An abstract illustration of a molecular or nanoscale structure. It features several green, semi-transparent spheres of varying sizes, connected by a network of orange, tube-like structures. The background is a solid blue color. The overall composition suggests a complex, interconnected network, possibly representing a polymer or a nanogel.

ZWITTERIONIC POLY(AMIDO AMINE) BASED NANOGEELS

TONY EKKELENKAMP

Zwitterionic poly(amido amine) based nanogels

Tony Ekkelenkamp

This research was financially supported by the provinces of Overijssel and Gelderland as part of the PET-MRI project, part of the Center for Medical Imaging North East Netherlands.

Printing of this thesis was supported by the Netherlands Society of Biomaterials & Tissue Engineering

Zwitterionic poly(amido amine)-based nanogels

Tony Ekkelenkamp

PhD thesis with references and summaries in English and Dutch
University of Twente, Enschede, the Netherlands

ISBN: 978-90-365-4255-5

DOI: 10.3990/1.9789036542555

Copyright © 2016 by Tony Ekkelenkamp. All rights reserved

Printed by Wörmann Print Service, Zutphen, the Netherlands

Cover design by Albert Wieringa (cover) and Ezra Theunissen (3D model)

ZWITTERIONIC POLY(AMIDO-AMINE) BASED NANOGELS

PROEFSCHRIFT

ter verkrijging van
de graad van doctor aan de Universiteit Twente,
op gezag van de rector magnificus,
Prof. dr. T.T.M. Palstra,
volgens besluit van het College voor Promoties
in het openbaar te verdedigen
op woensdag 7 december 2016 om 14:45 uur

Door

Antonie Everhard Ekkelenkamp

Geboren op 18 september 1986
te Zwolle

Dit proefschrift is goedgekeurd door:

Prof. dr. J.F.J. Engbersen (promotor)

Dr. J.M.J. Paulusse (Co-promotor)

© 2016 Tony Ekkelenkamp

ISBN: 978-90-365-4255-5

Samenstelling van de commissie:

Voorzitter: Prof.dr.ir. J.W.M. Hilgenkamp

Promotor: Prof. dr. J.F.J. Engbersen

Co-promotor: Dr. J.M.J. Paulusse

Leden: Prof. dr. ir. R.G.H. Lammertink
Universiteit Twente

Prof. dr. G.J. Vancso
Universiteit Twente

Prof. dr. P.H. Elsinga
Rijksuniversiteit Groningen

Dr. S. Leeuwenburgh
Radboud Universiteit Nijmegen

Dr. T. J. Visser
Syncom Groningen

"Maybe the sun will shine today,
the clouds will roll away.
Maybe I won't feel so afraid
and I will understand
that everything has its plan.
Either way."

Wilco, Either way

TABLE OF CONTENTS

Front matter	i
Table of Contents	ix
<u>Chapter 1</u>	1
General Introduction	
<u>Chapter 2</u>	9
Responsive Crosslinked Polymer Nanogels for Targeted Imaging and Therapeutics Delivery	
<u>Chapter 3</u>	63
Nanogel Formation by Inverse Nanoprecipitation of Zwitterionic Poly(amido amine)s	
<u>Chapter 4</u>	85
Functionalization of Poly(amido amine) Nanogels	
<u>Chapter 5</u>	101
Surfactant-free Preparation of Highly Stable Zwitterionic Poly(amido amine) Nanogels with Minimal Cytotoxicity	
<u>Chapter 6</u>	127
Poly(amido amine) Nanogels for Prostate Cancer Targeting	
Summary	149
Samenvatting	153
Dankwoord	157
Curriculum Vitae	161

CHAPTER 1

General Introduction

1. Nanoparticles for medical diagnostics and therapeutics

This thesis describes the development of zwitterionic nanogels as tool for diagnostic and therapeutic applications in prostate cancer. Over the past decades, the introduction of nanotechnology into therapeutic and diagnostic applications, i.e. nanomedicine, has generated considerable attention, as well as promising results, predominantly for cancer diagnosis and treatment.^{1,2} Many nanomedicine applications incorporate delivery of molecules for diagnostic or therapeutic purposes to specific locations of the body. Consequently, the delivery of many of these molecules requires effective delivery vehicles.¹⁻³ In this respect, polymeric nanostructures offer a versatile solution, since polymeric nanostructures show great variety in structure and physicochemical properties.^{4,5} Moreover, incorporation of biodegradable moieties into the polymer nanostructure may serve as a release mechanism, while also avoiding particle retention in the body, lowering possible toxic side-effects.^{5,6} Nano-sized hydrogels, or nanogels, are a particular class of polymeric nanostructures with interesting properties, such as high water content and high payload capacity, making them suitable for therapeutic delivery and targeted imaging.^{7,8} Similar to hydrogels, nanogels are composed of hydrophilic, crosslinked, polymer networks, which offer ample possibilities for carrying drug molecules,⁹ proteins¹⁰, nucleic acids,¹¹ and imaging contrast agents.¹² Due to their swollen state, nanogels can be designed with responsive properties for triggered payload release or contrast generation upon exposure to specific physicochemical cues.¹³

The concept of targeted delivery of therapeutics to specific diseased sites has already been proposed by Paul Ehrlich in the 19th century.¹⁴ It has received ample attention in the literature over the past decades.^{4,15,16} Targeted medicine allows maximization of therapeutic efficacy, while minimizing adverse effects for the patient.¹⁴ Therefore, specific and efficacious targeting of nanomedicines has become an important subject within the field of nanomedicine. Passive targeting can be achieved by size-specific accumulation of nanoparticles in tumor tissue and inflammation sites, due to an increased permeability of the local vasculature to nanoparticles smaller than 400 nm in diameter.^{15,17} Furthermore, the absence of lymphatic drainage in tumor tissue causes passive accumulation of nanoparticles within the tumor tissue. Active targeting can be achieved by conjugation of molecules, such as small targeting ligands,¹⁸ aptamers¹⁹ and antibodies,²⁰ onto nanoparticles to promote interaction with specific cells and tissues. Effective targeting and delivery of nanomedicines therefore involves minimization of non-specific interactions with proteins and cells, while promoting specific interactions with targeted cells and tissues.¹⁷ Moreover, targeting efficacy is increased when nanoparticles are retained within the bloodstream for an extended period of time. This is generally achieved by coating nanoparticles with a hydrophilic poly(ethylene glycol) (PEG) layer, which reduces non-specific protein adsorption onto the nanoparticle,

resulting in a delayed immunologic response and subsequent nanoparticle removal from the bloodstream.²¹

Poly(amido amine)s are a class of hydrophilic, peptidomimetic polymers, which have been extensively studied as an efficient nucleic acid delivery vehicle, while exhibiting minimal toxicity.^{22–24} Their facile, yet highly modular synthesis via a Michael polyaddition between bisacrylamides and bi-functional amines enables the incorporation of specific chemical moieties. Incorporation of ethylenediamine-*N,N'*-diacetic acid into the poly(amido amine) backbone grants the polymer zwitterionic properties, which are potentially advantageous for application in biological environments.²⁵ Similar to PEG, zwitterionic materials have shown to prevent non-specific interactions with proteins or cells. Moreover, due to their ionic and highly hydrophilic nature, zwitterionic materials bind water more tightly than PEG, which leads to only minimal interactions with proteins and cells.²⁶ Therefore, zwitterionic nanogels have shown high stability in serum²⁷ and extended retention within the bloodstream.²⁸ Zwitterionic poly(amido amine) nanogels therefore potentially offer opportunities for application as a highly stable carrier for application in imaging and drug delivery.

2. Aim and outline of this thesis

The content described in this thesis aims to: (i) prepare zwitterionic nanogels, composed of poly(amido amine)s, for application as a delivery vehicle for diagnostics and therapeutics; (ii) evaluate the chemical and physical properties of zwitterionic poly(amido amine) nanogels and their *in-vitro* stability and cytotoxicity; (iii) evaluate the potential of zwitterionic poly(amido amine) nanogels for targeted imaging of prostate cancer.

This thesis describes the formation and characterization of zwitterionic poly(amido amine) nanogels. These zwitterionic polymers were prepared via Michael addition between *N,N'*-methylenebis(acrylamide) and ethylenediamine-*N,N'*-diacetic acid. Nanogels were prepared by inverse nanoprecipitation of these polymers and subsequent crosslinking with ethylenediamine. The potential for conjugation and functionalization of these nanogels was evaluated, and their stability and cytotoxicity was studied through *in-vitro* cell experiments. The ability of targeting prostate-specific membrane antigen-expressing prostate cancer cells was assessed.

In **Chapter 2** a general overview of nanogels is given, as well as their applications as responsive materials in the delivery of therapeutics and imaging agents.

In **Chapter 3** the formation of zwitterionic poly(amido amine) nanogels via inverse nanoprecipitation is described. The synthesis of zwitterionic poly(amido amine) is described, as well as the formation of nanogels by inverse nanoprecipitation and subsequent crosslinking in a number of different non-solvents. Size and polydispersity of

the nanogels were evaluated during and after inverse nanoprecipitation and the results were compared to existing data on nanoprecipitation of polymers, to better understand the nanogel formation process.

In **Chapter 4** strategies for the functionalization of zwitterionic poly(amido amine) nanogels are evaluated. Nanogels were modified with cationic surface charges via *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) coupling, while nanogel functionalization with a fluorophore was carried out via isothiocyanate coupling. Azide moieties were introduced onto the nanogels via a diazo-donor and subsequently functionalized with fluorophores via isothiocyanate coupling and copper-free Huisgen azide-alkyne cycloaddition.

In **Chapter 5** the preparation and characterization of zwitterionic poly(amido amine) nanogels and poly(amido amine) nanogels modified with cationic charges is described. Their stability in fetal bovine serum and their cytotoxicity in three cell lines were evaluated. Cellular uptake of both nanogels, functionalized with a fluorescent label, was compared.

Chapter 6 describes the preparation of prostate-cancer targeting nanogels by functionalization of zwitterionic poly(amido amine) nanogels with ligands targeting the prostate-specific membrane antigen (PSMA). Their functionalization degree was determined and the ligand-content on these nanogels was estimated. Finally, the targeting ability of the nanogels was evaluated *in-vitro* with two different prostate cancer cell lines, of which only one expressed PSMA as a membrane protein.

References

- (1) Kim, B. Y. S.; Rutka, J. T.; Chan, W. C. W. *N. Engl. J. Med.* **2010**, 363 (25), 2434–2443.
- (2) Riehemann, K.; Schneider, S. W.; Luger, T. A.; Godin, B.; Ferrari, M.; Fuchs, H. *Angew. Chemie Int. Ed.* **2009**, 48 (5), 872–897.
- (3) Elsabahy, M.; Heo, G. S.; Lim, S.-M.; Sun, G.; Wooley, K. L. *Chem. Rev.* **2015**, 115 (19), 10967–11011.
- (4) Nicolas, J.; Mura, S.; Brambilla, D.; Mackiewicz, N.; Couvreur, P. *Chem. Soc. Rev.* **2013**, 42 (3), 1147–1235.
- (5) Elsabahy, M.; Wooley, K. L. *Chem. Soc. Rev.* **2012**, 41 (7), 2545.
- (6) Zhang, X.; Malhotra, S.; Molina, M.; Haag, R. *Chem. Soc. Rev.* **2015**, 44 (7), 1948–1973.
- (7) Kabanov, A. V.; Vinogradov, S. V. *Angew. Chemie Int. Ed.* **2009**, 48 (30), 5418–5429.
- (8) Oh, J. K.; Drumright, R.; Siegwart, D. J.; Matyjaszewski, K. *Prog. Polym. Sci.* **2008**, 33 (4), 448–477.
- (9) Ju, C.; Mo, R.; Xue, J.; Zhang, L.; Zhao, Z.; Xue, L.; Ping, Q.; Zhang, C. *Angew. Chemie Int. Ed.* **2014**.
- (10) Nochi, T.; Yuki, Y.; Takahashi, H.; Sawada, S.; Mejima, M.; Kohda, T.; Harada, N.; Kong, I. G.; Sato, A.; Kataoka, N.; Tokuhara, D.; Kurokawa, S.; Takahashi, Y.; Tsukada, H.; Kozaki, S.; Akiyoshi, K.; Kiyono, H. *Nat. Mater.* **2010**, 9 (7), 572–578.
- (11) Averick, S. E.; Paredes, E.; Irastorza, A.; Shrivats, A. R.; Srinivasan, A.; Siegwart, D. J.; Magenau, A. J.; Cho, H. Y.; Hsu, E.; Averick, A. A.; Kim, J.; Liu, S.; Hollinger, J. O.; Das, S. R.; Matyjaszewski, K. *Biomacromolecules* **2012**, 13 (11), 3445–3449.
- (12) Wu, W.; Mitra, N.; Yan, E. C. Y.; Zhou, S. *ACS Nano* **2010**, 4 (8), 4831–4839.
- (13) Molina, M.; Asadian-Birjand, M.; Balach, J.; Bergueiro, J.; Miceli, E.; Calderón, M.; Calderon, M. *Chem. Soc. Rev.* **2015**, 44 (17), 6161–6186.
- (14) Strebhardt, K.; Ullrich, A. *Nat. Rev. Cancer* **2008**, 8 (6), 473–480.
- (15) Albanese, A.; Tang, P. S.; Chan, W. C. W. *Annu. Rev. Biomed. Eng.* **2012**, 14, 1–16.
- (16) Bertrand, N.; Wu, J.; Xu, X.; Kamaly, N.; Farokhzad, O. C. *Adv. Drug Deliv. Rev.* **2014**, 66, 2–25.
- (17) Alexis, F.; Pridgen, E.; Molnar, L. K.; Farokhzad, O. C. *Mol. Pharm.* **2008**, 5 (4), 505–515.
- (18) Hrkach, J.; Von Hoff, D.; Mukkaram Ali, M.; Andrianova, E.; Auer, J.; Campbell, T.; De Witt, D.; Figa, M.; Figueiredo, M.; Horhota, A.; Low, S.; McDonnell, K.; Peeke, E.; Retnarajan, B.; Sabnis, A.; Schnipper, E.; Song, J. J.; Song, Y. H.; Summa, J.; Tompsett, D.; Troiano, G.; Van Geen Hoven, T.; Wright, J.; LoRusso, P.; Kantoff, P. W.; Bander, N. H.; Sweeney, C.; Farokhzad, O. C.; Langer, R.; Zale, S. *Sci. Transl. Med.* **2012**, 4 (128), 128ra39.
- (19) Lao, Y.-H.; Phua, K. K. L.; Leong, K. W. *ACS Nano* **2015**, 9 (3), 2235–2254.
- (20) Nelson, A. L.; Dhimolea, E.; Reichert, J. M. *Nat. Rev. Drug Discov.* **2010**, 9 (10), 767–774.
- (21) Knop, K.; Hoogenboom, R.; Fischer, D.; Schubert, U. S. *Angew. Chemie Int. Ed.* **2010**, 49 (36), 6288–6308.
- (22) Lin, C.; Engbersen, J. F. J. *J. Control. Release* **2008**, 132 (3), 267–272.
- (23) Martello, F.; Piest, M.; Engbersen, J. F. J.; Ferruti, P.; Gonçalves, C. M. B.; Tomé, L. C.; Garcia, H.; Brandão, L.; Mendes, A. M.; Marrucho, I. M. *J. Control. release* **2012**, 164 (3), 372–379.

- (24) van der Aa, L. J.; Vader, P.; Storm, G.; Schiffelers, R. M.; Engbersen, J. F. J. *J. Control. release* **2011**, *150* (2), 177–186.
- (25) Keefe, A. J.; Jiang, S. *Nat. Chem.* **2011**, *4* (1), 59–63.
- (26) Zhang, L.; Cao, Z.; Bai, T.; Carr, L.; Ella-Menye, J.-R.; Irvin, C.; Ratner, B. D.; Jiang, S. *Nat. Biotechnol.* **2013**, *31* (6), 553–556.
- (27) Cheng, G.; Mi, L.; Cao, Z.; Xue, H.; Yu, Q.; Carr, L.; Jiang, S. *Langmuir* **2010**, *26* (10), 6883–6886.
- (28) Zhang, L.; Cao, Z.; Li, Y.; Ella-Menye, J.-R.; Bai, T.; Jiang, S. *ACS Nano* **2012**, *6* (8), 6681–6686.

CHAPTER 2

Responsive Crosslinked Polymer Nanogels for Imaging and Therapeutics Delivery

Antonie E. Ekkelenkamp, M. Rachèl Elzes, Johan F.J. Engbersen
and Jos M.J. Paulusse

Manuscript submitted to Journal of Materials Chemistry B

1. Nanomedicine

Technological advances in the past few decades have fueled a revolution in the world of medicine. New technologies, such as advanced biomedical imaging,¹ production of sophisticated biomaterials,² molecular imaging,³ gene editing,⁴ targeted medicine,⁵ stem cell technology,⁶ theranostics⁷ and DNA sequencing,⁸ enable detailed monitoring and controlling of physiological processes. Therefore, diagnostics and therapy are dealing increasingly with processes occurring on a cellular level.^{9,10} These developments have also increased the need for diagnostic and therapeutic tools acting on the molecular level,¹⁰ where specific molecular interactions become of the utmost importance. Solutions to fulfill these needs are offered in the field of nanomedicine, where these problems are approached from a nanotechnology perspective.⁹⁻¹¹ A central part to nanomedicine is the treatment and detection of diseases by applying nanoparticles, which exist in an enormous variety,¹² ranging from inorganic nanoparticles, such as gold nanoparticles¹³ and silica nanoparticles,¹⁴ to polymer nanoparticles.⁵ The application of macromolecules and polymers in nanomedicine is widespread, whether as a stabilizer for e.g. inorganic nanoparticles¹³ or as a nanocarrier.⁵ Due to their tremendous variability and versatility, macromolecular nanostructures play a pivotal role in the areas of controlled drug delivery and imaging.^{11,15}

2. Macromolecular architectures

Nano-sized macromolecular nanostructures, such as polymer nanospheres,^{5,15-17} polymersomes,^{18,19} dendrimers,²⁰⁻²² polymeric micelles,²³⁻²⁵ and nanogels,²⁶⁻²⁹ show great variety in structure, physical properties and opportunities for functionalization.³⁰ Their variability in size and physiochemical properties^{31,32} makes these structures applicable in a wide range of biomedical applications, from controlled release to targeted imaging of tissues.^{11,15,33} Several macromolecular nanostructures and their typical size-regimes are shown in Figure 1 and will be discussed below

Figure 1: Macromolecular nanostructures and their size-range.

