 \mu\text{m}^2$ onto the surface of PTMC₃₇₃, the biomaterial remained untreated while a full composition cell culture medium was used to represent realistic *in vivo* conditions when using such biomaterials. These experimental conditions are different from literature studies where either detergents are added to or 10% FBS is left out from the medium or

where surfaces exposed to the medium are blocked prior to cell seeding.^[3b, 3c, 11] As a first step, the relationship between adhesive FN arrays and recruitment of focal adhesion components (i.e. vinculin) was studied. To this end, vinculin was immunostained after 1h of cell seeding on the printed FN arrays on PTMC₃₇₃ and inspected using fluorescence microscopy (Figure 7.4). When cells were seeded on a PTMC₃₇₃ substrate that was homogeneously covered with FN using a flat PDMS stamp, cells were widely spread showing small dots or rod-shaped adhesions (indicated with an arrow in the inset to Figure 7.4a), specially at the cell periphery. However, when cells were seeded on PTMC₃₇₃ with printed FN arrays, single focal adhesions were not observed while vinculin was present throughout the cells with a higher intensity at the periphery. These observations suggest that, in contrast to other literature studies where focal contacts were micropatterned,^[3b] the density and area of our patterns is too large to observe single focal contacts corresponding to the printed feature. Moreover, the cell adopts a more round-like shape that hampers the visualization of vinculin clusters as in cells seeded on homogeneously covered FN films. This suggests that there is an effect of the FN distribution in terms of local complex formation (usually at the leading edge of migrating cells). While in homogeneous FN films defined complexes are already formed, cells on the printed FN arrays are still in a dynamic state, readjusting their cytoskeleton to adapt to the features and form more mature stable focal adhesions.

In addition, when comparing Figures 7.4b and c, a large difference in terms of spreading on patterns with different spacing was observed. While a spacing of 20 μm allowed the bridging of two neighboring features, as can be seen in the overlay of the channels for vinculin and Rh-FN, cells on the same feature but with a larger spacing were confined on the feature area adopting the morphology dictated by the dots. These results indicate that even though cells were cultured with a background allowing protein adsorption from the cell culture medium and therefore cell adhesion, at an early stage the cells were able to sense the printed FN and to be instructed by the information included in the geometry of the printed FN microarrays.

Figure 7.4 – Vinculin distribution (after 1 h) of C2C12 cells seeded on FN-functionalized PTMC₃₇₃ substrates: a) fully covered FN (10% Rh-FN) layer with inset showing a magnification of the cell periphery with multiple focal contacts (white arrow); b) printed FN micro-array of 1:1 dots with "short" spacing and c) printed FN micro-array of 1:1 dots with "long" spacing. a-c are fluorescence microscopy images recorded using a green filter and a'-c' are the corresponding overlays with images recorded using a red filter. Values describe the aspect ratio, the area and the spacing of the arrays.

As a next step cell adhesion and spreading were quantified for the different geometries of the printed FN arrays using the fluorescence microscopy images of stained cells for their actin cytoskeleton and nuclei (Figure 7.5a-g). The results are summarized in the Table 7.5h. From the data a number of trends in the behavior of cell adhesion and spreading can be delineated with respect to the geometry of the array. The results of these experiments suggest that the extent of cell spreading is not related to the shape of the features, while no correlation between cell spreading and effective FN coverage was found. The latter finding is in contrast to reported work of Lehnert et al. [3b] However, our arrays represent a much narrower range of coverages and this effect could be less prominent when working in culture conditions favoring protein adsorption and consequently cell adhesion. Upon inspection of the images recorded in the case of the printed FN arrays with small features ($\sim 25 \mu\text{m}^2$) that are closely spaced ($\sim 20 \mu\text{m}$), the cytoskeleton was able to rapidly spread and reorganize, adapting itself to the geometrical pattern with sharp angles and straight edges (Figure 7.5a) in

agreement with observations made by Lehnert et al. for B16 F1 melanoma cells.^[3b] The spreading was restricted to the FN array when compared to a full layer of FN (Figure 7.4a), whereas the cells were spreading far more when compared to a control film without FN. When the features are more spatially separated ($\sim 50\text{ }\mu\text{m}$), the spreading was further restricted to parts of a printed FN array (Figure 7.5b). On this type of printed FN arrays, some cells were able to bridge only a few neighboring FN features building very tensioned cell geometries (Figure 7.5b) and further cases were observed of single cells adhering to an individual FN feature. When the area of the individual features was 4-fold increased to $100\text{ }\mu\text{m}^2$, while keeping the spatial separation constant at $50\text{ }\mu\text{m}$, most of the adhered cells were isolated on individual FN features in an almost 1:1 ratio (Figure 7.5c). In contrast, when the spacing of FN features in a printed FN array was reduced to $20\text{ }\mu\text{m}$, while keeping the area of FN constant at $100\text{ }\mu\text{m}^2$, cells were bridging individual FN features regularly, but in this case on average to a much less extent (Figure 7.5d) when compared to the previous case when the FN feature were smaller and further apart. The latter observation might indicate that when a minimum number of fibronectin proteins is interacting with their cell receptors, a reduced cell spreading will be observed.

Taken together, for a given shape and size of FN feature, cell spreading was more pronounced to cover large areas with a clear directionality when larger aspect ratios were present (Figure 7.5e-g) and when large spacings of individual FN features were used. These findings indicate a relation between cell motility and the aspect ratio of fibronectin areas and can be related to previous studies where cell motility has been related to aspect ratios of topological cues.^[12]

In contrast to cell spreading, cell adhesion was observed to correlate well with effective FN-coverage as plotted in Figure 7.5i. At higher effective FN coverage values (including size and spacing effects) a higher number of cells adhered, whereas the average number of adhered cells for all FN arrays studied was lower (8 ± 3) when compared to a control sample lacking FN (22 ± 4). The combined observation that more cells adhered to a control film but showed less spreading, reflects the potential of controlling the adhesive properties of cells to FN arrays on the same films even when proteins from the cellular medium might be adsorbed onto the background. Even though cells that are seeded on printed FN arrays sense the same background as on a control film, the printed FN arrays are able to regulate cell motility.

Figure 7.5 – Cell spreading on different micropatterned geometries: 1:2 circles with different area and spacing (a-d); Squares with equal area and spacing but different aspect ratio (e-g). Insets show a magnified area and values describe the aspect ratio, the area and the spacing of the patterns. Table h) summarizes the spreading values of cells seeded on all the printed micropatterns and graph i) shows cell number vs. effective FN coverage as an indication of cell adhesion for both squares and circles. The line serves as a guide to the eye. Error bars indicate standard deviations of the mean of three independent experiments.

7.2.4 Printing an hBMP-6 array for early cellular differentiation studies

Arrays of hBMP-6 were printed on PDMS blocks (approx. 1 x 1 x 0.5 cm) that were functionalized with poly(glycidyl methacrylate) (PGMA) brushes following the procedure described in Chapter 6 (Scheme 7.2). To avoid strong background fluorescence of PDMS,^[13] for some fluorescence microscopy experiments hBMP-6 arrays were printed on PTMC₃₇₃ films that were functionalized with GPTMS as previously described (*vide supra*). To print the hBMP-6 arrays on PTMC₃₇₃-GPTMS the two-step printing procedure involving a transfer printing step (Figure 7.2a) was employed, while the hBMP-6 arrays on the soft blocks of PDMS-PGMA could be printed directly (Figure 7.2b). The epoxide functionalities in the brushes allow the formation of a covalent bond between the printed proteins and the brush.

Scheme 7.2 – Schematic procedure of the surface functionalization of PDMS with PGMA by pre-activation using O₂ plasma. hBMP-6 (ellipse) can be subsequently immobilized via direct printing on the activated film via the reaction between the primary amine groups and the epoxide moieties.

Upon surface functionalization, different protein solutions were prepared and employed for loading the reservoirs of the Si stamp. Different hBMP-6 concentrations (0.1, 0.45 and 1.75 μM) were used while a constant concentration of FN (2 μM) and Rh-FN (0.2 μM) was added. The addition of FN serves to confine cells to the printed hBMP-6 areas and for visualizing the printed array. According

to the methodology displayed in Figure 7.2b, a group of 4 reservoirs of the stamp was manually loaded with each of the hBMP-6 solutions and a reference solution containing only FN and Rh-FN. After 2h, the loaded Si stamp was placed in conformal contact with a PDMS-PGMA surface to print the hBMP-6/FN array. The quality of the hBMP-6/FN array was first evaluated using fluorescence microscopy experiments before cell seeding experiments. The printed hBMP-6/FN array was visualized following an immunofluorescence staining protocol for the hBMP-6, while the labeled fibronectin allowed direct localization of the printed array. Figure 7.6a shows a series of fluorescence microscopy images of successfully immunostained hBMP-6 and printed Rh-FN. When no hBMP-6 was present in the array, the immunofluorescence was absent. The corresponding immunofluorescence intensity profiles were averaged over the printed hBMP-6 arrays each comprising 144 individual spots (Figure 7.6b). From these profiles, the intensity of the stained hBMP-6 clearly increases while increasing the concentration of the solutions used to load the Si stamp. The immunofluorescence profile saturates (Figure 7.6c), while concomitantly a constant red fluorescence intensity corresponding to Rh-FN was observed in the same spots. These results confirm a good correlation between the protein solutions used for loading the Si stamp and the printed protein arrays. Although the individual spots show a fairly constant intensity across the entire array for the different concentrations used, a low protein concentration produces the lowest standard deviation in intensity. In addition, an excellent printing fidelity of the dots with respect to the original design of the stamp was observed as was observed in the case of printing FN arrays as described in the previous paragraph. Moreover, the results also show that the printed hBMP-6 proteins are folded correctly.

Figure 7.6 – a) Fluorescence microscopy images of printed arrays with Si stamps loaded with solutions (concentrations indicated) of FN (with 10% Rh-FN) (left) and hBMP-6 (right). b) The average fluorescence intensity of rhodamine (top) and immunofluorescence (bottom) in dots across the array. Error bars represent the standard deviation of the mean of four individual printed arrays each containing 144 dots. c) the average fluorescence intensity of the different arrays with hBMP-6 and FN (corrected for background intensity). The curve serves as a guide to the eye. Error bars represent the standard deviation of the mean immunofluorescence of all dots of an array (measured in quadruplicate).

With these printed hBMP-6/FN arrays in hand, as a next step, mouse C2C12 myoblastic cells were subsequently seeded on the hBMP-6/FN array on PDMS-PGMA. Each group of four individual hBMP-6/FN arrays is separated by 640 μm , while individual hBMP/FN arrays are separated by 320 μm . These distances are sufficient to avoid cross-talk by paracrine communications^[14] between cells on arrays made of different hBMP-6 concentration. Prior to cell seeding however, unreacted epoxide moieties were capped with NH_2 -PEG to further improve specific cell adhesion to the printed hBMP-6 arrays. From the optical microscopy image in Figure 7.7a, a clear confinement of cells on the printed hBMP/FN arrays was observed already after 4 h after seeding the cells. After 4 days of cell

culture, only a minimal amount of cells died demonstrating a good biocompatibility of the printed hBMP-6/FN array on the PDMS surface (not shown). To explore the dependence of the signal transduction on the amount of hBMP-6 in the printed hBMP-6/FN arrays, ALP produced after 4 days was stained histochemically as a marker of early cell differentiation (Figure 7.7b). The stained areas match with printed hBMP-6/FN arrays presenting the different groups where hBMP-6 was present in the arrays, which increase in intensity upon increasing the quantity of immobilized hBMP-6. In the array lacking the hBMP-6 no stains were observed on the adhered cells. When plotting the ALP intensity versus the quantity of printed hBMP-6 a clear dose-dependent relationship was observed (Figure 7.7c) in agreement with literature data.^[10] As the plot in Figure 7.7c shows that the ALP staining intensity increases linearly with increasing hBMP-6 concentrations, while the plot in Figure 7.6c shows that the hBMP-6 immunostaining intensity saturates over this range of hBMP-6 concentrations. These results indicate that the BMP cell receptors were not yet saturated by the printed hBMP-6 array. Although the highest hBMP-6 concentration used for loading the Si stamp is 1.75 μ M, the total amount of immobilized hBMP-6 in an array could be estimated to be ca. 1.5 pg (3.6% patterned area) when using the results of Chapter 6. This amount is far below the amount of hBMP-6 needed to reach receptor saturation as was estimated in Chapter 6 to be ca. 190 ng (100% patterned area).

Figure 7.7 – a) Optical microscopy image of cells confined onto printed hBMP-6 arrays after 4 h of cell seeding. b) Images of ALP enzymatic histochemical staining and hematoxylin counterstaining on printed hBMP-6/FN arrays (concentrations indicated). c) Average ALP staining of C2C12 cells seeded on different concentrations of arrayed hBMP-6. The line serves to guide the eye. Error bars represent the standard deviation of the average intensity of each region in the optical microscopy images.