2.1. Dendrimers

Dendrimers are very well-defined branched macromolecules and offer accurate control over end-group functionalization.^{20,21} Moreover, due to the employed synthetic approaches, these materials are essentially still molecules, facilitating the identification of potential therapeutic activity and hence acceptance for clinical application. Therefore, these structures are promising as imaging carriers^{22,34–38} and specific targeting with small molecules.^{39–41} However, a distinctive disadvantage is that dendrimers are synthesized by consecutive multi-step syntheses, often with low yields and high costs.^{42,43} Therefore, as an alternative, the design and synthesis of hyperbranched polymers have received increasing interest, which allow the facile synthesis of well de-fined dendritic polymer structures with a low polydispersity.^{44,45}

2.2. Self-assembled nanostructures

Nanostructures such as polymeric micelles,^{46,47} polymersomes^{48,49} and liposomes,⁵⁰ depend on hydrophobic interactions of amphiphilic macromolecules.^{48,51} Owing to their amphiphilic nature, these nanostructures are very suitable for the encapsulation of either hydrophobic or hydrophilic drugs.^{48,50} Furthermore, the encapsulation of contrast agents for medical imaging enables the application of these nanostructures for imaging purposes.^{12,52,53} Moreover, the combination of imaging and therapy, i.e. theranostics, is often an application of these self-assembled nanostructures.^{47,54}

2.3. Polymer nanospheres

Polymer nanospheres can be considered as “solid spheres” in aqueous environments, consisting of water-insoluble polymer particles dispersed in aqueous solution.^{55,56} Many polymers, such as polystyrene,⁵⁷ poly(*n*-butyl cyanoacrylate)⁵⁸ and poly(lactic-*co*-glycolic acid)⁵ have been extensively used for nanosphere preparation for biomedical applications.¹⁵ Poly(lactic-*co*-glycolic acid) (PLGA) nanospheres have been researched extensively throughout the past two decades.^{5,15–17} PLGA is a relatively safe and biodegradable polymer, which is approved for medical use by the FDA as well as the EMA.⁵⁹ The incorporation of hydrophobic as well as hydrophilic drugs, proteins, antigens and nucleic acids has been shown to increase drug bioavailability and efficacy.⁵⁹ Langer and coworkers have recently reported on highly stable docetaxel-loaded PLGA nanospheres, which were functionalized with targeting ligands for prostate cancer treatment.⁵ Moreover, after very successful and promising results in mice, phase I clinical trials have been started on this formulation.

3. Nanogels - preparation and characterization

The formation of large intramolecularly crosslinked macromolecules was recognized as early as the late 1940's in synthetic rubber emulsions, where also the term microgel was coined.⁷⁰ However, nano-sized hydrogels did not emerge until the mid-1980's,⁷¹ and the potential for controlled release of therapeutics was indicated in the first decade of this century.²⁹ Similar to hydrogels,⁶⁵ nanogels can – depending on their chemical composition – respond to stimuli in a reversible or irreversible manner. Incorporation of degradable moieties into the nanogel network, such as esters,^{72,73} acetals,⁷⁴ and disulfide bonds^{75,76} triggers nanogel degradation under the influence of specific stimuli. Moreover, in their aqueous state, nanogels can also be responsive to reversible chemical processes, enabling reversible nanogel swelling and shrinkage,²⁹ as shown in Figure 2A. Attractive and repulsive forces within the nanogel network cause shrinkage, such as temperature-induced aggregation of thermoresponsive monomers,^{77–79} or swelling by charge repulsion due to protonation and deprotonation of the polymer network.²⁹ As a consequence, nanogels can be designed to be responsive to physical stimuli, such as pressure,⁸⁰ ionic strength,⁸¹ temperature^{77–79} and pH.^{27,75,82,83}

Figure 2: Swelling of nanogels is influenced by the nanogels' composition, such as presence of weakly acidic and basic groups, the crosslinking density and incorporation of thermoresponsive moieties (A, reproduced from ref. 29). The crosslinking density also influences the nanogel's swelling ability and particle rigidity (B, adapted from ref. 67). Jiang et al. illustrated this with 250 nm gels with high crosslinking density, which could not pass through a 0.22 μm syringe filter (shown on the left), whereas 250 nm gels with a low crosslinking density did successfully pass (shown on the right). Moreover, incorporation of thermoresponsive moieties in the nanogel matrix causes the nanogel to collapse upon heating above the volume-phase transition temperature (VPTT) (C, adapted from ref. 69).

3.1. Nanogel formation

Nanogels can be obtained via various synthetic routes, ranging from emulsification to self-assembly. Several methods to obtain covalently crosslinked nanogels are discussed below. Physically crosslinked nanogels can also be obtained, but are outside the scope of this review.

3.1.1. Emulsion polymerization

A commonly applied technique for nanogel preparation is radical (co) polymerization in water-in-oil emulsions, which can be categorized into inverse (mini) emulsions and microemulsions.^{84,85} An aqueous solution of monomers and bifunctional crosslinkers is emulsified by ultrasonication or mechanical stirring in a nonpolar solvent, resulting in an emulsion of monomer-containing water droplets in an oil phase. Surfactants are used to stabilize the emulsion, and subsequently the monomer-containing aqueous phase is polymerized. Inverse (mini)emulsions are kinetically stable systems to prepare nanogels below or around the critical micelle concentration (CMC) of the surfactant. Mechanical stirring and ultrasonication are used to prepare inverse emulsions and miniemulsions, respectively. Microemulsions are thermodynamically stable emulsions, in which surfactants are added above their CMC.⁸⁴ After polymerization and subsequent removal of the oil phase surfactants and unreacted monomer, a crosslinked polymer network is obtained, which can be resuspended in water as a nanogel.^{86–89} To obtain meticulous control over nanogel formation, Oh et al. used atom-transfer polymerization (ATRP) to prepare nanogels in a mini-emulsion, which resulted in nanogels with highly uniform molecular-weight distributions.⁹⁰ Moreover, ATRP allowed the formation of a uniform network, where degradation products maintained an $M_w/M_n < 1.5$, and precise control over end-group functionalization.

3.2. Precipitation and dispersion polymerization

Another technique involving two phases for nanoparticle preparation is precipitation polymerization.^{84,85} In this technique a solvent is selected which is able to dissolve both the monomer and initiator, but in which the formed polymer becomes insoluble above a critical degree of polymerization. Normally this leads to macroscopic precipitation and production of irregular-shaped polydisperse particles,^{84,91} but here a stabilizer is added to achieve a stable colloidal dispersion.⁹² With this classic dispersion polymerization, micrometer-sized particles can be readily prepared.^{84,91} This method has also been adapted in the synthesis of nanometer-sized structures, often by applying controlled radical polymerization techniques, such as reversible addition-fragmentation chain transfer (RAFT).^{93–96} Dispersion polymerization has been performed using a macromolecular RAFT agent that simultaneously acts as a chain transfer agent and

a steric stabilizer for the formed polymer block.^{93–98} When a bifunctional monomer is added during the polymerization process, the growing fraction of the block-copolymers are crosslinked and core-shell nanogels are obtained.^{99–105} The solvent can be organic^{93,106} or water^{99–101} and the formed nanogels generally have diameters of around 30–150 nm. Moreover, employing cyclic vinyl monomers allows introduction of responsive and biodegradable groups into the carbon backbone of the polymer.⁹⁸ Thermoresponsive monomers such as *N,N*-diethyl acrylamide (DEAAM), *N*-isopropyl acrylamide (NIPAM) and short oligo-ethylene glycol chains, as well as the non-responsive monomer *N*-(2 hydroxypropyl) methacrylamide, are inherently miscible with water, while the corresponding polymers precipitate at higher temperatures owing to their lower critical solution temperature (LCST). These properties make aqueous dispersion polymerization particularly attractive for the preparation of thermally sensitive nanogels.^{97,99,102,104}

3.2.1. Controlled polymerization with multi-functional monomers

Owing to advances in the field of polymer chemistry, formation of sophisticated well-defined polymeric structures such as star copolymers with crosslinked cores¹⁰⁷, (hyper)branched polymers¹⁰⁸ and nano-networks¹⁰⁹ have been become available (Figure 3). With a sufficient degree of branching and crosslinking, these structures obtain nanogel properties, such as swelling and responsiveness.^{110,111} An interesting approach to make such nanogels is by controlled radical cross-linking copolymerization, which allows the direct formation of nanoparticles in a one- or two-step in a single-phase.¹¹² Since this process is not limited by the formation of emulsion droplets or precipitation, it is particularly suitable for the preparation of small (sub-100 nm) nanostructures. Furthermore, size and structure of the formed nanogels can be controlled by varying reaction conditions such as temperature, monomer concentration and reaction time. Often, this approach involves ATRP or reversible-addition fragmentation polymerization (RAFT) polymerization of a monovinyl monomer and a divinyl crosslinker.^{113–116} The resulting nanogels contain unreacted double bonds, which may be used for functionalization. For example, Wang and coworkers investigated the degree of branching in nanogels based on a disulfide divinyl crosslinker and 2-(Dimethylamino)ethyl methacrylate (DMAEMA) for gene delivery, where the unreacted vinyl groups were used for post-functionalization.¹¹⁶ The authors showed that an increased branching degree facilitated more efficient gene delivery. Alternatively, linear polymers with pendant reactive groups can be crosslinked in a second step to yield nano-networks with low polydispersity.^{109,117,118} Harth and coworkers were able to prepare several linear polyester chains bearing alkene, alkyne or epoxide functionalities.¹⁰⁹ Epoxide crosslinking using a diamine yielded particles with good control over size and narrow polydispersities. Other approaches include the use of inimers¹¹⁹ or multifunctional initiators¹²⁰.

Figure 3: Examples of polymeric structures: star copolymers with crosslinked core (left), (hyper)branched polymers (middle) and crosslinked nano-networks (right).

3.2.2. Inverse nanoprecipitation/solvent displacement

Nanoparticles are frequently prepared by nanoprecipitation of water-insoluble polymers: a polymer solution in an organic, water-miscible, solvent is added to a large volume of water.^{55,56} Under specific conditions the polymers will aggregate into clusters ranging from 100 to 1000 nm and surfactants are often used to stabilize the formed solution, as shown in Figure 4.¹²¹ After the organic solvent is removed by dialysis, ultrafiltration or similar techniques, a solution of solid polymer nanoparticles is obtained.^{17,122} Similarly, hydrophilic polymers can also precipitate into nano-sized clusters in an inversed system: the polymer is dissolved in an aqueous solution, which is added to a large volume of a water-miscible organic solvent.^{74,83,123} After chemical crosslinking and subsequent removal of unreacted polymer, crosslinker and used surfactants, a solution of nanogels is obtained.

Figure 4: Nanogel preparation by inverse nanoprecipitation involves adding an aqueous polymer solution to a water-miscible non-solvent. Nano-sized polymer clusters form upon solvent and non-solvent mixing. Crosslinking of these polymer clusters and subsequent non-solvent removal results in an aqueous nanogel solution.

3.3. Self-assembly

Another commonly-used method to fabricate nanogels is by self-assembly, requiring the polymeric components to spontaneously aggregate in an aqueous environment.²⁹ This can be achieved by incorporating functional groups into a polymer which are capable of forming physical crosslinks with each other, with other polymer chains or with crosslinkers. Nanogels can also be stabilized by physical crosslinks that are assembled through hydrophobic interactions¹²⁴ or inclusion complex formation.^{125–127} In a similar fashion, covalently crosslinked nanogels can be obtained by aggregation and subsequent crosslinking, such as disulfide formation^{128–130} and chemical conjugation (Figure 5).¹³¹

Figure 5: Disulfide-crosslinked nanoparticles prepared by self-assembly of amphiphilic block-co-polymers containing hydrophobic cyclic carbonate (A) and pyridyl-disulfide (B) moieties. Hydrophobic self-assembled micelles were crosslinked by cystamine (A) and a catalytic amount of dithiothreitol (DTT) (B). Crosslinking of these micelles causes conversion of the hydrophobic micelle core into a hydrophilic nanogel matrix for protein (A) and drug delivery (B). A and B are adapted from refs. 128 and 129, respectively.

3.3.1. Microcontact printing

DeSimone and coworkers have used templating techniques (particle replication in non-wetting templates, PRINT) using etched nanostructures for the fabrication of nanogels.¹³² A major advantage of PRINT over the aforementioned preparation techniques is that it grants unique control over particle size, dispersity and shape,¹³³ which also influences its interaction with biomolecules, proteins and cells.³² In addition, PRINT has been demonstrated to be a scalable process, through roll-to-roll production (shown in Figure 6).¹³⁴

Figure 6: Illustration of PRINT nanogel production to roll-to-roll production. Clockwise, a perfluoropolyether mold is prepared via a nanostructured silicon wafer template, then polymer precursor added to the PRINT mold and the cavities are filled by rolling out the precursor material through roll-to-roll process. Nanogels are formed by photocuring and additional processes, and removed from the mold by a degradable adhesive layer, which is dissolved to obtain PRINT nanogels. Adapted from ref. 134.

3.4. Nanogel characterization

3.4.1. Light scattering

Characterization of nanoparticles by dynamic light scattering (DLS) provides valuable information on their size and size distribution. DLS measures fluctuations in scattering intensity caused by the Brownian motion of particles in a sample, while being illuminated by a laser. By applying an autocorrelation function on the measured fluctuations in scattering intensity on a specific time-scale, the diffusional coefficient (D) is obtained, which is used to calculate the particle's hydrodynamic radius (R_h) with the Stokes-Einstein equation (Equation 1), where k_B , T and η are Boltzmann's constant, temperature and medium viscosity, respectively.^{135,136}

$$R_h = \frac{k_B T}{6\pi\eta D} \quad \text{Equation 1}$$

Nanotracking analysis (NTA) also offers analysis of particle size distribution, by tracking the motion of nanoparticles in a flow cell, while illuminated by a laser.¹³⁷ Nanoparticles are visualized by monitoring the particle's light scattering coronas under a microscope. From analysis of the monitored nanoparticle tracks, the diffusional coefficient is obtained, which can be used to calculate the hydrodynamic radius of the nanoparticle (Equation 1). Although the same parameters are obtained from DLS and NTA, NTA offers a more direct visualization of nanoparticle size and concomitant size distribution.¹³⁷ Furthermore, NTA measurements are less sensitive to large particles and therefore enable measurement of more polydisperse samples.¹³⁸

Static light scattering (SLS) can be used to obtain molecular weight as well as the size of particles in suspension. SLS is, however, predominantly used to measure the molecular weight of a polymer or a nanoparticle,¹³⁵ which is achieved by measuring the average light scattering intensity over time.

Small-angle-X-ray and neutron scattering (SAXS and SANS, respectively) has been applied to obtain information on the structure of nanoparticles and polymers.¹³⁹ SAXS and SANS studies, on the internal structure of nanogels, have however only been sparsely reported.^{80,140–143}

3.4.2. Zeta potential

Nanoparticle surface charge is a key characteristic for biomedical applications, and is measured by determining the zeta potential, which can be achieved relatively fast through electrophoretic light scattering.¹⁴⁴ However, interpretation of zeta potential for nanoparticles smaller than 100 nm is far from straightforward, which requires consideration of the measurement conditions and the nature of the analyzed nanoparticle.¹⁴⁴ Zeta potential measurements have become an essential tool for nanomedicine characterization, since surface charge is an important factor in biological environments.¹⁴⁵ Furthermore, zeta potential can be a useful tool for evaluation of the nanoparticle functionalization-degree.¹⁴⁶

3.4.3. Electron and atomic force microscopy

Scanning and transmission electron microscopy (SEM and TEM, respectively), as well as atomic force microscopy (AFM) have risen to become well-established characterization techniques for nanostructured materials and nanoparticles. TEM^{147–150} and SEM,^{67,79,83} can both image nanoparticles with great detail, allowing analysis of nanoparticle size, shape and even morphology. Since nanogels generally consist of light elements, e.g. carbon, oxygen and nitrogen, often staining of samples with heavier elements is required to achieve appropriate contrast. In this respect, AFM analysis can also analyze size and shape of nanoparticles, without the requirement of using staining agents.^{72,147,151} AFM, and regular SEM and TEM analysis of nanogels requires water removal, which solely allows imaging of nanogels in a collapsed state, resulting in smaller observed particle sizes than in aqueous environments, such as measured by light scattering techniques. However, imaging of nanogels is possible via cryo-TEM,^{152,153} allowing imaging of nanogels in their swollen state.¹⁴⁹

4. Nanogels in biological environments

Biomedical application of nanoparticles poses several challenges with respect to the design of nanoparticles, since colloidal stability of nanoparticles in complex biological fluids is often quite different from aqueous conditions.^{154,155} Biological media, such as serum and cell culture media, present environments with high ionic strength and high concentrations of proteins and biomolecules, which can diminish repulsive forces between nanoparticles.^{155,156} Colloidal stability of nanoparticles is an important characteristic since it influences retention of nanoparticles within the bloodstream, particle mobility and even toxicity.¹⁵⁷ Nanoparticle toxicity is dramatically increased when particles aggregate in the bloodstream and cause capillary obstruction, thrombosis and embolism.^{31,155,157} Furthermore, nanoparticles may have undesirable interactions with molecules and cells present in biological environments, depending on their size, surface potential, surface chemistry and hydrophobicity.¹⁵⁶ Yet, the aforementioned characteristics also determine a nanoparticle's effectiveness in applications where a cell-nanoparticle interaction is desired, such as in targeted drug delivery⁵ and gene delivery.⁵¹

4.1. Nanoparticle stabilization

Under aqueous conditions, attractive van der Waal (vdW) forces between nanoparticles (Figure 7A) destabilize a colloidal suspension.^{154,155} Steric repulsion (Figure 7B) and electrostatic repulsion (Figure 7C) are the main forces which can stabilize a colloidal suspension.¹⁵⁵ Electrostatic stabilization is an important factor in the colloidal stability of a nanoparticle solution.¹⁵⁵ However, biological environments contain high concentrations of ions, causing compression of the electric double layer, decreasing the effective length scale of the electrostatic repulsion, which can result in colloidal destabilization.¹⁵⁶ Steric stabilization is a frequently applied strategy to stabilize nanoparticles in biological media, which is easily achieved by grafting polymers onto a nanoparticle. Poly(ethylene glycol) (PEG) coatings are often used in this manner, by shielding the nanoparticle surface and inhibiting interaction between nanoparticles.^{154,155} Moreover, the hydrophilicity of PEG also induces binding of water molecules, further stabilizing the nanoparticles' colloidal stability by preventing hydrophobic interactions between nanoparticles.^{154,158} Incorporation of PEG considerably reduces non-specific interactions between proteins and nanoparticles, although PEG still exhibits some degree of non-specific protein interaction.¹⁵⁸ Jiang and coworkers have studied extensively the application of zwitterionic polymers, and showed not only extensive reductions in protein adsorption, but also that zwitterionic materials do not cause a foreign body reaction.¹⁵⁹

Figure 7: Depiction of colloidal interactions. Attractive vdW forces can de-stabilize colloidal suspensions at very close range (A). Addition of steric stabilization, such as polymers, stabilize nanoparticles (B). Moreover, surface charge can stabilize nanoparticles even further (C), which depends on the ionic strength of the medium, which determine the thickness of the Stern layer and diffuse layer. The potential energy (ψ) of repulsion between nanoparticles as a function of the distance (d) for vdW forces, steric repulsion, EDL and extended DLVO theory (D), which incorporates several forces besides the ones arising from vdW, EDL and steric repulsion, such as hydration and depletion forces. Adapted from ref. 155.