7.3 Conclusions

In this chapter it has been shown that novel Si stamps can be successfully used to fabricate protein microarrays for the study of cellular function. With the device, different strategies have been developed to print protein arrays on relevant biomaterials yielding active proteins. Transfer printing has been used to array FN onto epoxide terminated PTMC₃₇₃-GPTMS films yielding patterns of high fidelity according to the stamp design. These biomaterials with arrays of FN having varied shapes and aspect ratios have been used for systematically exploring the geometrical limits of the ECM for C2C12 mouse myoblastic cells. In addition, the Si stamp has been successfully employed to fabricate protein arrays of different concentrations of hBMP-6 at constant concentration of FN on soft PDMS substrate functionalized with PGMA brushes via direct printing. In this case, after good confinement of C2C12 cells was ensured, ALP activity has been related to the amount of hBMP-6 at the surface.

These examples illustrate the potential of the novel printing device to speed up cell-interface investigations using biochemical cues.

7.4 Acknowledgements

The major part of the work presented in this chapter was done in collaboration with Dr. E. Bat and Dr. P.F. Moonen.

7.5 Experimental Section

Materials

Commercial grade reagents were purchased from Sigma-Aldrich and used without further purification unless stated otherwise. Solvents were dried over molecular sieves. Water was of MilliQ quality. PTMC₃₇₃ was kindly provided by Dr. E. Bat. Fibronectin from bovine plasma was purchased from Sigma and rhodamine-fibronectin from bovine plasma (labeled with 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester) (0.6 dyes per protein molecule) was purchased from Cytoskeleton, Inc. These proteins were reconstituted in water (1 g/L) as indicated by the providers and further diluted to the desired working concentration in PBS buffer. Recombinant Human BMP-6 (hBMP-6) was purchased from R&D Systems as a carrier-free, 0.2 µm filtered solution (0.04 g/L) in acetonitrile and TFA and further diluted to the desired working concentration in PBS buffer.

Preparation, activation and functionalization of poly (trimethylene carbonate) (PTMC₃₇₃) films

PTMC₃₇₃ was allowed to dissolve in chloroform (1% w/v) by stirring for 4 h at room temperature. The solution was then spin-coated (3 s at 500 rpm followed by 30 s at 3000 rpm) onto clean microscope glass slides (76 x 26 mm, MENZEL-GLÄSER), which were cut to approx. 1.5 cm² pieces. For surface activation, an O₂-plasma treatment was applied for 15 sec by tuning the current to 40 mA of a Plasma-Prep II plasma etcher (SPI supplies, USA). The oxidized films were immediately used for the functionalization with (3-glycidyloxypropyl)trimethoxysilane (GPTMS). To this end, the films were immersed in 1% v/v in dry hexane overnight at room temperature under a N₂ atmosphere using oven-dried glassware (see Scheme 7.1). Following monolayer formation, the films were removed from the solution, rinsed several times with hexane to remove unbound material, rinsed with ethanol and dried in a flow of dry N₂. The samples were immediately used to prevent the ring-opening reaction of the epoxide groups.

Preparation of poly(glycidyl methacrylate) (PGMA) brush layers on poly(dimethylsiloxane) (PDMS)

Poly(dimethylsiloxane) (PDMS) (Dow Corning) blocks were fabricated by casting a degassed 10:1 (v/v) mixture (30 mL) of poly(dimethylsiloxane) and curing agent (Sylgard 184, Dow Corning) on the surface of a cleaned featureless Si wafer, which was previously treated with 1H,1H,2H,2H-perfluorododecyltrichlorosilane (PFDTs) to facilitate the removal of the PDMS. After overnight curing at 60 °C, PDMS was cut into approx. 1.5 cm² pieces and oxidized by oxygen plasma for 20 sec (current tuned to 40 mA). The oxidized PDMS substrates were immediately functionalized following procedures adapted from literature (see Scheme 7.2).^[15] Briefly, the PDMS substrates were placed in a desiccator together with a vial containing 100 µL of (3-aminopropyl)-triethoxysilane (APTES). After evacuation for 10 min using a diaphragm vacuum pump, the vapor of APTES was allowed to react overnight with the oxidized PDMS. Subsequently, the substrates were thoroughly rinsed with ethanol and dried under a flow of dry N₂. The cleaned amino-functionalized substrates were immersed into a solution of 5.5 mL triethylamine and 40 mL of ethanol and allowed to cool to 0 °C. Subsequently, 300 µL of ATRP initiator 2-bromo-2-methylpropionyl bromide was added dropwise to the solution. After complete addition, the solution was left for 1.5 h under a N₂ atmosphere. Afterwards, the initiator-functionalized substrates were rinsed with ethanol and water and dried under a flow of dry N₂ and stored in an argon-degassed flask. Afterwards, a solution of glycidyl methacrylate (5 mL, 5.21 g, 36.7 mmol), 2,2'-dipyridyl (bpy) (141 mg, 0.904 mmol) in methanol (4 mL) and water (1 mL) was prepared and purged with argon for 30 min in a round-bottom flask sealed with a septum. CuCl (36.4 mg, 0.368 mmol) and CuBr₂ (3.9 mg, 0.017 mmol) were added into another flask and purged with argon for 30 min. Monomer, ligand, and catalyst were then combined, stirred and degassed for another 30 min to facilitate the formation of the organometallic complex. Subsequently, this solution was transferred with a degassed syringe into the argon-degassed flask that contained the initiator-functionalized PDMS substrates. After 1 h at room temperature, the substrates were removed from the flask and thoroughly rinsed with water followed by gentle sonication in ethanol. After rinsing with ethanol, the PGMA-brush functionalized PDMS substrates were dried under a flow of dry N₂ and immediately used to prevent the ring-opening reaction of epoxide groups.

Substrate characterization

Modified surfaces were examined by dynamic contact angle (sessile droplet) measurements using a Krüss G10 contact angle measuring instrument, equipped with a CCD camera. Droplets (1.5-2 μL) of water (MilliQ, Millipore, $R = 18.2 \text{ M}\Omega\text{cm}$) were placed randomly on the surface and advancing and receding (Θ_a and Θ_r) contact angles were automatically determined during growth and shrinkage of the droplet using a drop shape analysis routine. All reported values were an average of at least three measurements.