4.2. Protein adsorption

Nanoparticles in biological media can interact with proteins in a non-specific manner, eventually resulting in coverage of the nanoparticle surface, forming a protein corona,¹⁵⁴ altering the physical properties of the nanoparticle and influencing its colloidal stability, and subsequently the particle's circulation properties.^{154–156} Moreover, in *in-vivo* applications, complement proteins in the protein corona cause removal of the nanoparticle from the circulation through opsonization.^{31,154} Factors affecting the adsorption of proteins include nanoparticle size, surface curvature, surface charge and hydrophobicity.^{31,154,155} Particle functionalization with PEG provides steric stabilization as well as reducing non-specific interactions with proteins, by introducing a hydrophilic layer around the nanoparticle.^{154,155}

4.3. Cellular interactions

Interactions between nanoparticles and cells are fundamental for applications such as controlled drug and gene delivery, and targeted imaging. These applications require specific interaction of nanoparticles with specific cell-types. At the foundation of

these interactions lie physical properties of the nanoparticle,¹⁶⁰ such as the size,¹⁶¹ shape,³² rigidity¹⁶² and surface charge¹⁶¹ of the nanoparticle, as well as the chemical composition of the nanoparticle, including hydrophilicity and hydrophobicity.³² Nanoparticles with a more positive surface charge interact with the negatively-charged cellular membrane, which increases cellular uptake of positively charged nanoparticles.¹⁶¹ Size and shape of nanoparticles have a combined effect on their rate of uptake in cells.³² For instance, it has been found that elongated nanoparticles show higher cellular uptake than spherical nanoparticles.¹⁶³ Nanogel rods of different size, but comparable aspect ratio, have shown similar cellular uptake.¹⁶⁴ Yet, cellular uptake of nanoparticles in terms of shape combined with their size is poorly understood.³²

Cellular targeting of nanogels can be achieved by conjugation with targeting ligands^{32,165} such as, antibodies¹⁶⁶ (and fragments), targeting peptides,¹⁶⁷ aptamers¹⁶⁸ and small molecules.^{5,169} Monoclonal antibodies can selectively target specific membrane proteins on cellular surfaces, but require extensive purification and highly selective and sensitive conjugation.^{43,166} Targeting peptides, aptamers and small molecules are therefore interesting alternatives, despite their lower selectivity.^{15,43}

4.4. Systemic circulation

Many nanoparticles for nanomedicine are designed to be administered intravenously, where it has to be taken in account that the aforementioned interactions with proteins and cells influence the circulatory properties of the nanocarriers,^{32,160–162} which further influences targeting efficacy.³¹ In addition, intravenous colloidal destabilization of nanoparticles can have toxic effects.^{31,155,157}

The aforementioned steric nanoparticle stabilization by PEG-ylation reduces protein adsorption, and as a consequence lowers cellular uptake and extends retention in the blood circulation.¹⁵⁸ However, it has been demonstrated that after multiple administrations of PEG-ylated nanocarriers, these nanocarriers are cleared at an increased rate from the blood stream (accelerated blood clearance – ABC – phenomenon).¹⁷⁰ PEG has also been shown to cause an immunological response, including hypersensitivity to PEG.^{158,170} Another drawback of PEG is its non-biodegradability.¹⁵⁸ In recent years, zwitterionic polymers have been opted as an anti-fouling polymer, as demonstrated in hydrogels,¹⁷¹ surface coatings^{172,173} and nanogels.¹⁵⁸ Jiang and coworkers showed that zwitterionic poly(carboxybetaine) (pCB) is more hydrated and therefore a more effective hydrophilic stabilizer than PEG, caused by ionic-dipole interaction between the polymer and water. Low protein fouling was demonstrated in pCB zwitterionic nanogels⁸⁶ and pCB-functionalized enzymes.¹⁴⁸

Mechanical properties of nanogels, which are mainly determined by the crosslinking density, have been shown to markedly influence the cellular uptake of nanogels, as more rigid nanoparticles show higher cellular uptake by macrophages.¹⁶² This was also

demonstrated in in-vivo studies with PEG⁶⁸ and zwitterionic pCB nanogels,⁶⁷ which showed that nanogels with a lower crosslinking density were increasingly retained within the blood circulation, as depicted in Figure 8. It was recently shown that nanogels are even able to penetrate the skin deep into the stratum corneum, which was attributed to the hydrophilic nature of the employed nanogels as well as their mechanical properties.¹⁴⁹

Figure 8: Depending on their crosslinking density, and consequently their rigidity, nanogels show differences in blood circulation retention, as demonstrated with PEG (A) pCB nanogels (B). Poly(ethylene glycol) nanogels with a low crosslinking density (circles) were more slowly removed from the bloodstream than their counterparts with a higher crosslinking density (squares) (A). Similarly, zwitterionic pCB nanogels with decreasing mechanical stiffness: 1.35, 0.87, 0.58, 0.26 and 0.18 MPa, denoted by pCB 15%, pCB 10%, pCB 5%, pCB 2% and pCB 2%-, respectively (B) showed retention of pCB nanogels for a long time in the bloodstream, where lower crosslinking density correlated with longer retention within the bloodstream. A and B are adapted from refs. 68 and 67, respectively.

5. Responsiveness

The use of polymers as a responsive material has received increasing attention during the last decade.¹⁷⁴ Especially the field of controlled drug delivery has benefitted greatly from the application of responsive polymers.^{175–177} However, the application of responsive polymers in imaging/sensing has only started receiving attention in recent years.^{66,176,178} In comparison to the amount of responsive small molecular probes, the application of responsive polymers for molecular sensing is still in its infancy.^{176,178} Nevertheless, there has been a number of studies where polymer nanoparticles have been successfully used as stimuli-responsive imaging moieties.^{77,179–181}

5.1. Temperature responsiveness

Polymeric conformational changes in hydrogels result in volume phase transitions, leading to shrinking or expansion of the hydrogel. Temperature-responsive polymers show lower (LCST) or upper critical solution temperature (UCST) behavior, becoming insoluble or soluble upon heating, respectively. The potential of poly(*N*-isopropylacrylamide) (pNIPAM) in biomedical applications already has long been recognized, because its LCST

is close to body temperature and is independent of molecular weight.¹⁸³ pNIPAM has been successfully incorporated in nanogels in multiple variations, often by copolymerization with other monomers to add functionality to the nanogels.¹⁷⁷ Moreover, the volume-phase transition temperature (VPTT) of nanogels can be adjusted by copolymerization with other monomers, for example by acrylic acid to improve drug incorporation.^{79,82,184–187} Peng et al. studied the effect of the incorporation of PEG-methacrylate and methacrylic acid functional groups into a pNIPAM-based nanogel, which showed that acrylic acid lowers the VPTT of the nanogels, while PEG reduces size and increases solubility of the nanogels.⁸²

Polymers based on oligo ethylene glycol (OEGA) exhibit lower immunogenicity than pNIPAM¹⁸⁸ and also display thermoresponsive behavior, but have a higher LCST of 90 °C.¹⁸⁹ However, the incorporation of functional monomers into the nanogel enables adjusting of the VPTT to 25–35 °C.^{72,128,188,189} Poly(vinylcaprolactam) (pVCl) is another thermoresponsive polymer with an LCST in the temperature range 30–50 °C, depending on the pVCl concentration in solution and the molecular weight.¹⁸³ Incorporation of other monomers alters the VPTT of pVCl nanogels^{190–192} and their temperature dependent drug release rate.^{190,192}

5.2. pH responsiveness

Throughout the body the local pH can vary considerably.^{33,51} Therefore, nanogels in biomedical applications have been designed to respond to these differences by swelling or degradation. Under normal conditions the physiological pH is maintained around 7.4 in almost all tissues,³³ whereas the pH in the gastro-intestinal tract, for example, can vary between 1 and 7.5.⁵¹ Local pH differences are also observed on the cellular level, though to a much smaller extent. Whereas the extracellular environment is maintained around pH 7.4, the pH of endosomes can vary between pH 5–7.2.^{33,193} These variations in local pH can be used as triggers for release or response in therapeutic delivery or imaging, respectively. In many tumors, the pH of the tumor extracellular environment is acidified as compared to healthy tissue, due to lactic acid production. This process is enhanced by poor tumor perfusion, which leads to lowering of the pH to 6.5–6.9 in the tumor microenvironment.^{33,193–195} Acidification is often also observed in inflamed and infected tissues.¹⁹⁵ Weakly basic, proton-accepting polymers with high buffering capacity have been successfully employed in intracellular gene delivery.^{196,197}

5.2.1. Reversible swelling

As discussed earlier, swelling and shrinking are intrinsic properties of nanogels.²⁹ Incorporation of weakly acidic or basic groups grants pH-responsive swelling properties to the nanogel.¹⁹³ The generation of local charges within the network causes electrostatic repulsion within the polymer network, leading to swelling. This process renders the

nanogel responsive to local pH changes, which is illustrated in Figure 2A and 9.²⁹ The acidified microenvironment of tumors therefore offers possibilities for pH-responsive drug delivery and pH-responsive imaging.^{29,33} By choosing the pK_a of the polymer, the pH-responsivity of the nanogel can be tuned. An overview of pH-responsive monomers is shown in Table 1, which are applicable in different polymerization techniques, among which radical polymerization and NCA ring-opening polymerization.^{177,182,198} pH-responsive nanogels prepared by radical polymerization are often prepared by incorporation of acrylic acid^{79,187,188} or methacrylic acid.¹⁹⁹ The pK_a of acrylic acid moieties lies around 7.3, which enables complexation of cationic drugs, such as doxorubicin, which is released upon protonation of the acrylic acid group below physiological pH.¹⁸⁷ 2-Diethylaminoethyl acrylate and 2-dimethylaminoethyl acrylate-based nanogels have been investigated by Oishi et al.^{196,200} and Wu et al.,¹⁸¹ who demonstrated increased nanogel swelling in acidified media, enabling drug release and responsive imaging. However, incorporation of these cationic monomers also increases the toxicity of these nanogels.¹⁹⁶

Figure 9: Some examples of responsive functional groups, which show responsiveness to pH, molecules present in biological systems or temperature. The depicted thermosensitive moieties undergo a conformational change upon heating above their LCST. For more detailed information on responsive groups in nanogels references 72 and 138 are highly recommended.

Table 1: pH-responsive monomers and crosslinkers used for nanogel formation. Protecting groups of protected monomers are indicated in orange.

Structure	Responsiveness	References
	pH-responsive	Methacrylic acid: 169,180,190,201 Acrylic acid: 78,79,82,185,186
	pH-responsive, hydrophobic switch	(Dimethylamino)ethyl methacrylate: 181 (Diethylamino)ethyl methacrylate: 202–204
	pH-responsive, protected NCA monomer	205
	pH-responsive, protected NCA monomer	205
	pH-degradable, switchable monomer	72
	pH-degradable crosslinker	28,203
	pH-degradable crosslinker	192

5.2.2. Hydrolysis

Local pH differences within the body and cellular compartments can be utilized for degradation of specific chemical bonds. Since responses below physiological pH³³ are interesting from a biomedical perspective,³³ pH-sensitive hydrolysable bonds, such as ester, acetal and hydrazone bonds, are frequently incorporated into nanogels.⁷⁶ pH-triggered release of a nanogel's payload can be achieved by including bonds into the nanogel matrix which are hydrolyzed at a certain pH value, leading to swelling and ultimately degradation into macromolecular/polymeric fragments, depending on the design of the nanogel.^{72,192,196} These bonds are included into the nanogel matrix by incorporation in the polymer backbone, or by including pH-labile crosslinkers, as depicted in Table 1. The preparation of hydrolysis-sensitive nanogels can, for instance, be achieved via precipitation polymerization of NIPAM, OEGMA or VCl in the presence of hydrolysable crosslinkers, such as ethylenglycoldimethacrylate¹⁹⁶ and 2,2-dimethacroyloxy-1-ethoxypropane.¹⁹² A brief overview of hydrolysable crosslinkers is given in Table 1.

Another strategy is to combine functional groups within a polymer is shown by Lu et al., who prepared zwitterionic poly(succinimide) polypeptide nanogels, crosslinked by pH-sensitive hydrazone bonds.²⁰⁶ Qian et al. included hydrophobic monomers with hydrophobic ortho-ester bonds into nanogels, which hydrolyzed below physiological pH, rendering the nanogel hydrophilic, inducing swelling and facilitating drug release.⁷²

Triggered release of drugs from a nanogel can also be achieved by utilizing a pro-drug strategy, where the drug is conjugated to the nanogel with a pH-labile bond, such as the conjugation of doxorubicin via a hydrazone linkage.^{207,208}

5.2.3. Charge conversion

Surface charge is an important parameter in directing cellular uptake of nanoparticles.^{145,161} Surface-charge switching has therefore received interest as a drug delivery mechanism.^{28,209–211} Charge conversion of anionic nanoparticles into cationic nanoparticles, greatly enhances their cellular uptake.¹⁴⁵ Du et al. reported a PEG-diacrylate-crosslinked 2-aminoethyl methacrylate nanogel, of which the primary amine groups were functionalized with 2,3-dimethylmaleic anhydride.²⁸ The nanogels displayed a negative surface charge at physiological pH, but the charge changes to positive at pH 6.8, which is typical for a tumor microenvironment. MDA-MB-435s (breast cancer) cells showed significantly more nanogel uptake at pH 6.8, compared to 7.4. Similarly, Chen et al. formed polyvinylalcohol nanogels by click chemistry; azide-functionalized polyvinyl alcohol was crosslinked with polyvinyl alcohol with a disulfide-linked alkyne moiety.²¹² Afterwards, the remaining azide groups were reacted with dimethylmaleic propargylamide, to provide the charge-conversion properties to the nanogel.

5.3. Biomolecule sensitivity

5.3.1. Redox-responsiveness

In healthy tissue, the intracellular redox potential is regulated by an elevated concentration of glutathione (GSH) to regulate damaging reactive oxygen species, such as H_2O_2 and NO.³³ Intracellular GSH concentrations are reported to vary between 2–10 mM,^{33,213} whereas the GSH concentration in the extracellular space is estimated between 2–20 μ M.²¹³ Taking advantage of the elevated intracellular GSH concentrations, reduction-sensitive disulfide bonds are often incorporated in drug and gene delivery systems to achieve intracellular release.²¹³ Moreover, drug and gene carriers which combine high buffering capacity with bioreducibility, have shown to be very effective due to the proton sponge effect.^{33,197} This involves protonation of the polymer in the endosome during endocytosis. The increased influx of protons, accompanied by chloride ions, eventually leading to the osmotic bursting of the endosome. Bioreducible nanogels can be prepared by including bioreducible, bifunctional, monomers (shown in Table 2)

into the reaction mixture during emulsion^{72,214,215} or precipitation polymerization.^{186,188,190} Nanogel crosslinking via NCA polymerization with bifunctional, bioreducible monomers (shown in Table 2), was also demonstrated by Chen and coworkers.²¹⁶ Another method to include disulfide bonds into the nanogel matrix is by crosslinking free thiols in a polymer backbone with each other.¹²⁹ Disulfide polymer crosslinking can be achieved by polymerizing a pyridylsulfide monomer, which can then crosslink with itself via disulfide exchange.^{128,201,217}

Table 2: bioreducible monomers and crosslinkers used for nanogel formation. Protecting groups of protected monomers are indicated in orange

Structure	Responsiveness	References
Protected monomer for disulfide crosslinker formation	Methacrylate: 128,201
Acrylate: 217
Bioreducible crosslinker	72, 218
Bioreducible crosslinker	186,188,190,191,197,219–221
Bioreducible NCA crosslinker	216
Dual responsive crosslinker, bioreducible and lipase degradable	222

5.3.2. Enzyme responsiveness

The incorporation of enzyme-cleavable sites into polymeric materials has been an interesting approach for the degradation of biomaterials.^{195,223,224} Enzyme-triggered release from polymer nanoparticles has the advantage that enzymatic cleavage is highly specific²²³ and is strongly dependent on the type of cell and the state of the targeted tissue.^{195,223} Furthermore, upregulation of specific enzymes is commonly encountered in inflamed or cancerous tissues^{223,224} Generally, nanomaterials are designed for hydrolase sensitivity, where proteases, lipases and glycosidases are currently the most relevant hydrolase targets in drug delivery.²²³ Thornton et al. reported a PEG-based nanogel for enzyme-triggered release of proteins, as shown in Figure 10, where zwitterionic

enzymatically cleavable peptides caused the nanogel to swell upon degradation by specific proteases.²²⁵ The sites for enzymatic degradation were shown to be enzyme-specific, and subsequent protein-release was confirmed.

Figure 10: PEG-based nanogels containing zwitterionic peptide moieties with a protease cleavage site between the anionic and cationic part of the moiety (A). Upon enzymatic degradation of the linker, the charge conversion within the gel leads to swelling and subsequent release of its protein payload (B). Adapted from ref. 225.

Glycosidases are enzymes which degrade specific naturally occurring polysaccharides, making polysaccharide-based materials by definition enzymatically biodegradable.²²³ One example is the hyaluronic acid degrading enzyme hyaluronidase, which activity is upregulated by several cancer types.²²⁶ Hyaluronic acid has frequently been used to prepare nanogels, because of its biocompatibility.^{78,227,228} Moreover, the enzymatic degradation of hyaluronic acid is interesting for a variety of applications, including responsive imaging.²²⁹ PEI-pullan cationic nanogels loaded with paclitaxel were reported by Yim et al, which were coated with hyaluronic acid to shield the positive surface charge in order to reduce non-specific uptake of the nanogel.²³⁰ Degradation of the hyaluronic acid coating resulted in charge conversion resulting in increased cellular uptake, as shown in Figure 11. *In-vivo* experiments showed dramatic reductions in tumor volume (Figure 11B and C). Similarly, dextran has been investigated heavily as

a material to prepare nanogels,^{89,231–234} which also contain other functional groups for nanogel degradation, such as disulfide bonds.^{89,232} Dextran degradation has also been investigated as a means of nanogel degradation.^{235,236} Proteases are able to cleave specific peptide sequences. Matrix metalloproteases (MMPs) have been identified as target for controlled release, because of their upregulation in cancerous tissue.²²³ Despite receiving considerable attention in the field of controlled drug delivery,^{14,223} MMP sensitive nanogels have only been reported sparsely.²³⁷

Figure 11: Enzyme-responsive paclitaxel-loaded cationic nanogels, coated with hyaluronic acid to prevent non-specific cellular uptake: hyaluronidase, which is overexpressed by some tumors, degrading the hyaluronic acid layer and exposes the cationic nanogel, which is easily taken up by local cells. Paclitaxel delivery in a tumor-graft model led to a considerable reduction in tumor volume (B and C). Adapted from ref. 230.

Enzymatic activity of specific lipases has been identified as an interesting therapeutic target, since phospholipases are, for instance, upregulated during infection and inflammation.²²³ In this respect, polyphosphoesters are promising materials, since they are degraded by phospholipases, secreted by bacteria.^{26,238}

5.3.3. Glucose responsiveness

Monitoring glucose concentrations is critical in treating diabetes mellitus.²³⁹ Responsiveness of a material towards glucose concentrations may allow on-demand release of insulin.²²⁴ The design of glucose-responsive polymers is based on the incorporation of phenylboronic acid moieties in the polymer backbone.^{177,240,241} Boronic acids are hydrophobic in their neutral form, while the anionic form is hydrophilic, which enables the formation of a boronate ester with 1,2-diols (as depicted in Figure 9), as presented by glucose and polysaccharides. Phenylboronic acids are commonly used in biomedical applications, since its pK_a enables glucose-responsiveness at physiological pH.²⁴⁰

6. Nanogels in imaging

Recent developments in contrast enhancement for medical imaging attributes an important role to nanoparticles,²⁴² among which polymeric nanoparticles.¹¹ The versatile nature of polymer nanoparticles offers, for instance, control over their biodegradability and biodistribution properties.^{51,242} Furthermore, there are numerous strategies for conjugation of functional imaging or targeting groups to the nanoparticle^{15,243} and the combination of therapy and imaging, i.e. theranostics, focuses the attention towards multi-functional nanocarriers.^{7,11,242} Responsive polymeric materials are likewise gaining more attention,^{174,195,244} since stimuli-responsive nanoparticles can enable triggered release upon changes in local physiochemical parameters. Furthermore, stimuli-responsive imaging or sensing, can reveal information about the bio-environment of diseased areas in terms of pH, enzyme activity, and temperature, among others.^{11,66,176}

6.1. MRI

Magnetic resonance imaging (MRI) has developed to one of the most important imaging modalities since its introduction in the 1970's.²⁴⁵ Image contrast in MRI relies on the environment-dependent proton relaxation in magnetic gradients and is generated by influencing the spin-lattice (longitudinal, T_1) or spin-spin (transversal, T_2) relaxation times of water protons in the imaged tissue.^{246,247} Paramagnetic ions, such as gadolinium ions, shorten the T_1 relaxation time and provide positive contrast, while superparamagnetic iron oxide nanoparticles (SPIONs) lengthen the T_2 relaxation time and therefore give negative contrast. However, MRI contrast generation is also greatly influenced by many other factors, such as scan sequence.²⁴⁸ More recently, upconversion nanoparticles, CEST and ^{19}F -MRI have been applied for enhanced MRI contrast generation.^{245,247}

6.1.1. Longitudinal (T_1) contrast agents

Chelated gadolinium compounds (Figure 12) are widely applied in the clinic for MRI contrast enhancement. However, these compounds generate relatively low contrast enhancement and are cleared rapidly from the body, hence requiring high dose administration (0.1 mmol/kg body weight).²⁴⁸ Strategies to increase the relaxivity and contrast generated by gadolinium-based contrast agents have been described by Caravan,²⁴⁶ which include increasing the gadolinium payload, increasing the exposure of gadolinium complexes to water molecules, and restricting the complex' mobility.^{246,248,249} The attachment of gadolinium chelates to nanoparticles is therefore interesting, because these particles can deliver a high payload to a specific site of interest. Careful design of a nanostructure can increase the generated MRI contrast.^{245,246,249} Nanoparticles are able to remain in the bloodstream for longer durations than small molecules, such as gadolinium chelates.²⁴⁷ These features make targeting more effective and inhibits unwanted vascular extravasion.³¹

Figure 12: Gadolinium chelated by diethylenetriaminepentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)

Due to their high water content and the possibility to attach gadolinium chelates, nanogels are very suitable as carriers of MRI contrast agents. Co-polymers of oligo(ethylene glycol) methylether acrylate and pentafluorophenyl acrylate have been synthesized by Li et al. via RAFT polymerization to yield three different architectures (linear, hyperbranched and star-like nanogels), following functionalization with DOTA (Figure 13).²⁵⁰ Figure 13B shows the hyperbranched structure exhibited the largest relaxivity, despite the increased rotational diffusion time of the star-like polymers (794 ps vs. 651 ps for the hyperbranched structure). This was attributed to the enhanced exposure of the gadolinium complex to water, which was also shown by the same authors to be an important factor.²⁵¹ Lux et al. reported the formation of crosslinked poly(acrylamide) nanogels, where the crosslinker included the chelators gadolinium-loaded DOTA and DTPA.²⁵² The resulting nanogels displayed a 3-4 times higher relaxivity than the separate crosslinkers. Furthermore, the DTPA-based crosslinkers displayed a higher stability when they were incorporated into a nanogel. Similarly, well-defined single-chain polymer nanoparticles, crosslinked by DTPA-containing crosslinkers, were reported by Perez-Baena et al., combining fidelity of the nanostructure with ease of preparation.²⁵³

Figure 13: Investigated DOTA-functionalized polymer structures and the influence of the location of the gadolinium moieties (A), which revealed that hyperbranched structures offer the highest increase in relaxivity per gadolinium moiety (B). However, the star-shaped polymer allowed higher gadolinium loading. Adapted from ref. 250.

Transversal (T_2) contrast agents

Compared to gadolinium-based contrast agents, SPIONs for enhancing MRI contrast are a relatively new development, which are currently under clinical investigation.^{245,254} SPIONs have been successfully employed in applications, such as hyperthermia treatment of tumors and magnetic drug delivery.²⁵⁴ Encapsulation of SPIONs into nanogels has been reported on a few occasions.^{86,255–258} Zhang et al. showed the encapsulation of SPIONs in bioreducible zwitterionic nanogels, which remained stable under physiological conditions and quickly released encapsulated SPIONs and drugs after exposure to dithiothreitol (DTT).⁸⁶ Recently, Liu et al. prepared theranostic nanogels, crosslinked by boronate-coated SPIONs and a poly(vinyl alcohol)-pVCl copolymer, which exhibited drug release upon exposure to glucose.²⁵⁹

6.1.2. Responsive MRI contrast agents

Nanogels are by nature responsive nanomaterials, which is advantageous in MRI, since MRI contrast is influenced by environmental factors. pH-sensitive gadolinium chelates have been reported in the past.^{260,261} Nanogels offer the possibility of responsive MRI contrast by shrinking in response to their environment, while the resulting motion restriction of gadolinium chelates results in contrast enhancement.²⁴⁹ This concept was reported by Okada et al. who incorporated gadolinium chelates into the backbone of pH-responsive poly(acrylic acid) nanogels.²⁶² However, the same effect was not observed by Almutairi and coworkers, which was attributed to the existence of too much remaining mobility of the gadolinium chelates.²⁵² T_1 switchable nanogels have been reported by Santra et al.²⁶³ (Figure 14A). A poly(acrylic acid) nanogel with an iron oxide core was loaded with gadolinium chelates, of which the generated MRI contrast was quenched due to its proximity to the SPION core. Upon protonation of the nanogel matrix the gadolinium chelates were released and generate T_1 contrast. Similarly, Wang et al.²⁶⁴ reported a glycol chitosan nanogel containing small SPION clusters and Mn^{2+} ions (Figure 14B). Upon acidification, the Mn^{2+} ions were released and the MRI signal was switched on. A pH-responsive ratiometric sensor based on a Gd^{3+} -DTPA coated SiO_2 core, surrounded by a poly(methacrylic) acid nanogel was reported by Okada et al.¹⁸⁰, as depicted in Figure 14C. In its shrunken state, the nanogel restricts the mobility of bound water molecules, shortening the T_2 relaxation time of these protons. Since the T_1 relaxation time arising from the gadolinium chelates is not affected by the pH, the ratio between the T_2 and T_1 relaxation time can be used to determine the sensor's concentration.

The use of nanogels with alternative contrast enhancement strategies, such as ^{19}F MRI²⁶⁵ and magnetic nanocrystal structures,²⁶⁶ has also been reported.

Figure 14: MRI-based nanogel pH sensors: pH-responsive nanogels, which contain SPIONs and gadolinium (A) or manganese (B), leaving the generated T1 contrast by gadolinium and manganese ions in a quenched state due to the proximity of the SPIONs. However, release of the paramagnetic ions at a pH lower than physiological conditions, T1 MRI contrast is restored. Ratiometric MRI pH sensor, consisting of a pH-responsive nanogels with a silicon core, with DTPA-Gd³⁺ grafted to the surface. The relaxivity of the nanogel is influenced by swelling and shrinking of the pH responsive nanogel matrix. Measurement of T1-relaxativity, which remains constant, and T2 relaxivity, allows for ratiometric measurement of the local pH via MRI. A, B and C are adapted from refs. 263, 264 and 180, respectively.

6.2. Nuclear imaging

Medical imaging utilizing ionizing radiation (X-ray imaging) has been part of medical practice for more than a century and has evolved into more modern imaging techniques, resulting in the introduction of computed tomography (CT), positron emission tomography (PET) and single-photon emission computed tomography (SPECT).²⁶⁷ Labeling of nanogels for nuclear imaging techniques offers the possibility to obtain highly sensitive imaging probes, since these techniques require much less imaging agent as compared to MRI. The use of colloidal gold nanoparticles is a promising new method to generate contrast in CT images.^{38,268} Nanogels with embedded gold nanoparticles have been reported frequently, however, mostly for optical imaging techniques.^{67,78,233,269,270} Gold nanoparticle-loaded sucrosebutyrate acetate nanogels for image-guided radiation therapy have been reported by Jølk et al.,²⁷¹ which can potentially enhance the effects of radiation therapy. Other nuclear imaging techniques such as PET and SPECT require

the labelling with radioactive tracers such as ^{68}Ga , ^{18}F , ^{89}Zr and ^{64}Cu for PET and ^{111}In for SPECT.²⁶⁷ Conjugation of PET and SPECT tracers to nanoparticles is often used for image-guided drug delivery,²⁷² or assessing the *in-vivo* fate of nanoparticles.^{152,273} Majmudar et al. showed that 13 nm ^{89}Zr -labelled dextran nanogels can be used to target macrophages in arteriosclerotic plaque, which were imaged by PET/MRI.²⁷⁴ Macrophage content in arteriosclerotic plaque is a major determinant for plaque rupture. The highly sensitive PET imaging of these cells was, however, not exclusively specific to macrophages alone. Acrylamide nanogels with metal chelating crosslinkers were prepared by Lux et al. (as discussed earlier for MRI²⁵²) and loaded with ^{64}Cu for PET/CT imaging, revealing a higher accumulation of the nanoparticles in tumor tissue than the freely chelated ^{64}Cu tracer.²⁷⁵

6.3. Fluorescence imaging

Incorporation of fluorophores into nanoparticles for biomedical applications has enabled numerous possibilities for nanoparticle tracking¹⁷ and detection, as well as biochemical sensing.^{276,277} The possibilities of fluorescent imaging are extended further by Förster resonance energy transfer (FRET) to visualize the proximity of two separate fluorophores.¹²⁸ Integration of fluorescence probes into nanogels enables sensitive stimuli-responsive imaging.²⁷⁸ Since fluorescence imaging techniques are widely accessible, nanogels to measure temperature,^{77,78,279} pH,^{276,280,281} glucose concentration,¹⁸¹ and enzyme activity^{203,282} via fluorescence imaging have been reported. Incorporation of fluorophores into a nanogel matrix can also provide an environment-sensitive sensor. One strategy employed by Gota et al. was to prepare a nanogel based on a copolymer of NIPAM and a fluorophore-containing monomer.⁷⁷ Due to the thermosensitive chain collapse of pNIPAM, the water content of the nanogel was regulated by the environmental temperature. Another method is fluorescence quenching as demonstrated by Oishi et al, who included AuNPs into a pNIPAM nanogel, functionalized with FITC by a caspase-3 degradable linker.²⁰³ The FITC fluorescence was in a quenched state, which was activated by the degradation of the linker. Similarly, Peng et al. included the fluorophores coumarin-6 and Nile Red and the pH indicator bromothymol blue into a polyurethane nanogel.²⁸³ The fluorescence of coumarin-6 is quenched by bromothymol blue at low pH, whereas at high pH the excitation signal of coumarin-6 activates Nile Red by FRET, resulting in a NIR emission. Cao et al. demonstrated the ratiometric pH sensing by incorporating a highly sensitive fluorescent organic dye into a similar polyurethane nanogel.²⁷⁶ Furthermore, they demonstrated extremely sensitive intracellular pH sensing and H_2O_2 responsivity. Hyaluronic acid nanogels were functionalized with ICG, a NIR fluorescent dye, which intensity increased upon enzymatic degradation of hyaluronic acid.²²⁹ Since hyaluronidase is overexpressed by a lot of cancers,²²⁶ the nanogel can serve as an imaging tool for cancer detection. This mechanism was demonstrated in several cell lines, where signal intensity was increased in cancer cell lines.

Zhou and coworkers have developed several core-shell nanogels with temperature⁷⁸, glucose¹⁸¹ and pH-sensitive²⁸⁰ optical imaging capabilities in combination with drug delivery. Metal nanoparticles^{78,181} and quantum dots²⁸⁰ were included into the stimuli-responsive nanogels by dispersion polymerization. Responsiveness was included by copolymerization of acrylic acid, oligo(ethylene glycol)methyl ether methacrylate or 4-vinylphenylboronic acid, to achieve pH, temperature and glucose sensitivity, respectively. Figure 15 shows the reversible sensitivity of quantum dot luminescence under influence of glucose concentration and temperature, where the sensitivity to glucose was shown to be only minimally influenced by other metabolites and ions. Similar nanogel systems, where quantum dots and metal nanoparticles embedded into a responsive nanogel matrix to achieve glucose sensitivity have also been reported.²⁸⁴

Figure 15: Photoluminescence response and reversible responsiveness of nanogels with a quantum dot core, sensitive to glucose (A and C, respectively) and temperature (B and D, respectively). The conformational changes of the nanogel matrix influences the photoluminescence of the quantum dot. Both nanogels proved responsive to glucose concentrations (A, decreasing photoluminescence upon exposure to glucose) and temperature (B, increasing photoluminescence upon increasing temperature), respectively. Moreover, both nanogels showed reversible responsivity to glucose and temperature (C and D, respectively). Adapted from refs. 181 (A and C) and 78 (B and D).

7. Nanogels in Therapeutic delivery

Controlled delivery of molecules for therapeutic purposes is a central part of the nanomedicine field, which has originated bright perspectives for current and future clinical application.^{5,285,286} In this respect, nanogels promise to combine their advantageous properties, such as high water content, responsiveness and biocompatibility, for efficient therapeutic delivery.^{29,84}

7.1. Drug delivery

Nano-sized drug delivery systems have been shown to be very effective, predominantly in delivering chemotherapeutics to tumors.⁵¹ Some of these drug delivery vehicles have entered the clinical trial-stage of drug development.²⁸⁷ In this respect, application of nanogels for drug delivery is very interesting and because of their responsive nature, nanogels can release drugs upon exposure to specific stimuli.⁷⁶

7.1.1. Chemotherapeutics

Targeted delivery of chemotherapeutics is of utmost importance in the treatment of cancer. The application of stimuli-responsive drug delivery vehicles, among which nanogels, has shown encouraging results.²²⁴ As described earlier, the VPTT behavior of thermoresponsive polymers is influenced by incorporation of hydrophilic monomers.^{82,128,190,191} Drug release is influenced by the VPTT,^{79,191} which is potentially useful under hyperthermia conditions.¹⁹¹ Moreover, copolymerization with acrylic acid monomers facilitates binding of cationic molecules, such as doxorubicin^{82,184–187} and cisplatin⁷⁹ at physiological pH, and triggering release at lower pH values. For instance, it has been demonstrated that p(NIPAM-co-acrylic acid) nanogels could be loaded efficiently with doxorubicin and cisplatin, respectively.^{79,186} Both systems showed an increased drug release rate at lower pH values and in-vivo tumor graft models showed that both nanogels were able to achieve a considerable reduction in tumor volume, while having minimal influence on the body-weight of the used animals, demonstrating effective drug delivery without adverse health effects on the animals.^{79,186} Moreover, incorporating crosslinkers with disulfide bonds^{72,186,188,288} or crosslinking by disulfide formation,¹²⁸ enables specific intracellular drug release, further increasing the therapeutic efficacy.¹⁸⁶ Drug retention of disulfide-crosslinked p(OEGA-pyridyl)disulfide methacrylate) nanogels was determined by Ryu et al.¹²⁸ The nanogels were demonstrated to efficiently deliver doxorubicin into cells. Drug retention inside the nanogels was examined in the presence of phospholipid membranes by FRET. Figure 16 shows that encapsulation of a FRET pair in a densely crosslinked nanogel (37%) retains its FRET emission, whereas a lower crosslinking density (7%) results in the loss of FRET emission. However, cell toxicity was reduced to the same level by both nanogels, which was attributed to lower cellular uptake of the densely crosslinked nanogel.