Si stamp functionalization with hydrogel

A Si stamp with reservoirs and channels was fabricated by photolithography and deep reactive ion-etching (DRIE) according to a procedure reported by our group as published elsewhere.^[7] Before use, the stamps were activated in piranha solution (3:1 concentrated H_2SO_4 / 30% H_2O_2) for at least 30 min (**Warning!** *Piranha solution should be handled with caution. It has been reported to detonate unexpectedly*) and then rinsed several times with water, ethanol and then dried under a flow of dry N_2 . The stamps were subsequently functionalized overnight in a desiccator by evaporating PFDS as described above, rinsed with acetone and dried under a flow of dry N_2 . The stamp was pressed on a flat PDMS substrate until conformal contact was achieved while keeping the reservoir side on top. The system was subsequently exposed to O_2 plasma for 5 minutes, under the conditions previously described, to remove the PFDS layer everywhere except at the printing side, which was protected by the PDMS substrate. Subsequently, the inner walls of the reservoirs and channels were functionalized overnight by vapor deposition of 75 μL of 3,3-trichlorosilylpropylmethacrylate (TPM) in a desiccator as previously described. After washing the functionalized stamps with acetone and ethanol, the stamps were dried in a flow of N_2 . Then, the stamps were placed in a glass vial containing a solution of 1900 μL H_2O , 24 v% 2-hydroxyethylmethacrylate (HEMA) (600 μL) and 0.5 mol% ethylene glycol dimethacrylate (EGDMA) and vortexed to solubility. After purging the solution with N_2 , 33.34 μL of the initiator mixture (ammoniumpersulfate (APS) (7.5 mg / 100 μL) and 2.5 μL of N,N,N',N'-tetramethylethylenediamine (TEMED) (Acros) was added. Polymerization was allowed to proceed overnight after which excess polymer was removed from the outside of the Si stamps. After thorough washing with water the stamps were ready to use.

Printing of protein solutions

After removing the excess of water of the Si stamps with dust-free paper, all reservoirs of the stamp were either loaded with 10 μ L of FN (2 μ M, containing 10% of Rh-FN) in PBS or groups of 4 reservoirs were loaded with 2 μ L of a solution containing FN (2 μ M, containing 10% of Rh-FN) and hBMP-6 (0, 0.1, 0.45 or 1.75 μ M) in PBS. After applying the solutions to the reservoirs, the stamps were left in the fridge for 2 h. Prior to printing, the stamps were shock dried to remove excess solution. Subsequently, the loaded stamps were brought in conformal contact with either the epoxide-functionalized PDMS films (as described above) or with a featureless unfunctionalized PDMS substrate (non-oxydized) for 1 min. By carefully removing the Si stamp, the printing could be repeated up to 75 times. After 1 h, the protein patterned epoxide-functionalized PDMS substrates were rinsed with water and used for further experiments, while the protein patterned unfunctionalized PDMS substrates were further used for transfer printing to replicate the protein patterns onto the functionalized PTMC-GPTMS films (as described above). To this end, the protein patterned PDMS substrates were brought in conformal contact with the PTMC-GPTMS surfaces for 1 min. After 1 h, the protein patterned PTMC-GPTMS substrates were rinsed with water and used for further experiments.

For the immobilization of hBMP-6 only, loaded Si stamps were used for printing onto the same substrates following the same steps as described above. The quality of the printing was assessed by using the free software ImageJ.

SEM characterization

For assessing the morphology of the hydrogel in the bulk and inside the silicon chips, the gels were dehydrated in graded alcohols and subjected to critical point drying followed by sputter-coating with gold. Their surfaces were analyzed by scanning electron microscopy (SEM, Philips XL 30 ESEM-FEG, The Netherlands).

hBMP-6 immunostaining

Following hBMP-6 immobilization, the epoxide-terminated background was passivated by incubating the samples for 3 h in a solution of 0.1 wt% O-(2-aminoethyl)polyethyleneglycol 3,000 (NH₂-PEG) in PBS buffer. Subsequently, the substrates were washed with PBS buffer, rinsed with high-purity water and dried with dry nitrogen. A solution of 5 μ g/mL of monoclonal anti-hBMP-6 antibody produced in mouse (mouse IgG2B isotype, clone 74219.11, Invitrogen) was prepared in PBS buffer including 1% BSA and 200 μ L of the solution were used to incubate the protein arrays. After 1 h, the substrates were washed three times 10 min with PBS buffer including 1mM Tween-20, rinsed with water and dried with dry

nitrogen. Finally, 200 μL of a solution (2 $\mu\text{g/mL}$) Alexa Fluor® 488 goat anti-mouse IgG2a ($\gamma 2a$) (Invitrogen) in PBS buffer including 1% BSA were used to incubate the samples for 1 h. The substrates were then washed again three times 10 min with PBS buffer including 1 mM Tween-20, rinsed with water and dried with dry nitrogen. The arrays were inspected with fluorescence microscopy imaging.

Cell culturing

Substrates were sterilized prior to cell seeding with 70% ethanol and subsequently washed twice with PBS to remove any excess of ethanol. C2C12 (Mouse myoblast) cells were plated at a density of 25000 cells/ cm^2 in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich), supplemented with 10% FBS, 4.5 g/L glucose, L-glutamine and NaHCO_3 ; 1% (v/v) penicillin-streptomycin solution (with 10,000 units penicillin and 10 mg streptomycin/mL, Sigma-Aldrich) and cultured at 37 °C in a humidified 5% CO_2 atmosphere for 1 h. Subsequent to incubation, adhered cells were carefully washed twice with sterile PBS to remove loosely attached cells. Cells were fixed with a 4% formaldehyde solution in PBS for 10 min at room temperature and stained for F-actin with Alexa Fluor® 488-labeled phalloidin (Invitrogen) and DAPI dilactate (Invitrogen) following the protocol from the provider. Vinculin staining was performed by incubating the samples with a solution containing Monoclonal Anti-Vinculin FITC (Clone hVIN-1), diluted 50x from the supplied solution in PBS containing 1% BSA for 1h. Afterwards, the substrates were washed twice with PBS and water and carefully dried in a flow of N_2 and inspected using fluorescence microscopy imaging. For determining cell number and area of spreading, free software CellProfiler was used. For differentiation studies, the medium was supplemented with 0.2 mM L-ascorbic acid-2-phosphate (AsAP) and a seeding density of 10000 cells/ cm^2 was used. For ALP staining, cells were fixed in 10% buffered formalin for 1 h. ALP and counter hematoxylin stainings were performed using the Leukocyte Alkaline Phosphatase kit (Sigma Aldrich) according to manufacturer's protocol.

Live/Dead assay (LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells, Molecular Probes®) was performed according to the protocol from the provider.

Fluorescence Microscopy

Fluorescence microscopy images were taken using an Olympus inverted research microscope IX71 equipped with a mercury burner U-RFL-T as light source and a digital Olympus DR70 camera for image acquisition using the following Olympus filter cubes: $300 \text{ nm} \leq \lambda_{\text{ex}} \leq 400 \text{ nm}$ for blue emission ($410 \text{ nm} \leq \lambda_{\text{em}} \leq 510 \text{ nm}$), $510 \text{ nm} \leq \lambda_{\text{ex}} \leq 550 \text{ nm}$ for red emission ($\lambda_{\text{em}} \geq 580$) and $460 \text{ nm} \leq \lambda_{\text{ex}} \leq 490$

nm for green emission ($\lambda_{\text{em}} \geq 520$). All fluorescence microscopy images were acquired in air.