Figure 16: Disulfide-crosslinked nanogels containing a FRET pair (A) were incubated together with phospholipid vesicles. Nanogels with a low crosslinking density showed loss of FRET emission (B and D), while FRET emission remained stable in nanogels with a high crosslinking density (C and D). FRET emission was however lost after exposing the nanogels to a reducing agent (DTT). Adapted from ref. 128.

The biocompatibility and biodegradability of polypeptides makes these materials very interesting for application in nanomedicine and the variety of monomers and possibilities for post-modification allows the incorporation of stimuli-responsive functional groups.²⁸⁹ Di-block polypeptide-based polymers can be used to form polyionic complexes by counterion-induced self-aggregation, which has been researched extensively by Kataoka and coworkers.⁴⁷ Nanogels can be obtained by crosslinking these polyionic complexes: Bronich and coworkers prepared PEG-polyglutamic acid nanogels, which were formed by initial self-aggregation by Ca^{2+} and subsequent crosslinking with cystamine.²⁹⁰ The hydrophobicity of the nanogel was increased by partial phenylalanine modification of the polymer to increase drug loading efficacy. Combination therapy of DOX and a chemotherapy-resistance inhibitor in a nanogel formulation showed tumor size-reduction and a diminished loss of body weight in mice. Polypeptide nanogels have also been prepared by a single-step NCA polymerization of mono and bifunctional monomers and PEG-amine as initiator.^{291,292} The disulfide bond in the bifunctional crosslinker rendered the nanogels bio-reducible and treatment of tumor-bearing mice with these nanogels resulted in a decreased tumor volume, while minimizing body-weight loss.²¹⁶ Furthermore, Chen and coworkers showed that incorporation of lysine and glutamic acid moieties gave the nanogels pH-responsive properties.²⁰⁵ Similar to pNIPAM-acrylic acid nanogels,⁸² lysine-containing nanogels encapsulated doxorubicin very effectively by ionic interaction and were able to deliver doxorubicin effectively into HeLa and HepG2

cells.²⁰⁵ Doxorubicin release was shown to be facilitated by disulfide cleavage upon exposure to glutathione, as well as breaking the ionic interaction between doxorubicin and the nanogel below physiological pH. Doxorubicin-loaded zwitterionic polypeptide nanogels with pH-labile hydrazone crosslinks have also been reported.²⁰⁶

Tightly crosslinked polypeptide nanogels were prepared by Lee et al, who crosslinked a phenyl-alanine-*co*-histidine-*b*-PEG polypeptide by micelle formation and subsequent crosslinking of the outer PEG shell with bovine serum albumin (BSA).²⁹³ The histidine moieties caused swelling of the nanogel and concomitant doxorubicin release below pH 6.4. In a similar system, Ju et al. prepared a nanogel by crosslinking *N*-lysinal-*N'*-succinyl chitosan, *N,N'*-methylenebis(acrylamide) and NIPAM by radical polymerization. After purification, the nanogel was further crosslinked on the outer shell by BSA (Figure 17A).²⁷ The resulting nanogel showed a reversible high degree of swelling and the nanogel was shown to be able to deliver doxorubicin sequentially to tumor cells. Furthermore, the reversible nature of the nanogels enabled intercellular delivery of doxorubicin. Figure 17B shows that after co-incubation with previously infected cells, doxorubicin was continuously released in the freshly exposed cells.

Figure 17: Doxorubicin-loaded nanogels consisting of pNIPAM and *N*-lysinal-*N'*-succinyl chitosan and coated by bovine serum albumin, was shown to release doxorubicin below physiological pH (A). Moreover, cells, which were exposed to nanogels were sequentially introduced to new cell cultures (B, left-to-right). This revealed that doxorubicin was delivered intercellularly by the nanogels. Adapted from ref. 27.

Dendritic polyglycerol exhibits lower immunogenicity than PEG⁸⁸ and hyperbranched dendritic polyglycerol-based nanogels have likewise been researched extensively, among others by Haag and coworkers and Frey and coworkers.^{294,295} DOX encapsulation was achieved by disulfide²⁰⁷ or boronate ester²⁹⁶ crosslinking. Furthermore, Sousa-Herves et al. used acrylate-functionalized hyperbranched polyglycerol and several acrylate-based monomers

as well as bis(2-methacryloyl)oxyethyl disulfide to form a DOX-loaded nanogel in a one-pot-synthesis, which is sensitive to bioreduction, as well as to pH changes.²¹⁸ Incorporation of therapeutics into nanogels can also be achieved by a pro-drug approach: dendritic polyglycerol-based nanogels have been used in the controlled release of doxorubicin.²⁰⁷ Acid-labile hydrazine linkers were used to include doxorubicin on the nanogel exterior or inside the nanogel matrix. Furthermore, the nanogel was crosslinked by disulfide exchange, rendering the nanogel responsive to both pH and bioreduction.

Nanogels sensitive to bacterial-secreted lipases have been reported by Wang and coworkers, who developed a macrophage-targeted mannose-functionalized polyphosphoester-based nanogel, which proved sensitive to bacteria-produced phospholipase as well as to reducing environments to deliver antibiotics.²⁶ To improve the targeting efficiency towards macrophages, the nanogels were functionalized with mannosamine (Figure 18A). After loading with vancomycin, the nanogels were tested in a methicillin-resistant *Staphylococcus aureus*-infected RAW 264.7 culture and in an MRSA-infected zebrafish model; on both occasions the mannose-targeted nanogel performed significantly better. Figure 18B shows that GFP-expressing MRSA was completely quenched after 5 hours post injection. Furthermore, administration of low concentrations of vancomycin encapsulated in the mannosylated nanogels shows a substantially higher survival-rate when compared to free vancomycin and the non-targeted nanogel.

7.2. Gene Delivery

Delivery of nucleic acids for therapeutic purposes is generally very inefficient and requires a suitable delivery vehicle to protect the nucleic acids against DNase and RNase mediated degradation.²⁹⁷ Furthermore, the therapeutic gene needs to be transported into the cells of interest. Despite their high efficiency, viral gene delivery vectors bring some critical issues, such as the required extensive purification, limited genetic payload, and safety issues. Hence, the use of polymeric gene delivery vectors as an alternative carrier is receiving ample interest.²⁹⁷ In this respect, nanogels offer very interesting properties as gene delivery vehicles, since their crosslinked structure offers the required stability (opposed to non-crosslinked delivery vehicles) for encapsulation of small nucleic acid fragments – such as miRNA and siRNA.²⁹⁸ pNIPAM nanogels have therefore been applied in the delivery of siRNA.^{147,299,300} Although these nanogels lacked a specific release mechanism, nucleic acids were still readily released from the nanogel matrix. Incorporation of cationic moieties in these pNIPAM nanogels, enables stable and efficient condensation of nucleic acids and the crosslinked nanogel structure facilitates the condensation of small nucleic acid fragments very efficiently.^{150,196,298} Polysaccharide nanogels, such as dextran,^{89,272} chitosan,^{301,302} amylose,³⁰³ and hyaluronic acid nanogels,²²⁸ have also been successfully applied in siRNA delivery for gene-silencing.³⁰⁴

Figure 18: Vancomycin-containing polyphosphoester nanogels, functionalized with mannose for targeting purposes (A) evaluated in a zebrafish methicillin-resistant *Staphylococcus aureus* (MRSA) infection model. Zebrafish infected with green fluorescent protein expressing MRSA and treated with PBS, empty mannose-functionalized nanogels (MNG), vancomycin (Van), vancomycin-loaded nanogels (NG-V) and vancomycin-loaded mannose-functionalized nanogels (MNG-V) (B). 9 hours post-infection MNG-V's treated zebrafish showed no fluorescence from MRSA infection. Survival-rate of zebrafish treated with 30, 15 and 3 ng vancomycin (C, D and E, respectively), showing significantly better survival rates for mannose-functionalized nanogels, at the lowest vancomycin concentration. Adapted from ref. 26.

pH-responsive and degradable monomers have been included into the nanogel matrix to improve nucleic acid condensation and triggered release.^{76,195,298} Tamura et al. prepared a 2-(*N,N*-diethylaminoethyl) methacrylate nanogel as a pH-responsive monomer with a pKa around 7.0, which could facilitate endosomal escape as well as siRNA complexation. Ethylene glycol dimethacrylate was used as a pH labile crosslinker and PEG-methacrylate for incorporation of stealth properties.¹⁹⁶ These nanogels showed superior stability as compared to non-crosslinked polyplexes and highly efficient RNA interference at N/P ratios as low as 2.¹⁹⁶ The ability to condensate siRNA was further improved by methylation of the nanogels' tertiary amines.³⁰⁵ A quaternization degree of 10% proved to be very effective, exhibiting gene silencing properties comparable to b-PEI

in HuH-7 cells.³⁰⁵ Raemdonck et al. used a similar approach, where quaternized 2-(*N,N*-dimethylaminoethyl) methacrylate was incorporated in pH-degradable dextran nanogels to facilitate siRNA-loading.⁸⁹ Averick et al. reported fully quaternized dimethylaminoethyl methacrylate bioreducible nanogels formed by ATRP in a miniemulsions (Figure 19A).²¹⁵ These nanogels could be complexed with siRNA, as well as with pDNA, which resulted in a stable complex formation. Furthermore, no cell toxicity was observed and Figure 19B and C show the transfection abilities of the nanogels for pDNA and SiRNA.

Figure 19: Bioreducible nanogels containing quaternized amines for controlled gene delivery (A). The nanogels were capable of delivering pDNA (B), as well as siRNA (C), with good transfection efficiencies. Adapted from ref. 215

Conjugation of siRNA to nanogels to further improve the complex stability has also been reported by Dunn et al. who initially prepared siRNA-loaded 2-aminoethyl methacrylate-based nanogels via a PRINT method.³⁰⁶ However, siRNA was readily released from the nanogel, limiting the RNA interference efficacy. Therefore, siRNA was conjugated to the nanogels by a disulfide linker, which dramatically improved RNA interference. A rather novel strategy is to use nucleic acids to crosslink delivery vehicles.³⁰⁷ Hong et al. crosslinked thiolated low molecular weight linear PEI with thiolated siRNA, which showed effective gene silencing.¹⁵¹ Moreover, the crosslinked siRNA-PEI nanogel exhibited a condensed structure and caused minimal to non-observable cell toxicity.

Treatment of (chronic) inflammatory diseases with siRNA shows great potential, because of the ability to (temporarily) knock down pro-inflammatory cytokines.^{202,308} In this respect, nanogels have been used to deliver siRNA for treatment of liver fibrosis¹⁵⁰ and rheumatoid arthritis.³⁰⁹ Using less invasive administration routes, such as oral delivery, expose the nanogel-siRNA complex to harsh conditions. Knipe et al. reported an oral formulation for inflammatory bowel disease treatment based on nanogels.²⁰² 2-(diethylamino)ethyl methacrylate-co-*tert*-butyl methacrylate nanogels were prepared by ATRP emulsion polymerization. siRNA-nanogels were incorporated in trypsin-degradable peptide-crosslinked PVP-MAA microgels to protect the nanogels during passage through the gastrointestinal tract. Figure 20B shows that after microgel degradation, transfection in RAW 264.7 cells was comparable to non-encapsulated nanogels and Lipofectamine (a transfection agent for in-vitro applications).

Figure 20: Microgel-encapsulated 2-(diethylamino)ethyl methacrylate-co-*tert*-butyl methacrylate nanogels for oral siRNA-delivery: after degradation of the microgel matrix, fluorescently labeled nanogels were added to murine macrophages, showing cellular uptake (A, cell nuclei are stained in blue, the cell wall in red and the nanogels in green). Delivery of TNF- α knockdown siRNA showed no significant difference between the nanogels and Lipofectamine (B). Moreover, nanogels released from degraded microgels, showed no significant change in TNF- α knockdown, compared to bare nanogels and Lipofectamine. Adapted from ref. 202.

7.3. Protein delivery

Delivery of recombinant proteins can be a low-risk alternative to gene delivery, as long-term risks arising from genetic modification are avoided. However, direct administration often gives dissatisfying results due to protein instability, immunogenicity, short half-life and low cellular uptake.³¹⁰ Encapsulation of therapeutic proteins into nanogel matrixes is a very promising strategy to overcome these problems.^{74,87,148,310,311} Moreover, the responsive properties of nanogels offer possibilities for triggered release by incorporating enzymatically degradable,²²⁵ bioreducible,^{129,312} and pH-labile bonds.^{73,74,211,310}

Mini-emulsion polymerization has been used to encapsulate enzymes into an oligoethylene glycol matrix.^{73,87} After release from pH-degradable oligoethylene glycol nanogels, only 25% of the enzyme activity was lost.⁷³ Steinhilber et al. showed the encapsulation of enzymes into pH-degradable polyglycerol nanogels, using nanoprecipitation in order to prevent enzyme denaturation.⁷⁴ During nanoprecipitation, the nanogels were immediately crosslinked by click chemistry and after degradation of the benzacetal bonds enzyme activity was not noticeably reduced. Delivery of apoptotic proteins into cancer cells can activate specific cell apoptosis pathways, such as apoptin. Zhao et al. prepared acrylamide-based nanogels where large anionic apoptin complexes served as a template material for cationic nanogels, as shown in Figure 21A.³¹² Addition of *N*-(3-aminopropyl)methacrylamide, acrylamide and *N,N'*-bis(acryloyl)cystamine and subsequent radical crosslinking resulted in 80 nm bio-reducible nanogels, which induced cell apoptosis after cellular uptake. Furthermore, in vivo studies in a mouse tumor graft model showed that intratumoral injections with the apoptin nanogels significantly reduced tumor volume (Figure 21D). Encapsulation and release of cytochrome C, an apoptotic peptide, has been reported. Chen et al. prepared a block copolymer via RAFT polymerization consisting of a PEG block and a copolymer of 2-hydroxyethyl methacrylate and a cyclic carbonate monomer.¹²⁹ The cyclic carbonate was ring-opened with cystamine and immediately oxidized in the presence of cytochrome C to obtain disulfide crosslinked nanogels. Boronic acid bearing polyglycerol nanogels, which were consecutively functionalized with primary amine and dimethylmaleic anhydride, giving them a zwitterionic character with charge conversion properties, have been reported for the delivery of cytochrome C.²¹¹ Cytochrome C was encapsulated via nanoprecipitation and nanogel crosslinking was achieved via boronate ester formation with the polyglycerol backbone. Both studies revealed protein release as well as intracellular release of cytochrome C and the induction of apoptosis.^{129,211}

Enzymes may also be applied as a bioscavenger to remove toxins from the bloodstream. However, due to their short half-life and immunogenicity, application of recombinant enzymes as bioscavengers is not viable, not even after PEG-ylation.¹⁷⁰ Zhang et al. crosslinked acrylate-modified butyrylcholinesterase into zwitterionic poly(carboxybetaine) nanogels for organophosphate removal.¹⁴⁸ The incorporated enzyme displayed a small drop in activity, but showed superior blood circulation compared to the native enzyme. Furthermore, no immunogenic response to the nanogel was observed in rats.

Figure 21: Encapsulation of apoptin into a bio-reducible cationic nanogel (A). Nanogels effectively reduced cell viability in cancer cell lines by apoptosis delivery (B), but not on human fibroblasts (C). Moreover, the apoptin-containing bio-reducible nanogels also significantly reduced tumor volume in a mouse xenograft model (D). Adapted from ref. 312.

7.3.1. Insulin delivery

On-demand delivery of insulin for treatment of diabetes mellitus may benefit from glucose-responsive delivery systems. Therefore, controlled release of insulin in the bloodstream has been investigated for phenylboronic acid-based glucose-sensitive nanogels.^{181,241,313,314} Poly(4-vinylphenylboronic acid-co-2-(dimethylamino)ethyl acrylate) nanogel were templated on gold nanoparticles.¹⁸¹ Insulin was loaded into the nanogel matrix, and released in response to increases in glucose concentration due to nanogel swelling.

Polypeptide nanogels for glucose-sensitive insulin release were prepared by crosslinking a glucose-functionalized PEG-polypeptide block copolymer with a bi-functional phenylboronic acid crosslinker in the presence of insulin.³¹⁵ Insulin-containing nanogels were formed by crosslinking with a bifunctional, which showed insulin release upon exposure to glucose. Furthermore, cell viability in HELA cells was barely affected, while no significant nanogel-induced blood hemolysis was observed.

7.3.2. Nanogel-based vaccines

Delivery of proteins and peptides to antigen presenting cells – predominantly dendritic cells – plays a key role in the immune response modified by vaccines.³¹⁶ Protection of these antigens and enhancements in specific uptake would promote the efficacy of vaccines, and provide important possibilities to trigger specific immune responses to cancer cells among others.^{124,317} Akiyoshi and coworkers have done extensive research on self-assembled cholesterylated pullan nanogels (CPNs).³¹⁸ For example, intranasal

administration of antigen-containing CPNs in mice induced immunity against a *clostridium botulinum* type-A neurotoxin.¹²⁴ Covalently crosslinked nanogels have also been explored for vaccine delivery.^{232,319,320} Disulfide crosslinked PEI-alginate nanogels could effectively encapsulate ovalbumin, a model antigen.³¹⁹ Ovalbumin-specific CD8⁺ T-cell counts were significantly increased after administration of disulfide crosslinked nanogels, compared to non-bioreducible nanogels or free ovalbumin. Li et al. incorporated thiol groups into dextran nanogels to covalently incorporate ovalbumin via disulfide linkages.²³² Loading capacities above 10% were achieved and delivery of encapsulated ovalbumin into dendritic cells resulted in antigen presentation able to activate T-cells, which was not observed in dendritic cells exposed to ovalbumin. Synthetic poly(hydroxyethylmethacrylate-*co*-methacrylic acid) nanogels have been loaded with ovalbumin for oral delivery,³²⁰ where the ovalbumin was retained in the nanogel at low pH (gastric conditions) and released at physiological pH.