7.6 References

- [1] For reviews see for example: a) E. Phizicky, P. I. H. Bastiaens, H. Zhu, M. Snyder, S. Fields, *Nature* **2003**, 422, 208. b) D. Weinrich, P. Jonkheijm, C. M. Niemeyer, H. Waldmann, *Angewandte Chemie International Edition* **2009**, 48, 7744. c) P. F. Predki, *Current Opinion in Chemical Biology* **2004**, 8, 8. d) C. M. Niemeyer, *Trends in Biotechnology* **2002**, 20, 395. e) H. Zhu, M. Snyder, *Current Opinion in Chemical Biology* **2003**, 7, 55.
- [2] For reviews see for example: a) N. Nath, J. Hyun, H. Ma, A. Chilkoti, *Surface Science* **2004**, 570, 98. b) D. Falconnet, G. Csucs, H. Michelle Grandin, M. Textor, *Biomaterials* **2006**, 27, 3044.
- [3] For examples see: a) R. Singhvi, A. Kumar, G. Lopez, G. Stephanopoulos, D. Wang, G. Whitesides, D. Ingber, *Science* **1994**, 264, 696. b) D. Lehnert, B. Wehrle-Haller, C. David, U. Weiland, C. Ballestrem, B. A. Imhof, M. Bastmeyer, *Journal of Cell Science* **2004**, 117, 41. c) N. D. Gallant, K. E. Michael, A. J. García, *Molecular Biology of the Cell* **2005**, 16, 4329. d) M. Théry, *Journal of Cell Science* **2010**, 123, 4201.
- [4] For examples see: a) R. S. Kane, S. Takayama, E. Ostuni, D. E. Ingber, G. M. Whitesides, *Biomaterials* **1999**, 20, 2363. b) J. F. Young, H. D. Nguyen, L. Yang, J. Huskens, P. Jonkheijm, L. Brunsveld *ChemBioChem* **2010**, 11, 180. c) P. I. Rozkiewicz, Y. Kraan, M. W. T. Werten, F. A. de Wolf, V. Subramaniam, B. J. Ravoo, D. N. Reinhoudt, *Chemistry – A European Journal* **2006**, 12, 6290. d) G. Csucs, R. Michel, J. W. Lussi, M. Textor, G. Danuser, *Biomaterials* **2003**, 24, 1713.
- [5] C. Wendeln, B. J. Ravoo, *Langmuir* **2012**, 28, 5527.
- [6] For examples see: a) J. P. Renault, A. Bernard, D. Juncker, B. Michel, H. R. Bosshard, E. Delamarche, *Angewandte Chemie International Edition* **2002**, 41, 2320. b) A. Bernard, J. P. Renault, B. Michel, H. R. Bosshard, E. Delamarche, *Advanced Materials* **2000**, 12, 1067. c) M. Geissler, A. Bernard, A. Bietsch, H. Schmid, B. Michel, E. Delamarche, *Journal of the American Chemical Society* **2000**, 122, 6303. d) E. Delamarche, A. Bernard, H. Schmid, B. Michel, H. Biebuyck, *Science* **1997**, 276, 779. e) D. Juncker, H. Schmid, U. Drechsler, H. Wolf, M. Wolf, B. Michel, N. de Rooij, E. Delamarche, *Analytical Chemistry* **2002**, 74, 6139. f) J. Tien, C. M. Nelson, C. S. Chen, *Proceedings of the National Academy of Sciences* **2002**, 99, 1758. g) H.-W. Li, B. V. O. Muir, G. Fichet, W. T. S. Huck, *Langmuir* **2003**, 19, 1963. h) J. D. Gerding, D. M. Willard, A. Van Orden, *Journal of the American Chemical Society* **2005**, 127, 1106.
- [7] E. Bat, P. Jonkheijm, A. van Amerongen, J. Huskens: *Stamp for making a microarray of biomolecules*, **12186681.8**-1270 Filed on 28.09. 2012.
- [8] a) B. D. Martin, S. L. Brandow, W. J. Dressick, T. L. Schull, *Langmuir* **2000**, 16, 9944. b) M. Markowitz, D. Turner, B. Martin, B. Gaber, *Applied Biochemistry and Biotechnology* **1997**, 68, 57. c) B. D. Martin, B. P. Gaber, C. H. Patterson, D. C. Turner, *Langmuir* **1998**, 14, 3971.
- [9] M. A. Meißl, Z.-T. Zhu, V. Kumar, K. J. Lee, X. Feng, Y. Y. Huang, I. Adesida, R. G. Nuzzo, J. A. Rogers, *Nature Materials* **2006**, 5, 33.
- [10] T. Ebisawa, K. Tada, I. Kitajima, K. Tojo, T. K. Sampath, M. Kawabata, K. Miyazono, T. Imamura, *Journal of Cell Science* **1999**, 112, 3519.
- [11] C. Yan, J. Sun, J. Ding, *Biomaterials* **2011**, 32, 3931.
- [12] H. Jeon, H. Hidai, D. J. Hwang, K. E. Healy, C. P. Grigoropoulos, *Biomaterials* **2010**, 31, 4286.
- [13] S. Cesaro-Tadic, G. Dernick, D. Juncker, G. Buurman, H. Kropshofer, B. Michel, C. Fattinger, E. Delamarche, *Lab on a Chip* **2004**, 4, 563.

- [14] K. Francis, B. O. Palsson, *Proceedings of the National Academy of Sciences* **1997**, *94*, 12258.
- [15] a) X. Sui, A. Di Luca, M. K. Gunnewiek, E. S. Kooij, C. A. van Blitterswijk, L. Moroni, M. A. Hempenius, G. J. Vancso, *Australian Journal of Chemistry* **2011**, *64*, 1261. b) S. Edmondson, W. T. S. Huck, *Journal of Materials Chemistry* **2004**, *14*, 730.

Development of novel methodologies for tethering growth factors (GFs) to materials is highly desired for the generation of biomaterials with improved tissue repair properties. Progress in the development of biomaterials that incorporate GFs and the *in vivo* performance of such biomaterials in tissue engineering applications, such as stents, orthopaedic implants, sutures and contact lenses, is still challenged by the required control over the mobility of growth factors in biomaterials. Many of the current methodologies to introduce GFs in biomaterials suffer from a lack of control over the spatiotemporal delivery of GFs.

The aim of the work described in this thesis is the functional tethering of GFs to biomaterials using reversible chemical strategies with spatiotemporal control, thus following nature's paradigm. This work consisted of three parts: a) non-covalent strategies have been used to capture GFs to surfaces by employing nanobodies and peptides. In this part of the research considerable attention has been paid as well to fundamental aspects on controlling protein orientation in densely packed layers; b) reversible covalent chemistry has been used to control the spatiotemporal availability of GFs in the extracellular matrix (ECM) by using hydrolysable siloxane and imine bonds as examples and c) a protein array technology has been introduced to create functional platforms of various shapes and content for studying cell behavior.