8. Conclusions

Polymeric nanostructures have opened up numerous avenues for the controlled delivery of therapeutics and targeted imaging. Nanogels are a relatively new delivery vehicle, which uniquely consists of a homogeneous hydrophilic matrix. Nanogels are characterized by high water content, with potentially high drug-loading capacity. Nanogels also offer interesting opportunities for the delivery of contrast agents for imaging. Moreover, the use of highly hydrophilic materials to prepare nanogels, such as zwitterionic polymers, reduces non-specific interactions with cells and proteins and consequently improves their colloidal stability in biological environments. Manipulating the nanogel mechanical properties by controlling their crosslinking density, allows for enhancing blood circulation. By careful monomer choice, responsive properties can be incorporated into nanogels. As a result, reversibly-responsive nanogels have been prepared for triggered payload release as well as for responsive imaging. Moreover, breaking covalent bonds under specific conditions renders nanogels irreversibly responsive to pH, reducible environments and enzyme activity, which triggers payload release, biodegradation and charge conversion. All of these properties are beneficial for increasing targeting efficacy and therapeutic release. Besides triggered release, nanogels can also be used in responsive imaging to monitor, for instance, local pH, glucose concentration or temperature in biological systems. Despite the promising results obtained in controlled drug delivery, nanogels require high crosslinking degrees for efficient encapsulation, often leading to premature drug leakage. Moreover, long-term stability of therapeutic nanogel formulations are yet to be evaluated before transition into clinical applications. Nevertheless, nanogels have shown great versatility in mechanisms for release and responsiveness, allowing specific design of nanogel structures for a wide

range of applications. Prospective application of nanogels for imaging is paving the way for sensitive and location-specific measurement of pH, temperature, enzyme activity and metabolite concentrations, providing unprecedented detailed and localized information in a minimally invasive manner. Moreover, nanogels in therapeutic delivery promise highly efficient drug delivery and reversible responsiveness to physiological stimuli, allowing on/off switching of release to facilitate true on-demand delivery.

9. References

- (1) Judenhofer, M. S.; Wehr, H. F.; Newport, D. F.; Catana, C.; Siegel, S. B.; Becker, M.; Thielscher, A.; Kneilling, M.; Lichy, M. P.; Eichner, M.; Klingel, K.; Reischl, G.; Widmaier, S.; Röcken, M.; Nutt, R. E.; Machulla, H.-J.; Uludag, K.; Cherry, S. R.; Claussen, C. D.; Pichler, B. J. *Nat. Med.* **2008**, *14* (4), 459–465.
- (2) Nair, L. S.; Laurencin, C. T. *Prog. Polym. Sci.* **2007**, *32* (8), 762–798.
- (3) Vinegoni, C.; Botnaru, I.; Aikawa, E.; Calfon, M. A.; Iwamoto, Y.; Folco, E. J.; Ntziachristos, V.; Weissleder, R.; Libby, P.; Jaffer, F. A. *Sci. Transl. Med.* **2011**, *3* (84), 84ra45–84ra45.
- (4) Makarova, K. S.; Haft, D. H.; Barrangou, R.; Brouns, S. J. J.; Charpentier, E.; Horvath, P.; Moineau, S.; Mojica, F. J. M.; Wolf, Y. I.; Yakunin, A. F.; van der Oost, J.; Koonin, E. V. *Nat. Rev. Microbiol.* **2011**, *9* (6), 467–477.
- (5) Hrkach, J.; Von Hoff, D.; Mukkaram Ali, M.; Andrianova, E.; Auer, J.; Campbell, T.; De Witt, D.; Figa, M.; Figueiredo, M.; Horhota, A.; Low, S.; McDonnell, K.; Peeke, E.; Retnarajan, B.; Sabnis, A.; Schnipper, E.; Song, J. J.; Song, Y. H.; Summa, J.; Tompsett, D.; Troiano, G.; Van Geen Hoven, T.; Wright, J.; LoRusso, P.; Kantoff, P. W.; Bander, N. H.; Sweeney, C.; Farokhzad, O. C.; Langer, R.; Zale, S. *Sci. Transl. Med.* **2012**, *4* (128), 128ra39.
- (6) Bolli, R.; Chugh, A. R.; D'Amario, D.; Loughran, J. H.; Stoddard, M. F.; Ikram, S.; Beache, G. M.; Wagner, S. G.; Leri, A.; Hosoda, T.; Sanada, F.; Elmore, J. B.; Goichberg, P.; Cappetta, D.; Solankhi, N. K.; Fahsah, I.; Rokosh, D. G.; Slaughter, M. S.; Kajstura, J.; Anversa, P. *Lancet* **2011**, *378* (9806), 1847–1857.
- (7) Kelkar, S. S.; Reineke, T. M. *Bioconjug. Chem.* **2011**, *22* (10), 1879–1903.
- (8) Lander, E. S.; Linton, L. M.; Birren, B.; Nusbaum, C.; Zody, M. C.; Baldwin, J.; Devon, K.; Dewar, K.; Doyle, M.; FitzHugh, W.; Funke, R.; Gage, D.; Harris, K.; Heaford, A.; Howland, J.; Kann, L.; Lehoczy, J.; LeVine, R.; McEwan, P.; McKernan, K.; Meldrim, J.; Mesirov, J. P.; Miranda, C.; Morris, W.; Naylor, J.; Raymond, C.; Rosetti, M.; Santos, R.; Sheridan, A.; Sougnez, C.; Stange-Thomann, N.; Stojanovic, N.; Subramanian, A.; Wyman, D.; Rogers, J.; Sulston, J.; Ainscough, R.; Beck, S.; Bentley, D.; Burton, J.; Clee, C.; Carter, N.; Coulson, A.; Deadman, R.; Deloukas, P.; Dunham, A.; Dunham, I.; Durbin, R.; French, L.; Grafham, D.; Gregory, S.; Hubbard, T.; Humphray, S.; Hunt, A.; Jones, M.; Lloyd, C.; McMurray, A.; Matthews, L.; Mercer, S.; Milne, S.; Mullikin, J. C.; Mungall, A.; Plumb, R.; Ross, M.; Shownkeen, R.; Sims, S.; Waterston, R. H.; Wilson, R. K.; Hillier, L. W.; McPherson, J. D.; Marra, M. A.; Mardis, E. R.; Fulton, L. A.; Chinwalla, A. T.; Pepin, K. H.; Gish, W. R.; Chisoe, S. L.; Wendl, M. C.; Delehaunty, K. D.; Miner, T. L.; Delehaunty, A.; Kramer, J. B.; Cook, L. L.; Fulton, R. S.; Johnson, D. L.; Minx, P. J.; Clifton, S. W.; Hawkins, T.; Branscomb, E.; Predki, P.; Richardson, P.; Wenning, S.; Slezak, T.; Doggett, N.; Cheng, J.-F.; Olsen, A.; Lucas, S.; Elkin, C.; Uberbacher, E.; Frazier, M.; Gibbs, R. A.; Muzny, D. M.; Scherer, S. E.; Bouck, J. B.; Sodergren, E. J.; Worley, K. C.; Rives, C. M.; Gorrell, J. H.; Metzker, M. L.; Naylor, S. L.; Kucherlapati, R. S.; Nelson, D. L.; Weinstock, G. M.; Sakaki, Y.; Fujiyama, A.; Hattori, M.; Yada, T.; Toyoda, A.; Itoh, T.; Kawagoe, C.; Watanabe, H.; Totoki, Y.; Taylor, T.; Weissenbach, J.;

- Heilig, R.; Saurin, W.; Artiguenave, F.; Brottier, P.; Bruls, T.; Pelletier, E.; Robert, C.; Wincker, P.; Rosenthal, A.; Platzer, M.; Nyakatura, G.; Taudien, S.; Rump, A.; Smith, D. R.; Doucette-Stamm, L.; Rubenfield, M.; Weinstock, K.; Lee, H. M.; Dubois, J.; Yang, H.; Yu, J.; Wang, J.; Huang, G.; Gu, J.; Hood, L.; Rowen, L.; Madan, A.; Qin, S.; Davis, R. W.; Federspiel, N. A.; Abola, A. P.; Proctor, M. J.; Roe, B. A.; Chen, F.; Pan, H.; Ramser, J.; Lehrach, H.; Reinhardt, R.; McCombie, W. R.; de la Bastide, M.; Dedhia, N.; Blöcker, H.; Hornischer, K.; Nordsiek, G.; Agarwala, R.; Aravind, L.; Bailey, J. A.; Bateman, A.; Batzoglou, S.; Birney, E.; Bork, P.; Brown, D. G.; Burge, C. B.; Cerutti, L.; Chen, H.-C.; Church, D.; Clamp, M.; Copley, R. R.; Doerks, T.; Eddy, S. R.; Eichler, E. E.; Furey, T. S.; Galagan, J.; Gilbert, J. G. R.; Harmon, C.; Hayashizaki, Y.; Haussler, D.; Hermjakob, H.; Hokamp, K.; Jang, W.; Johnson, L. S.; Jones, T. A.; Kasif, S.; Kasprzyk, A.; Kennedy, S.; Kent, W. J.; Kitts, P.; Koonin, E. V.; Korf, I.; Kulp, D.; Lancet, D.; Lowe, T. M.; McLysaght, A.; Mikkelsen, T.; Moran, J. V.; Mulder, N.; Pollara, V. J.; Ponting, C. P.; Schuler, G.; Schultz, J.; Slater, G.; Smit, A. F. A.; Stupka, E.; Szustakowski, J.; Thierry-Mieg, D.; Thierry-Mieg, J.; Wagner, L.; Wallis, J.; Wheeler, R.; Williams, A.; Wolf, Y. I.; Wolfe, K. H.; Yang, S.-P.; Yeh, R.-F.; Collins, F.; Guyer, M. S.; Peterson, J.; Felsenfeld, A.; Wetterstrand, K. A.; Myers, R. M.; Schmutz, J.; Dickson, M.; Grimwood, J.; Cox, D. R.; Olson, M. V.; Kaul, R.; Raymond, C.; Shimizu, N.; Kawasaki, K.; Minoshima, S.; Evans, G. A.; Athanasiou, M.; Schultz, R.; Patrinos, A.; Morgan, M. J. *Nature* **2001**, 409 (6822), 860–921.
- (9) Riehemann, K.; Schneider, S. W.; Luger, T. A.; Godin, B.; Ferrari, M.; Fuchs, H. *Angew. Chemie Int. Ed.* **2009**, 48 (5), 872–897.
 - (10) Kim, B. Y. S.; Rutka, J. T.; Chan, W. C. W. *N. Engl. J. Med.* **2010**, 363 (25), 2434–2443.
 - (11) Elsabahy, M.; Heo, G. S.; Lim, S.-M.; Sun, G.; Wooley, K. L. *Chem. Rev.* **2015**, 115 (19), 10967–11011.
 - (12) Kunjachan, S.; Ehling, J.; Storm, G.; Kiessling, F.; Lammers, T. *Chem. Rev.* **2015**, 115 (19), 10907–10937.
 - (13) Kim, D.; Park, S.; Lee, J. H.; Jeong, Y. Y.; Jon, S. J. *Am. Chem. Soc.* **2007**, 129 (24), 7661–7665.
 - (14) Zhang, J.; Yuan, Z.-F.; Wang, Y.; Chen, W.-H.; Luo, G.-F.; Cheng, S.-X.; Zhuo, R.-X.; Zhang, X.-Z. *J. Am. Chem. Soc.* **2013**, 135 (13), 5068–5073.
 - (15) Nicolas, J.; Mura, S.; Brambilla, D.; Mackiewicz, N.; Couvreur, P. *Chem. Soc. Rev.* **2013**, 42 (3), 1147–1235.
 - (16) Cheng, J.; Teply, B. A.; Sherifi, I.; Sung, J.; Luther, G.; Gu, F. X.; Levy-Nissenbaum, E.; Radovic-Moreno, A. F.; Langer, R.; Farokhzad, O. C. *Biomaterials* **2007**, 28 (5), 869–876.
 - (17) Reisch, A.; Runser, A.; Arntz, Y.; Mély, Y.; Klymchenko, A. S.; Reisch, A.; Runser, A.; Arntz, Y.; Me, Y. *ACS Nano* **2015**, 9 (5), 5104–5116.
 - (18) Ahmed, F.; Pakunlu, R. I.; Brannan, A.; Bates, F.; Minko, T.; Discher, D. E. *J. Control. Release* **2006**, 116 (2), 150–158.
 - (19) Brinkhuis, R. P.; Stojanov, K.; Laverman, P.; Eilander, J.; Zuhorn, I. S.; Rutjes, F. P. J. T.; van Hest, J. C. M. *Bioconjug. Chem.* **2012**, 23 (5), 958–965.
 - (20) Lee, C. C.; MacKay, J. A.; Fréchet, J. M. J.; Szoka, F. C. *Nat. Biotechnol.* **2005**, 23 (12), 1517–1526.
 - (21) Helms, B.; Meijer, E. W. *Science* **2006**, 313 (5789), 929–930.

- (22) Huang, C.-H.; Nwe, K.; Al Zaki, A.; Brechbiel, M. W.; Tsourkas, A. *ACS Nano* **2012**, 6 (11), 9416–9424.
- (23) Sun, G.; Cui, H.; Lin, L. Y.; Lee, N. S.; Yang, C.; Neumann, W. L.; Freskos, J. N.; Shieh, J. J.; Dorshow, R. B.; Wooley, K. L. *J. Am. Chem. Soc.* **2011**, 133 (22), 8534–8543.
- (24) Joralemon, M. J.; O'Reilly, R. K.; Hawker, C. J.; Wooley, K. L. *J. Am. Chem. Soc.* **2005**, 127 (48), 16892–16899.
- (25) Shi, Y.; van Steenberg, M. J.; Teunissen, E. A.; Novo, L.; Gradmann, S.; Baldus, M.; van Nostrum, C. F.; Hennink, W. E. *Biomacromolecules* **2013**, 14 (6), 1826–1837.
- (26) Xiong, M.-H. H.; Li, Y.-J. J.; Bao, Y.; Yang, X.-Z. Z.; Hu, B.; Wang, J. *Adv. Mater.* **2012**, 24 (46), 6175–6180.
- (27) Ju, C.; Mo, R.; Xue, J.; Zhang, L.; Zhao, Z.; Xue, L.; Ping, Q.; Zhang, C. *Angew. Chemie Int. Ed.* **2014**.
- (28) Du, J.-Z. Z.; Sun, T.-M. M.; Song, W.-J. J.; Wu, J.; Wang, J. *Angew. Chemie Int. Ed.* **2010**, 49 (21), 3621–3626.
- (29) Kabanov, A. V.; Vinogradov, S. V. *Angew. Chemie Int. Ed.* **2009**, 48 (30), 5418–5429.
- (30) Thanh, N. T. K.; Green, L. A. W. *Nano Today* **2010**, 5 (3), 213–230.
- (31) Alexis, F.; Pridgen, E.; Molnar, L. K.; Farokhzad, O. C. *Mol. Pharm.* **2008**, 5 (4), 505–515.
- (32) Albanese, A.; Tang, P. S.; Chan, W. C. W. *Annu. Rev. Biomed. Eng.* **2012**, 14, 1–16.
- (33) Such, G. K.; Yan, Y.; Johnston, A. P. R.; Gunawan, S. T.; Caruso, F. *Adv. Mater.* **2015**, 27 (14), 2278–2297.
- (34) Ye, M.; Qian, Y.; Tang, J.; Hu, H.; Sui, M.; Shen, Y. J. *Control. Release* **2013**, 169 (3), 239–245.
- (35) Opina, A. C.; Wong, K. J.; Griffiths, G. L.; Turkbey, B. I.; Bernardo, M.; Nakajima, T.; Kobayashi, H.; Choyke, P. L.; Vasalatiy, O. *Nanomedicine (Lond.)* **2015**, 10 (9), 1423–1437.
- (36) Cheng, Z.; Thorek, D. L. J.; Tsourkas, A. *Angew. Chemie Int. Ed.* **2010**, 49 (2), 346–350.
- (37) Floyd, W. C.; Klemm, P. J.; Smiles, D. E.; Kohlgruber, A. C.; Pierre, V. C.; Mynar, J. L.; Fréchet, J. M. J.; Raymond, K. N. *J. Am. Chem. Soc.* **2011**, 133 (8), 2390–2393.
- (38) Wen, S.; Li, K.; Cai, H.; Chen, Q.; Shen, M.; Huang, Y.; Peng, C.; Hou, W.; Zhu, M.; Zhang, G.; Shi, X. *Biomaterials* **2013**, 34 (5), 1570–1580.
- (39) Kono, K.; Nakashima, S.; Kokuryo, D.; Aoki, I.; Shimomoto, H.; Aoshima, S.; Maruyama, K.; Yuba, E.; Kojima, C.; Harada, A.; Ishizaka, Y. *Biomaterials* **2011**, 32 (5), 1387–1395.
- (40) Lempens, E. H. M.; Merckx, M.; Tirrell, M.; Meijer, E. W. *Bioconjug. Chem.* **2011**, 22 (3), 397–405.
- (41) Li, X.; Qian, Y.; Liu, T.; Hu, X.; Zhang, G.; You, Y.; Liu, S. *Biomaterials* **2011**, 32 (27), 6595–6605.
- (42) Röglin, L.; Lempens, E. H. M.; Meijer, E. W. *Angew. Chemie Int. Ed.* **2011**, 50 (1), 102–112.
- (43) Cheng, Z.; Al Zaki, A.; Hui, J. Z.; Muzykantov, V. R.; Tsourkas, A. *Science* **2012**, 338 (6109), 903–910.
- (44) Wilms, D.; Stiriba, S.-E.; Frey, H. *Acc. Chem. Res.* **2010**, 43 (1), 129–141.
- (45) Khandare, J.; Calderón, M.; Dagia, N. M.; Haag, R. *Chem. Soc. Rev.* **2012**, 41 (7), 2824–2848.
- (46) Zhang, S.; Zou, J.; Zhang, F.; Elsabahy, M.; Felder, S. E.; Zhu, J.; Pochan, D. J.; Wooley, K. L. *J. Am. Chem. Soc.* **2012**, 134 (44), 18467–18474.