In summary, the reversibility of the tether has been found to play important roles in the biological activity. The results of the studies demonstrate the advantage of tethers that combine *immobilized* GFs, such as GF stability or the creation of locally highly concentrated GF reservoirs, with *released* mobile GFs, such as optimization of orientation for an optimal interaction with cellular receptors. Although such systems are attractive, knowledge about the application of such tethering strategies *in vivo* is limited and deserves detailed attention in future research and leaves ample room for synthesis. For example, stimuli responsive systems might provide tools for a breakthrough in the tissue engineering field.

De ontwikkeling van nieuwe methodes voor het koppelen van groeifactoren (GF) aan oppervlakken opent de deur voor een nieuwe generatie van biomaterialen met verbeterde weefselhersteleigenschappen. De ontwikkeling en *in vivo* evaluatie van GF-dragende biomaterialen zoals stents, orthopedische implantaten, hechtmateriaal en contactlenzen, wordt bemoeilijkt door een gebrek aan controle over de GF-mobiliteit in dergelijke substraten. Veel van de huidige routes om GF in biomaterialen te introduceren beschikken niet over de gewenste GF-afgifte over de tijd en in de ruimte. Het doel van het werk, beschreven in dit proefschrift, is de functionele koppeling van GF aan biomaterialen waarbij controle in tijd en ruimte, zoals die wordt aangetroffen in de natuur, mogelijk wordt. Hierbij is gebruik gemaakt van reversibele chemische verankeringsmethoden, onderverdeeld in: a) niet-covalente methoden om GF op oppervlakken in te vangen door middel van nanobodies en peptiden. Hierbij is veel aandacht besteed aan fundamentele aspecten zoals eiwitoriëntatie in dichtgepakte eiwitlagen; b) Reversibele covalente chemie is gebruikt om de beschikbaarheid van GF in de extracellulaire matrix (ECM) te moduleren in ruimte en tijd. Hydrolyseerbare siloxanen en imine-bindingen zijn gebruikt om controle daarover te verkrijgen; c) Gebruikmakend van 'eiwitarray-technologie' zijn functionele substraten bereid waarbij de vorm alsmede de oppervlaktesamenstelling gevarieerd kon worden om celinteracties nader te bestuderen.

Samenvattend is er gevonden dat de omkeerbaarheid van GF-oppervlakteverankering van grote invloed is op de biologische activiteit. De resultaten laten de voordelen zien van de stabiliteit en een hoge lokale oppervlakteconcentratie van immobiele GF gecombineerd met GF-afgifte, resulterend in optimale oriëntatie en interactie met celreceptoren. Ondanks het feit dat deze systemen erg interessant en veelbelovend zijn, moet opgemerkt worden dat de kennis over de *in vivo* effecten van oppervlakteverankering beperkt is. Verder onderzoek is dus noodzakelijk hetgeen de verdere ontwikkeling van interactieve biomaterialen zal stimuleren en mogelijk maken. Deze technologieën zouden voor een doorbraak kunnen zorgen in de regeneratieve geneeskunde.

List of publications

- Fluorogenic Detection of the Co-immobilization of Peptides that Combine Cartilage Targeting and Regeneration.
Jordi Cabanas-Danés, Carlo Nicosia, Ellie Landman, Marcel Karperien, Jurriaan Huskens and Pascal Jonkheijm. *Submitted to Journal of Materials Chemistry B*.
- Immobilization of Ferrocene-Modified SNAP-fusion Proteins.
Dorothee Wasserberg, Dana Uhlenheuer, Pauline Neiryneck, **Jordi Cabanas-Danés**, Jan Hendrik Schenkel, Bart Jan Ravoo, Jurriaan Huskens, Lech Milroy, Luc Brunsveld and Pascal Jonkheijm. *Submitted to International Journal of Molecular Sciences*.
- Reversible and Oriented Immobilization of Ferrocene-Modified Proteins.
Lanti Yang, Alberto Gomez-Casado, Jacqui F. Young, Hoang D. Nguyen, **Jordi Cabanas-Danés**, Jurriaan Huskens, Luc Brunsveld and Pascal Jonkheijm.
Journal of the American Chemical Society **2012**, *134*, 19199-19206
- A Fluorogenic Reactive Monolayer Platform for the Signaled Immobilization of Thiols.
Carlo Nicosia, **Jordi Cabanas-Danés**, Pascal Jonkheijm and Jurriaan Huskens.
ChemBioChem **2012**, *13*, 778-782
- Selective Immobilization of Biomolecules on PTMC Network Surfaces Using Micro Contact Printing.
Sandra Teixeira, Arancha González-Campo, **Jordi Cabanas-Danés**, Jurriaan Huskens, Pascal Jonkheijm, Dirk W. Grijpma.
Macromolecular Symposia **2011**, 309-310, 16-19

Publications in preparation:

- A Supramolecular Approach to Growth Factor Immobilization Using His₆-tagged VHH Single-domain Antibodies.
Jordi Cabanas-Danés, Emilie Rodrigues, Jasper van Weerd, Ellie Landman, Clemens van Blitterswijk, Theo Verrips, Jurriaan Huskens, Marcel Karperien and Pascal Jonkheijm.
- Spatiotemporal Regulation of hBMP-6 Growth Factor by Reversible Covalent Surface Tethering.
Jordi Cabanas-Danés, Ellie Landman, Clemens van Blitterswijk, Jurriaan Huskens, Marcel Karperien and Pascal Jonkheijm
- Controlling the Orientation of Self-assembled Proteins with Multiple His₆-tags.
Dorothee Wasserberg, **Jordi Cabanas-Danés**, Jurriaan Huskens, Vinod Subramaniam and Pascal Jonkheijm
- Orthogonal Supramolecular Assembly of His₆-tagged and Adamantyl-modified Proteins.
Jordi Cabanas-Danés, Dorothee Wasserberg, Vinod Subramaniam, Jurriaan Huskens and Pascal Jonkheijm
- Strategies for Mimicking, Recruiting, Delivering and Presentating Growth Factors at the Cell-Material Interface.
Jordi Cabanas-Danés, Tushar Satav, Jurriaan Huskens and Pascal Jonkheijm
- Cell Studies with Arrays of Multiple Proteins Fabricated Using a Hydrogel-Filled Silicon Stamp.
Jordi Cabanas-Danés, Erhan Bat, Pieter Moonen, Jurriaan Huskens and Pascal Jonkheijm
- Protein Microarrays Using Hydrogel-Filled Silicon Stamps.
Erhan Bat, **Jordi Cabanas-Danés**, Pieter Moonen, Truus Posthuma-Trumpie, Aart van Amerongen, Jurriaan Huskens and Pascal Jonkheijm

Acknowledgments

Four years and a bit ago, I (re)landed in the Netherlands. This time however, it was the start of a very long journey which still continues today. If when still sitting next to the plane window, anyone told me the amount of incredibly inspiring people I would get the chance to meet, most of them becoming crucial in my day-to-day life by listening to me, advising me and encouraging me in addition to the constant support of my family and friends, I would not have believed it. Most likely I'm gonna forget names but please, don't feel offended by that. I really appreciate what you did for me.