- (47) Mi, P.; Dewi, N.; Yanagie, H.; Kokuryo, D.; Suzuki, M.; Sakurai, Y.; Li, Y.; Aoki, I.; Ono, K.; Takahashi, H.; Cabral, H.; Nishiyama, N.; Kataoka, K. *ACS Nano* **2015**, 9 (6), 5913–5921.
- (48) Brinkhuis, R. P.; Rutjes, F. P. J. T.; van Hest, J. C. M. *Polym. Chem.* **2011**, 2 (7), 1449.
- (49) Tanner, P.; Baumann, P.; Enea, R.; Onaca, O.; Palivan, C.; Meier, W. *Acc. Chem. Res.* **2011**, 44 (10), 1039–1049.
- (50) Allen, T. M.; Cullis, P. R. *Adv. Drug Deliv. Rev.* **2013**, 65 (1), 36–48.
- (51) Elsabahy, M.; Wooley, K. L. *Chem. Soc. Rev.* **2012**, 41 (7), 2545.
- (52) Cheng, Z.; Thorek, D. L. J.; Tsourkas, A. *Adv. Funct. Mater.* **2009**, 19 (23), 3753–3759.
- (53) Kim, K. S.; Park, W.; Hu, J.; Bae, Y. H.; Na, K. *Biomaterials* **2014**, 35 (1), 337–343.
- (54) Mi, P.; Cabral, H.; Kokuryo, D.; Rafi, M.; Terada, Y.; Aoki, I.; Saga, T.; Takehiko, I.; Nishiyama, N.; Kataoka, K. *Biomaterials* **2013**, 34 (2), 492–500.
- (55) Lepeltier, E.; Bourgaux, C.; Couvreur, P. *Adv. Drug Deliv. Rev.* **2014**, 71, 86–97.
- (56) Schubert, S.; Delaney, Jr, J. T.; Schubert, U. S. *Soft Matter* **2011**, 7 (5), 1581–1588.
- (57) Kulkarni, S. A.; Feng, S.-S. *Pharm. Res.* **2013**, 30 (10), 2512–2522.
- (58) Kreuter, J.; Ramge, P.; Petrov, V.; Hamm, S.; Gelperina, S. E.; Engelhardt, B.; Alyautdin, R.; von Briesen, H.; Begley, D. J. *Pharm. Res.* **2003**, 20 (3), 409–416.
- (59) Danhier, F.; Ansorena, E.; Silva, J. M.; Coco, R.; Le Breton, A.; Préat, V. *J. Control. Release* **2012**, 161 (2), 505–522.
- (60) Hoffman, A. S. *Adv. Drug Deliv. Rev.* **2002**, 54 (1), 3–12.
- (61) Peppas, N. A.; Hilt, J. Z.; Khademhosseini, A.; Langer, R. *Adv. Mater.* **2006**, 18 (11), 1345–1360.
- (62) Seliktar, D. *Science* **2012**, 336 (6085), 1124–1128.
- (63) Li, Y.; Rodrigues, J.; Tomás, H. *Chem. Soc. Rev.* **2012**, 41 (6), 2193–2221.
- (64) Censi, R.; Di, P.; Vermonden, T.; Hennink, W. E. J. *Control. Release* **2012**, 161 (2), 680–692.
- (65) Qiu, Y.; Park, K. *Adv. Drug Deliv. Rev.* **2001**, 53 (3), 321–339.
- (66) Molina, M.; Asadian-Birjand, M.; Balach, J.; Bergueiro, J.; Miceli, E.; Calderón, M.; Calderon, M. *Chem. Soc. Rev.* **2015**, 44 (17), 6161–6186.
- (67) Zhang, L.; Cao, Z.; Li, Y.; Ella-Menye, J.-R.; Bai, T.; Jiang, S. *ACS Nano* **2012**, 6 (8), 6681–6686.
- (68) Anselmo, A. C.; Zhang, M.; Kumar, S.; Vogus, D. R.; Menegatti, S.; Helgeson, M. E.; Mitragotri, S. *ACS Nano* **2015**, 9 (3), 3169–3177.
- (69) Ramos, J.; Forcada, J.; Hidalgo-Alvarez, R. *Chem. Rev.* **2014**, 114 (1), 367–428.
- (70) Baker, W. O. *Rubber Chem. Technol.* **1949**, 22 (4), 935–955.
- (71) Quillet, C.; Eicke, H. F. *J. Phys. Chem.* **1987**, 91 (15), 4211–4212.
- (72) Qiao, Z.-Y. Y.; Zhang, R.; Du, F.-S. S.; Liang, D.-H. H.; Li, Z.-C. C. *J. Control. Release* **2011**, 152 (1), 57–66.
- (73) Molla, M. R.; Marcinko, T.; Prasad, P.; Deming, D.; Garman, S. C.; Thayumanavan, S. *Biomacromolecules* **2014**, 15 (11), 4046–4053.
- (74) Steinhilber, D.; Witting, M.; Zhang, X.; Staegemann, M.; Paulus, F.; Friess, W.; Küchler, S.; Haag, R. *J. Control. Release* **2013**, 169 (3), 289–295.

- (75) Urakami, H.; Hentschel, J.; Seetho, K.; Zeng, H.; Chawla, K.; Guan, Z. *Biomacromolecules* **2013**, *14* (10), 3682–3688.
- (76) Zhang, X.; Malhotra, S.; Molina, M.; Haag, R. *Chem. Soc. Rev.* **2015**, *44* (7), 1948–1973.
- (77) Gota, C.; Okabe, K.; Funatsu, T.; Harada, Y.; Uchiyama, S. *J. Am. Chem. Soc.* **2009**, *131* (8), 2766–2767.
- (78) Wu, W.; Shen, J.; Banerjee, P.; Zhou, S. *Biomaterials* **2010**, *31* (29), 7555–7566.
- (79) Peng, J.; Qi, T.; Liao, J.; Chu, B.; Yang, Q.; Li, W.; Qu, Y.; Luo, F.; Qian, Z. *Biomaterials* **2013**, *34* (34), 8726–8740.
- (80) Hofmann, C. H.; Grobelny, S.; Erlkamp, M.; Winter, R.; Richtering, W. *Polymer (Guildf)*. **2014**, *55* (8), 2000–2007.
- (81) Kazakov, S.; Kaholek, M.; Gazaryan, I.; Krasnikov, B.; Miller, K.; Levon, K. *J. Phys. Chem. B* **2006**, *110* (31), 15107–15116.
- (82) Peng, J.; Qi, T.; Liao, J.; Fan, M.; Luo, F.; Li, H.; Qian, Z. *Nanoscale* **2012**, *4* (8), 2694–2704.
- (83) Ekkelenkamp, A. E.; Jansman, M. M. T.; Roelofs, K.; Engbersen, J. F. J.; Paulusse, J. M. J. *Acta Biomater.* **2016**, *30*, 126–134.
- (84) Oh, J. K.; Drumright, R.; Siegwart, D. J.; Matyjaszewski, K. *Prog. Polym. Sci.* **2008**, *33* (4), 448–477.
- (85) Nayak, S.; Lyon, L. A. *Angew. Chemie Int. Ed.* **2005**, *44* (47), 7686–7708.
- (86) Zhang, L.; Xue, H.; Cao, Z.; Keefe, A.; Wang, J.; Jiang, S. *Biomaterials* **2011**, *32* (20), 4604–4608.
- (87) Siegwart, D. J.; Srinivasan, A.; Bencherif, S. A.; Karunanidhi, A.; Oh, J. K.; Vaidya, S.; Jin, R.; Hollinger, J. O.; Matyjaszewski, K. *Biomacromolecules* **2009**, *10* (8), 2300–2309.
- (88) Steinhilber, D.; Seiffert, S.; Heyman, J. A.; Paulus, F.; Weitz, D. A.; Haag, R. *Biomaterials* **2011**, *32* (5), 1311–1316.
- (89) Raemdonck, K.; Naeye, B.; Buyens, K.; Vandenbroucke, R. E.; Høgset, A.; Demeester, J.; De Smedt, S. C. *Adv. Funct. Mater.* **2009**, *19* (9), 1406–1415.
- (90) Oh, J. K.; Tang, C.; Gao, H.; Tsarevsky, N. V.; Matyjaszewski, K. *J. Am. Chem. Soc.* **2006**, *128* (16), 5578–5584.
- (91) Arshady, R. *Colloid Polym. Sci.* **1992**, *270* (8), 717–732.
- (92) Ali, A. M. I.; Pareek, P.; Sewell, L.; Schmid, A.; Fujii, S.; Armes, S. P.; Shirley, I. M. *Soft Matter* **2007**, *3* (8), 1003.
- (93) Zhou, J.; Zhang, W.-J.; Hong, C.-Y.; Pan, C.-Y. *Polym. Chem.* **2016**, *7* (19), 3259–3267.
- (94) Derry, M. J.; Fielding, L. A.; Armes, S. P. *Prog. Polym. Sci.* **2016**, *52*, 1–18.
- (95) An, Z.; Shi, Q.; Tang, W.; Tsung, C.-K.; Hawker, C. J.; Stucky, G. D. *J. Am. Chem. Soc.* **2007**, *129* (46), 14493–14499.
- (96) Williams, M.; Penfold, N. J. W.; Armes, S. P. *Polym. Chem.* **2016**, *7* (2), 384–393.
- (97) Warren, N. J.; Armes, S. P. *J. Am. Chem. Soc.* **2014**, *136* (29), 10174–10185.
- (98) Ratcliffe, L. P. D.; Couchon, C.; Armes, S. P.; Paulusse, J. M. J. *Biomacromolecules* **2016**, *17* (6), 2277–2283.

- (99) Rieger, J.; Gazon, C.; Charleux, B.; Alaimo, D.; Jérôme, C. *J. Polym. Sci. Part A Polym. Chem.* **2009**, *47* (9), 2373–2390.
- (100) Gazon, C.; Rieger, J.; Sanson, N.; Charleux, B. *Soft Matter* **2011**, *7* (7), 3482.
- (101) Liu, G.; Qiu, Q.; Shen, W.; An, Z. *Macromolecules* **2011**, *44* (13), 5237–5245.
- (102) Shen, W.; Chang, Y.; Liu, G.; Wang, H.; Cao, A.; An, Z. *Macromolecules* **2011**, *44* (8), 2524–2530.
- (103) Liu, G.; Qiu, Q.; An, Z. *Polym. Chem.* **2012**, *3* (2), 504–513.
- (104) Xu, Y.; Li, Y.; Cao, X.; Chen, Q.; An, Z. *Polym. Chem.* **2014**, *5* (21), 6244–6255.
- (105) Ma, K.; Xu, Y.; An, Z. *Macromol. Rapid Commun.* **2015**, *36* (6), 566–570.
- (106) Deng, L.; Zhai, Y.; Guo, S.; Jin, F.; Xie, Z.; He, X.; Dong, A. *J. Nanoparticle Res.* **2009**, *11* (2), 365–374.
- (107) Gao, H.; Tsarevsky, N. V.; Matyjaszewski, K. *Macromolecules* **2005**, *38* (14), 5995–6004.
- (108) Bachler, P. R.; Forry, K. E.; Sparks, C. A.; Schulz, M. D.; Wagener, K. B.; Sumerlin, B. S. *Polym. Chem.* **2016**, 4155–4159.
- (109) van der Ende, A. E.; Kravitz, E. J.; Harth, E. *J. Am. Chem. Soc.* **2008**, *130* (27), 8706–8713.
- (110) Xu, Y.; Bolisetty, S.; Drechsler, M.; Fang, B.; Yuan, J.; Ballauff, M.; Müller, A. H. E. *Polymer (Guildf)*. **2008**, *49* (18), 3957–3964.
- (111) Park, J. H.; Bae, S. Y.; Kim, B. K. *Polym. Bull.* **2013**, *70* (3), 859–869.
- (112) Gao, H.; Matyjaszewski, K. *Prog. Polym. Sci.* **2009**, *34* (4), 317–350.
- (113) Khan, A.; Malkoch, M.; Montague, M. F.; Hawker, C. J. *J. Polym. Sci. Part A Polym. Chem.* **2008**, *46* (18), 6238–6254.
- (114) Gao, H.; Li, W.; Matyjaszewski, K. *Macromolecules* **2008**, *41* (7), 2335–2340.
- (115) Ferreira, J.; Syrett, J.; Whittaker, M.; Haddleton, D.; Davis, T. P.; Boyer, C. *Polym. Chem.* **2011**, *2* (8), 1671–1677.
- (116) Zhao, T.; Zhang, H.; Newland, B.; Aied, A.; Zhou, D.; Wang, W. *Angew. Chemie Int. Ed.* **2014**, *53* (24), 6095–6100.
- (117) Bai, Y.; Xing, H.; Vincil, G. a.; Lee, J.; Henderson, E. J.; Lu, Y.; Lemcoff, N. G.; Zimmerman, S. C. *Chem. Sci.* **2014**, *5* (7), 2862.
- (118) Zhuang, J.; Jiwanich, S.; Deepak, V. D.; Thayumanavan, S. *ACS Macro Lett.* **2012**, *1* (1), 175–179.
- (119) Wang, K.; Peng, H.; Thurecht, K. J.; Puttick, S.; Whittaker, A. K. *Biomacromolecules* **2015**.
- (120) Çakir, P.; Cutivet, A.; Resmini, M.; Bui, B. T. S.; Haupt, K. *Adv. Mater.* **2013**, *25* (7), 1048–1051.
- (121) Beck-Broichsitter, M.; Nicolas, J.; Couvreur, P. *Nanoscale* **2015**, *7*, 9215–9221.
- (122) Akbulut, M.; Ginart, P.; Gindy, M. E.; Theriault, C.; Chin, K. H.; Soboyejo, W.; Prud'Homme, R. K. *Adv. Funct. Mater.* **2009**, *19* (5), 718–725.
- (123) Khan, S. A.; Schneider, M. *Macromol. Biosci.* **2013**, *13* (4), 455–463.
- (124) Nochi, T.; Yuki, Y.; Takahashi, H.; Sawada, S.; Mejima, M.; Kohda, T.; Harada, N.; Kong, I. G.; Sato, A.; Kataoka, N.; Tokuhara, D.; Kurokawa, S.; Takahashi, Y.; Tsukada, H.; Kozaki, S.; Akiyoshi, K.; Kiyono, H. *Nat. Mater.* **2010**, *9* (7), 572–578.
- (125) Chen, X. X.; Chen, L.; Yao, X.; Zhang, Z.; He, C.; Zhang, J.; Chen, X. X. *Chem. Commun. (Camb)*. **2014**, *50* (29), 3789–3791.

- (126) Zan, M.; Li, J.; Luo, S.; Ge, Z. *Chem. Commun.* **2014**.
- (127) Battistini, E.; Gianolio, E.; Gref, R.; Couvreur, P.; Fuzerova, S.; Othman, M.; Aime, S.; Badet, B.; Durand, P. *Chemistry (Easton)*. **2008**, 14 (15), 4551–4561.
- (128) Ryu, J.-H.; Chacko, R. T.; Jiwanich, S.; Bickerton, S.; Babu, R. P.; Thayumanavan, S. *J. Am. Chem. Soc.* **2010**, 132 (48), 17227–17235.
- (129) Chen, W.; Zheng, M.; Meng, F.; Cheng, R.; Deng, C.; Feijen, J.; Zhong, Z. *Biomacromolecules* **2013**, 14 (4), 1214–1222.
- (130) Rahimian, K.; Wen, Y.; Oh, J. K. *Polym. (United Kingdom)* **2015**, 72, 387–394.
- (131) Wu, H.; Dong, J.; Zhan, X.; Yang, H.; Zhao, Y.; Zhu, S.; Wang, G. *RSC Adv.* **2014**, 4 (67), 35757.
- (132) Rolland, J. P.; Maynor, B. W.; Euliss, L. E.; Exner, A. E.; Denison, G. M.; DeSimone, J. M. *J. Am. Chem. Soc.* **2005**, 127 (28), 10096–10100.
- (133) Petros, R. A.; DeSimone, J. M. *Nat. Rev. Drug Discov.* **2010**, 9 (8), 615–627.
- (134) Xu, J.; Wong, D. H. C.; Byrne, J. D.; Chen, K.; Bowerman, C.; DeSimone, J. M. *Angew. Chemie Int. Ed.* **2013**, 52 (26), 6580–6589.
- (135) Ramos, J.; Imaz, A.; Callejas-Fernández, J.; Barbosa-Barros, L.; Estelrich, J.; Quesada-Pérez, M.; Forcada, J. *Soft Matter* **2011**, 7 (11), 5067.
- (136) Hall, J. B.; Dobrovolskaia, M. A.; Patri, A. K.; McNeil, S. E. *Nanomedicine (Lond)*. **2007**, 2 (6), 789–803.
- (137) Troiber, C.; Kasper, J. C.; Milani, S.; Scheible, M.; Martin, I.; Schaubhut, F.; Küchler, S.; Rädler, J.; Simmel, F. C.; Friess, W.; Wagner, E. *Eur. J. Pharm. Biopharm.* **2013**, 84 (2), 255–264.
- (138) Filipe, V.; Hawe, A.; Jiskoot, W. *Pharm. Res.* **2010**, 27 (5), 796–810.
- (139) Genix, A.-C.; Oberdisse, J. *Curr. Opin. Colloid Interface Sci.* **2015**, 20 (4), 293–303.
- (140) Amamoto, Y.; Kikuchi, M.; Masunaga, H.; Sasaki, S.; Otsuka, H.; Takahara, A. *Macromolecules* **2010**, 43 (4), 1785–1791.
- (141) Yusa, S.; Sugahara, M.; Endo, T.; Morishima, Y. *Langmuir* **2009**, 25 (9), 5258–5265.
- (142) Park, C. W.; Yang, H.-M.; Lee, H. J.; Kim, J.-D. *Soft Matter* **2013**, 9 (6), 1781–1788.
- (143) Dubbert, J.; Honold, T.; Pedersen, J. S.; Radulescu, A.; Drechsler, M.; Karg, M.; Richtering, W. *Macromolecules* **2014**, 47 (24), 8700–8708.
- (144) Doane, T. L.; Chuang, C.-H.; Hill, R. J.; Burda, C. *Acc. Chem. Res.* **2012**, 45 (3), 317–326.
- (145) Fröhlich, E. *Int. J. Nanomedicine* **2012**, 7, 5577–5591.
- (146) Thielbeer, F.; Donaldson, K.; Bradley, M. *Bioconj. Chem.* **2011**, 22 (2), 144–150.
- (147) McAllister, K.; Sazani, P.; Adam, M.; Cho, M. J.; Rubinstein, M.; Samulski, R. J.; DeSimone, J. M. *J. Am. Chem. Soc.* **2002**, 124 (51), 15198–15207.
- (148) Zhang, P.; Jain, P.; Tsao, C.; Sinclair, A.; Sun, F.; Hung, H.-C.; Bai, T.; Wu, K.; Jiang, S. *J. Control. Release* **2016**, 230, 73–78.
- (149) Rancan, F.; Asadian-Birjand, M.; Dogan, S.; Graf, C.; Cuellar, L.; Lommatzsch, S.; Blume-Peytavi, U.; Calderón, M.; Vogt, A. *J. Control. Release* **2016**, 228, 159–169.
- (150) Kaps, L.; Nuhn, L.; Aslam, M.; Brose, A.; Foerster, F.; Rosigkeit, S.; Renz, P.; Heck, R.; Kim, Y. O.; Lieberwirth, I.; Schuppan, D.; Zentel, R. *Adv. Healthc. Mater.* **2015**, 4 (18), 2809–2815.