I would like to start by expressing my gratitude to my supervisors **Jurriaan** and **Pascal**.

Jurriaan, niet alleen ben ik je dankbaar voor dat je me de kans hebt gegeven om onderzoek te doen binnen de MnF groep en voor dat je mijn promotor was, maar ook voor het feit dat je me de vrijheid hebt gegeven om mijn wetenschappelijk interesses en motivaties te ontdekken en je me hierbij altijd onvoorwaardelijke steun en advies hebt gegeven. Hoewel ik regelmatig dichter bij biologische dan bij scheikundige onderwerpen met mijn onderzoek terecht kwam, heb ik veel plezier gehad in onze discussies over het modeleren van de thermodynamische gedrag van multivalente proteïne oppervlakte systemen.

Pascal, de laatste jaren had ik de eer om jou als mijn dagelijkse supervisor te hebben. Ik ben je erg dankbaar dat je mij elke keer dat ik alarmerend naar je kantoor kwam ("Pascal, I think we have a problem") met alweer een 'verbal waterfall' zoals je dat noemt, aanmoedigd en gemotiveerd hebt. Ik heb me hierdoor altijd comfortabel gevoeld om mijn wetenschappelijke twijfels en progressive aan jou te vertellen. Het voelde, vooral vanwege jouw geduld en inlevingsvermogen, alsof ik het tegen een vriend vertelde. Dankjewel!

Though not directly involved with the research presented in this book, I would also like to express my gratitude to the other staff members of the MnF/SMCT/BNT cluster for their support: **Aldrik**, **Jeroen**, **Wim**, **Natalie** and above all **Melissa**, whom I thank a lot for all the chats in the swimming pool. Even though we ended up talking more than swimming it was really useful against the stress during the writing time. Ik wil ook graag de technici **Regine**, **Marcel**, **Richard**, **Bianca** en **Tieme** bedanken. Jullie belangrijke taken hebben de voortgang van mijn onderzoek mogelijk gemaakt. Dank aan **Izabel**, **Nicole** en **Gerardine** voor hun hulp bij het verslaan van alle bureaucratische dreigementen.

During this journey, I have had the opportunity not only to join forces with many great people which enhanced both the quality and the efficiency of the research (the best proof of that is that without these collaborations this book would not exist) but also, today I can call every single one of them a friend of mine. **Dodo**, since the first moment I met you, you've been a constant support both scientifically and personally from whom I've learnt a lot, *danke sehr!* **Emilie**, a big *merci beacoup/obrigado* both for all our scientific and non-scientific conversations during the "koffiepauze" of the BMM meetings. **Carlo**, grazie mille per tutti i momenti condivisi entrambi al lavoro come fuori. Vi auguro tutto il meglio a te e a **Mariska**. **Ellie**, ik wil jou echt bedanken voor al je hulp, bijna al mijn kennis over het kweken van cellen en biologische activiteit testen heb ik aan jou te danken. Ik ben blij dat we nooit de motivatie verloren om tot het gaatje te gaan en te ontdekken wat de KS cellen ons wilden vertellen.

Erhan, *çok teşekkür ederim* for all your ideas, tips and tricks which have been indispensable for our research presented in chapter 7. I am sure that your calmness and kind personality will lead you to a very successful career and I wish you a lot of happiness with **Arzu**. I had a great time visiting you guys in Los Angeles!

Not many months after I started my PhD I was lucky to meet a great friend of mine who has offered me unconditional help at any time to finally become one of my *paranimfen*. **Raluca**, nu am crezut vreodată ca voi întâlni în Enschede o persoană care să îmi devină *paranimf*, prietenă, soră, mamă și bunică ;) în același timp. Îți mulțumesc pentru tot ce ai făcut pentru mine de lungul acestor ani. Îți doresc tot binele din lume și sper că ne vom aminti râzând atât de momentele bune cât și de cele rele petrecute împreună. Ik wil ook graag mijn andere *paranimf* **Jasper** bedanken voor de lol binnen en het buiten het lab. Zelfs dagen die compleet misliepen werden een *grrrrreat* success met een dosis humor. Bedankt voor alle vruchtbare discussies en je hulp bij het organiseren van mijn verdediging.

I am really thankful to my lab mates for creating the perfect "working atmosphere" which met the compromise between helping each other and joking about each other in equal amounts. From the Langezijds crew: **Pieter**, bedankt voor je hulp bij diverse situaties, van belastingdocumenten, een bezoek aan de ambassade van Ghana in Den Haag, tot het dragen van meubels van IKEA. **Sven**, bedankt voor alle lol die we hadden met het pesten van elkaar.

Alessandro e Francesca S., grazie per essere sempre pronti entrambi per aiutarmi nel laboratorio e per le seratte insieme. **Carmen**, since I met you during your master assignment I've always enjoyed the time with you both at work and outside. *Danke schön* to you and **Jan** for inviting us to your great wedding!

Shu Han, 謝謝 for all your smiles and positive thoughts that made you a role model for many of us. **Alberto** and **Melanie**, *muchísimas gracias* and *vielen Dank* for all the tricks and tips and all the dinners at your place and nights out. Ánimos y suerte con todo. **Arancha**, moltes gràcies per tot. Vull que sàpiques que encara que fés broma amb lo de dir-te "mama", el teu suport i els teus consells em van ajudar tal i com ho fan els d'una mare. **Kim**, thanks a lot for all your funny stories. You are a born entertainer, good luck in Singapore! **Jealemy**, muchas gracias por todos los momentos en que el "príncipe de la oscuridad" decidía animarse a echarse unas risas. Suerte en Italia! Vorrei anche ringraziare al resto della comunità italiana a Langezijds: **Albert**, **Francesca C.** and **Riccardo** per tutti i buoni momenti nel lavoro e nelle seratte fuori. Before moving to Carré, I still would like to thank a lot of people which in a way or another contributed to making my day-to-day life very pleasant in Langezijds: **Victoria**, **Ignacio**, **Xuexin**, **Lanti**, **Mariana**, **Chien-Ching**, **Yiping**, **Oktay**, **Daniel**, **Dae June**, **Anna**, **Mudassir**, **Nicolai**, **Vijay**, **Deniz**, **Denis** (спасибо for all the good moments we had in our flat!), and from MTP: **Janet** (çok teşekkür ederim for all the teas at your place and the good moments in San Diego!), **Oya**, **Joost**, **Eddy**, **Edit** and **Clemens**.

After two years since I started, we moved to a brand new building called Carré, where my luck to meet inspiring people didn't cease and to whom I'm very grateful.

Angel, 謝謝 for all the inspiring evening chats in the office. I always admired your passionate curiosity for learning new topics which was represented by the amount of books you read and which created a big pile/mess on your desk. I guess it was inspiring to me cause I also managed to create the same (or a bigger) mess of articles and books on my desk even without having read them!

Emanuela, anche se il nostro periodo insieme in MnF non è durato molto, come dicono a volte, è stato intenso. Grazie per tutte le chiacchierate nei vagoni di silenzio in treno. Ti auguro molta felicità. **Wies**, hoewel je nieuw in deze groep bent, wil ik je bedanken voor de goede sfeer die je op het werk bracht. Ik hoop dat we elkaar nog regelmatig zullen ontmoeten om te dansen en we in "ademnood" raken. **Rick** en **Tom**, jullie zouden een boek moeten schrijven met de titel: "How to get your PhD without losing your nerves". Ik hoop dat jullie gedurende de rest van jullie PhD zo kalm zullen blijven en ik ben ervan overtuigd dat jullie de PhD succesvol zullen afronden.

Roberto, voglio ringraziarti anche a te per tutte le conversazione che abbiamo avuto provando a capire come funziona il mondo. Buona fortuna con il resto del tuo PhD. **Rajesh**, also *Romba Nandri* for your support with your optimistic words. I'm also thankful to the new crew in Pascal's group: **Bettina**, **Jenny**, **Shirish** and **Tushar** even if you arrived at the end of my PhD years I always enjoyed the time

with you full of jokes and laughs. **Raquel**, qué te voy a decir a tí? Muchas gracias por el cariño que me has mostrado desde que llegaste. Aunque a veces las cosas no vayan como uno se imaginaba sé que tú tienes la fuerza para superar cualquier contratiempo. Suerte con todo y mucha felicidad con **Thomas**.

Alejandro y Laura gracias también a vosotros por las charlas nocturnas o de fin de semana. Special thanks to all the other members of the cluster MnF/BNT who helped me in multiple ways during my time in Carré: **Sarah, Supitchaya, Tibor, Rik, Anne, Martijn, Ye Liang, Bala, Shahina, Jiguang Liu, Chengfen, Yujie, Ben, Rianne, Maarten** (hartstikke bedankt voor het nakijken van mijn proefschrift!). Thanks to all the students (bachelor, master, hogeschool, erasmus, visiting PhD's and postdocs...) who spent some time working within our groups with whom I've always been happy to chat. Also, I'm grateful to all members of TR and DBE groups always willing to help me and answer the thousands of biological questions I asked them. Zo wil ik ook graag **Marcel Karperien** bedanken voor de vruchtbare discussies over het onderzoek beschreven in hoofdstuk 4 en 6. **Sandra**, obrigado for your support and advices in the time we worked together. **Dirk Grijpma**, ook bedankt voor jouw wetenschappelijke bijdrage. Per anar acabant, moltes gràcies als meus amics de l'Esquirol, els quals tot i la distancia, han sapigut estar sempre presents i recolzar-me en el que fes falta. Gràcies **Ivette i Joan** (merci per la portada també!), **Sara i Quico** (molta sort amb la petita Bruna), **Ferran, Tina, Gemma i Jordi**. També vull donar les gràcies als amics de l'institut amb qui més o menys sovint hem anat seguint la tradició dels nostres sopars: **Susanna, Montse, David, Guillem i Marta** així com a totes les meves companyes de pis: **Anna, Eli, Gemma, Sandra, Marina i Blanca**. Moltes gràcies **Javi** pel teu suport malgrat tots els alts i baixos. Gràcies també a la meva gran amiga **Núria**. Des de que ens vam conèixer a York a plogut molt però sempre he sentit que em feies costat en tot el que necessitava. Moltes gràcies i sort amb la teva defensa també. **Berta**, moltes gràcies per fer-me de germana gran ja durant l'erasmus i més tard quan vaig tornar per començar la tesi. El teu suport va ser essencial per encarrilar-me a Enschede.

Een van de moeilijkste dingen vond ik om mijn familie achter te laten. Maar ik had niet gelukkiger kunnen zijn, niet alleen kreeg ik onvoorwaardelijke steun van mijn familie in Catalonië, maar ik heb ook een tweede familie in Nederland leren kennen. **Nan, Richard, Cécile, Alie, Arie, Jet en Gijs**, bedankt voor het warme welkom in jullie familie en de steun die ik sindsdien van jullie gekregen heb.

No hi ha prou paraules per agrair el recolzament rebut per part de la meva família. No tan sols m'heu ajudat sempre durant aquests anys fora, si no que també sou els responsables de que hagi pogut tenir l'oportunitat de seguir aquesta carrera a través del vostre amor i suport incondicionals. Per això i molt

més us vull donar les gràcies: **Pare, Mare, Íngrid i Ariadna** així com també a tots els tiets, tietes, cosins, cosines, cunyats i en especial a la meva **àvia**, qui malauradament no podrà ser-hi en aquest dia tan especial però qui sempre em va fer sentir molt estimat i recolzat.

En als laatste, maar zeker niet de minste, is er **Danny**, jouw onophoudelijke liefde en steun hebben mij altijd vertrouwen gegeven in deze stressvolle periode waarin er niets anders in ons leven leek dan het proefschrift. Verrassend genoeg, na dit alles, heb je toch geaccepteerd om samen te gaan wonen in ons kleine huisje. Ik moet heel veel geluk hebben. Je hebt mijn liefde en eeuwige dankbaarheid en ik voorspel veel avonturen en geluk in deze nieuwe periode.

Jordi

Amsterdam, December 2012

About the Author

Jordi Cabanas Danés was born on January 28th, 1985 in Vic, Catalonia, Spain. He studied Chemistry at the *Universitat Autònoma de Barcelona (UAB)*, where he received his degree (*Llicenciatura en Química*) in June 2008. During his undergraduate period, he worked as Erasmus student on the characterization and the catalytic study of Platinum based catalysts for the steam reforming reactions of methane, ethane and ethylene under the supervision of Prof. Dr. Ir. L. Lefferts, Dr. B. L. Mojet and Dr. Ir. P. O. Graf. The work was carried out in 2006 at the Catalytic Processes & Materials Group (CPM), University of Twente (The Netherlands).

In October 2008, he started his PhD research under the supervision of Prof. Dr. Ir. Juriaan Huskens and Dr. Ir. P. Jonkheijm at the Molecular Nanofabrication (MnF) group, University of Twente (The Netherlands), on the subject of growth factor immobilization strategies for tissue regeneration applications. The results of this research are described in this thesis.