- (151) Hong, C. A.; Kim, J. S.; Lee, S. H.; Kong, W. H.; Park, T. G.; Mok, H.; Nam, Y. S. *Adv. Funct. Mater.* **2013**, 23 (3), 316–322.
- (152) Singh, S.; Bingöl, B.; Morgenroth, A.; Mottaghy, F. M.; Möller, M.; Schmaljohann, J. *Macromol. Rapid Commun.* **2013**, 34 (7), 562–567.
- (153) Singh, S.; Blöbbaum, J.; Möller, M.; Pich, A. J. *Polym. Sci. Part A Polym. Chem.* **2012**, 50 (20), 4288–4299.
- (154) Nel, A. E.; Mädler, L.; Velegol, D.; Xia, T.; Hoek, E. M. V.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M. *Nat. Mater.* **2009**, 8 (7), 543–557.
- (155) Moore, T. L.; Rodriguez-Lorenzo, L.; Hirsch, V.; Balog, S.; Urban, D.; Jud, C.; Rothen-Rutishauser, B.; Lattuada, M.; Petri-Fink, A. *Chem. Soc. Rev.* **2015**, 44, 6287–6305.
- (156) Mu, Q.; Jiang, G.; Chen, L.; Zhou, H.; Fourches, D.; Tropsha, A.; Yan, B. *Chem. Rev.* **2014**, 114 (15), 7740–7781.
- (157) Kettler, K.; Veltman, K.; van de Meent, D.; van Wezel, A.; Hendriks, A. J. *Environ. Toxicol. Chem.* **2014**, 33 (3), 481–492.
- (158) Knop, K.; Hoogenboom, R.; Fischer, D.; Schubert, U. S. *Angew. Chemie Int. Ed.* **2010**, 49 (36), 6288–6308.
- (159) Keefe, A. J.; Jiang, S. *Nat. Chem.* **2011**, 4 (1), 59–63.
- (160) Mitragotri, S.; Lahann, J. *Nat. Mater.* **2009**, 8 (1), 15–23.
- (161) He, C.; Hu, Y.; Yin, L.; Tang, C.; Yin, C. *Biomaterials* **2010**, 31 (13), 3657–3666.
- (162) Beningo, K. A.; Wang, Y. J. *J. Cell Sci.* **2002**, 115 (Pt 4), 849–856.
- (163) Banerjee, A.; Qi, J.; Gogoi, R.; Wong, J.; Mitragotri, S. J. *Control. Release* **2016**, 238, 176–185.
- (164) Gratton, S. E. A.; Ropp, P. A.; Pohlhaus, P. D.; Luft, J. C.; Madden, V. J.; Napier, M. E.; DeSimone, J. M. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, 105 (33), 11613–11618.
- (165) Bertrand, N.; Wu, J.; Xu, X.; Kamaly, N.; Farokhzad, O. C. *Adv. Drug Deliv. Rev.* **2014**, 66, 2–25.
- (166) Nelson, A. L.; Dhimolea, E.; Reichert, J. M. *Nat. Rev. Drug Discov.* **2010**, 9 (10), 767–774.
- (167) Broda, E.; Mickler, F. M.; Lächelt, U.; Morys, S.; Wagner, E.; Bräuchle, C. J. *Control. Release* **2015**, 213, 79–85.
- (168) Lao, Y.-H.; Phua, K. K. L.; Leong, K. W. *ACS Nano* **2015**, 9 (3), 2235–2254.
- (169) Nukolova, N. V.; Oberoi, H. S.; Cohen, S. M.; Kabanov, A. V.; Bronich, T. K. *Biomaterials* **2011**, 32 (23), 5417–5426.
- (170) Abu Lila, A. S.; Kiwada, H.; Ishida, T. J. *Control. Release* **2013**, 172 (1), 38–47.
- (171) Zhang, L.; Cao, Z.; Bai, T.; Carr, L.; Ella-Menye, J.-R.; Irvin, C.; Ratner, B. D.; Jiang, S. *Nat. Biotechnol.* **2013**, 31 (6), 553–556.
- (172) Nguyen, A. T.; Baggerman, J.; Paulusse, J. M. J.; van Rijn, C. J. M.; Zuilhof, H. *Langmuir* **2011**, 27 (6), 2587–2594.
- (173) Nguyen, A. T.; Baggerman, J.; Paulusse, J. M. J.; Zuilhof, H.; van Rijn, C. J. M. *Langmuir* **2012**, 28 (1), 604–610.

- (174) Stuart, M. A. C.; Huck, W. T. S.; Genzer, J.; Müller, M.; Ober, C.; Stamm, M.; Sukhorukov, G. B.; Szleifer, I.; Tsukruk, V. V.; Urban, M.; Winnik, F.; Zauscher, S.; Luzinov, I.; Minko, S. *Nat. Mater.* **2010**, 9 (2), 101–113.
- (175) de Las Heras Alarcon, C.; Pennadam, S.; Alexander, C. *Chem. Soc. Rev.* **2005**, 34 (3), 276–285.
- (176) Hu, J.; Liu, S. *Macromolecules* **2010**, 43 (20), 8315–8330.
- (177) Schattling, P.; Jochum, F. D.; Theato, P. *Polym. Chem.* **2014**, 5 (1), 25–36.
- (178) Li, C.; Liu, S. *Chem. Commun. (Camb)*. **2012**, 48 (27), 3262–3278.
- (179) Wang, X.; Niu, D.; Li, P.; Wu, Q.; Bo, X.; Liu, B.; Bao, S.; Su, T.; Xu, H.; Wang, Q. *ACS Nano* **2015**, 9 (6), 5646–5656.
- (180) Okada, S.; Mizukami, S.; Sakata, T.; Matsumura, Y.; Yoshioka, Y.; Kikuchi, K. *Adv. Mater.* **2014**, 26 (19), 2989–2992.
- (181) Wu, W.; Mitra, N.; Yan, E. C. Y.; Zhou, S. *ACS Nano* **2010**, 4 (8), 4831–4839.
- (182) Hu, J.; Zhang, G.; Ge, Z.; Liu, S. *Prog. Polym. Sci.* **2014**, 39 (6), 1096–1143.
- (183) Schmaljohann, D. *Adv. Drug Deliv. Rev.* **2006**, 58 (15), 1655–1670.
- (184) Quan, C.-Y.; Chang, C.; Wei, H.; Chen, C.-S.; Xu, X.-D.; Cheng, S.-X.; Zhang, X.-Z.; Zhuo, R.-X. *Nanotechnology* **2009**, 20 (33), 335101.
- (185) Su, S.; Wang, H.; Liu, X.; Wu, Y.; Nie, G. *Biomaterials* **2013**, 34 (13), 3523–3533.
- (186) Yang, H.; Wang, Q.; Huang, S.; Xiao, A.; Li, F.; Gan, L.; Yang, X. *ACS Appl. Mater. Interfaces* **2016**, 8 (12), acsami.6b01602.
- (187) Cuggino, J. C.; Molina, M.; Wedepohl, S.; Igarzabal, C. I. A.; Calderón, M.; Gugliotta, L. M. *Eur. Polym. J.* **2016**, 78, 14–24.
- (188) Tian, Y.; Bian, S.; Yang, W. *Polym. Chem.* **2016**, 7 (10), 1913–1921.
- (189) Lutz, J.-F.; Hoth, A. *Macromolecules* **2006**, 39 (2), 893–896.
- (190) Lou, S.; Gao, S.; Wang, W.; Zhang, M.; Zhang, J.; Wang, C.; Li, C.; Kong, D.; Zhao, Q. *Nanoscale* **2015**, 7 (7), 3137–3146.
- (191) Tian, Y.; Wang, Y.; Shen, S.; Jiang, X.; Wang, Y.; Yang, W. *Part. Part. Syst. Charact.* **2015**, 32 (12), 1092–1101.
- (192) Wang, Y.; Zheng, J.; Tian, Y.; Yang, W. *J. Mater. Chem. B* **2015**, 3 (28), 5824–5832.
- (193) Kanamala, M.; Wilson, W. R.; Yang, M.; Palmer, B. D.; Wu, Z. *Biomaterials* **2016**, 85, 152–167.
- (194) Gillies, R. J.; Gatenby, R. A. *Cancer J.* **2015**, 21 (2), 88–96.
- (195) Fleige, E.; Quadir, M. A.; Haag, R. *Adv. Drug Deliv. Rev.* **2012**, 64 (9), 866–884.
- (196) Tamura, A.; Oishi, M.; Nagasaki, Y. *Biomacromolecules* **2009**, 10 (7), 1818–1827.
- (197) Lin, C.; Engbersen, J. F. J. *J. Control. Release* **2008**, 132 (3), 267–272.
- (198) Shen, Y.; Fu, X.; Fu, W.; Li, Z. *Chem. Soc. Rev.* **2015**, 44 (3), 612–622.
- (199) Wu, W.; Shen, J.; Gai, Z.; Hong, K.; Banerjee, P.; Zhou, S. *Biomaterials* **2011**, 32 (36), 9876–9887.
- (200) Oishi, M.; Hayashi, H.; Iijima, M.; Nagasaki, Y. *J. Mater. Chem.* **2007**, 17 (35), 3720.
- (201) De Koker, S.; Cui, J.; Vanparijs, N.; Albertazzi, L.; Grooten, J.; Caruso, F.; De Geest, B. G. *Angew. Chemie Int. Ed.* **2016**, 55 (4), 1334–1339.

- (202) Knipe, J. M.; Strong, L. E.; Peppas, N. A. *Biomacromolecules* **2016**, 17 (3), 788–797.
- (203) Oishi, M.; Tamura, A.; Nakamura, T.; Nagasaki, Y. *Adv. Funct. Mater.* **2009**, 19 (6), 827–834.
- (204) Li, M.; Tang, Z.; Sun, H.; Ding, J.; Song, W.; Chen, X. *Polym. Chem.* **2013**, 4 (4), 1199–1207.
- (205) Shi, F.; Ding, J.; Xiao, C.; Zhuang, X.; He, C.; Chen, L.; Chen, X. *J. Mater. Chem.* **2012**, 22 (28), 14168.
- (206) Lu, C.; Li, B.; Liu, N.; Wu, G.; Gao, H.; Ma, J. *RSC Adv.* **2014**, 4 (92), 50301–50311.
- (207) Zhang, X.; Achazi, K.; Steinhilber, D.; Kratz, F.; Dervedde, J.; Haag, R. *J. Control. Release* **2014**, 174, 209–216.
- (208) Zhan, F.; Chen, W.; Wang, Z.; Lu, W.; Cheng, R.; Deng, C.; Meng, F.; Liu, H.; Zhong, Z. *Biomacromolecules* **2011**, 12 (10), 3612–3620.
- (209) Zhuang, J.; Gordon, M. R.; Ventura, J.; Li, L.; Thayumanavan, S. *Chem. Soc. Rev.* **2013**, 42 (17), 7421–7435.
- (210) Tian, H.; Guo, Z.; Lin, L.; Jiao, Z.; Chen, J.; Gao, S.; Zhu, X.; Chen, X. *J. Control. Release* **2014**, 174, 117–125.
- (211) Zhang, X.; Zhang, K.; Haag, R. *Biomater. Sci.* **2015**, 3 (11), 1487–1496.
- (212) Chen, W.; Achazi, K.; Schade, B.; Haag, R. *J. Control. Release* **2015**, 205, 15–24.
- (213) Cheng, R.; Feng, F.; Meng, F.; Deng, C.; Feijen, J.; Zhong, Z. *J. Control. Release* **2011**, 152 (1), 2–12.
- (214) Oh, J. K.; Siegwart, D. J.; Matyjaszewski, K. *Biomacromolecules* **2007**, 8 (11), 3326–3331.
- (215) Averick, S. E.; Paredes, E.; Irastorza, A.; Shrivats, A. R.; Srinivasan, A.; Siegwart, D. J.; Magenau, A. J.; Cho, H. Y.; Hsu, E.; Averick, A. A.; Kim, J.; Liu, S.; Hollinger, J. O.; Das, S. R.; Matyjaszewski, K. *Biomacromolecules* **2012**, 13 (11), 3445–3449.
- (216) Huang, K.; Shi, B.; Xu, W.; Ding, J.; Yang, Y.; Liu, H.; Zhuang, X.; Chen, X. *Acta Biomater.* **2015**, 27, 179–193.
- (217) He, H.; Cattran, A. W.; Nguyen, T.; Nieminen, A.-L.; Xu, P. *Biomaterials* **2014**, 35 (35), 9546–9553.
- (218) Sousa-Herves, A.; Wedepohl, S.; Calderón, M. *Chem. Commun. (Camb)* **2015**, 51 (25), 5264–5267.
- (219) Vijayaraghavalu, S.; Labhasetwar, V. *Cancer Lett.* **2013**, 331 (1), 122–129.
- (220) Li, Y.; Duong, H. T. T.; Laurent, S.; MacMillan, A.; Whan, R. M.; Elst, L. Vander; Muller, R. N.; Hu, J.; Lowe, A.; Boyer, C.; Davis, T. P. *Adv. Healthc. Mater.* **2015**, 4 (1), 148–156.
- (221) Frost, R.; Coué, G.; Engbersen, J. F. J.; Zäch, M.; Kasemo, B.; Svedhem, S. *J. Colloid Interface Sci.* **2011**, 362 (2), 575–583.
- (222) Xiong, M.-H.; Bao, Y.; Yang, X.-Z.; Zhu, Y.-H.; Wang, J. *Adv. Drug Deliv. Rev.* **2014**, 78, 63–76.
- (223) de la Rica, R.; Aili, D.; Stevens, M. M. *Adv. Drug Deliv. Rev.* **2012**, 64 (11), 967–978.
- (224) Mura, S.; Nicolas, J.; Couvreur, P. *Nat. Mater.* **2013**, 12 (11), 991–1003.
- (225) Thornton, P. D.; Mart, R. J.; Webb, S. J.; Ulijn, R. V. *Soft Matter* **2008**, 4 (4), 821.
- (226) McAtee, C. O.; Barycki, J. J.; Simpson, M. A. *Adv. Cancer Res.* **2014**, 123 (402), 1–34.
- (227) Zhong, Y.; Zhang, J.; Cheng, R.; Deng, C.; Meng, F.; Xie, F.; Zhong, Z. *J. Control. Release* **2015**, 205, 144–154.
- (228) Lee, H.; Mok, H.; Lee, S.; Oh, Y.-K.; Park, T. G. *J. Control. Release* **2007**, 119 (2), 245–252.
- (229) Mok, H.; Jeong, H.; Kim, S.-J.; Chung, B. H. *Chem. Commun. (Camb)* **2012**, 48 (69), 8628–8630.

- (230) Yim, H.; Park, S.-J.; Bae, Y. H.; Na, K. *Biomaterials* **2013**, 34 (31), 7674–7682.
- (231) Zhou, S.; Dou, H.; Zhang, Z.; Sun, K.; Jin, Y.; Dai, T.; Zhou, G.; Shen, Z. *Polym. Chem.* **2013**, 4 (15), 4103.
- (232) Li, D.; Kordalivand, N.; Fransen, M. F.; Ossendorp, F.; Raemdonck, K.; Vermonden, T.; Hennink, W. E.; van Nostrum, C. F. *Adv. Funct. Mater.* **2015**, 25 (20), 2993–3003.
- (233) Cai, H.; Yao, P. *Nanoscale* **2013**, 5 (7), 2892–2900.
- (234) Gonçalves, C.; Lalatonne, Y.; Melro, L.; Badino, G.; Ferreira, M. F. M.; David, L.; Geraldès, C. F. G. C.; Motte, L.; Martins, J. A.; Gama, F. M. J. *Mater. Chem. B* **2013**, 1 (42), 5853.
- (235) Heller, D. A.; Levi, Y.; Pelet, J. M.; Doloff, J. C.; Wallas, J.; Pratt, G. W.; Jiang, S.; Sahay, G.; Schroeder, A.; Schroeder, J. E.; Chyan, Y.; Zurenko, C.; Queres, W.; Manzano, M.; Kohane, D. S.; Langer, R.; Anderson, D. G. *Adv. Mater.* **2013**, 25 (10), 1449–1454.
- (236) Aguirre, G.; Ramos, J.; Forcada, J. *Soft Matter* **2013**, 9 (1), 261–270.
- (237) Nazli, C.; Demirel, G. S.; Yar, Y.; Acar, H. Y.; Kizilel, S. *Colloids Surfaces B Biointerfaces* **2014**, 122, 674–683.
- (238) Lim, Y. H.; Tiemann, K. M.; Heo, G. S.; Wagers, P. O.; Rezenom, Y. H.; Zhang, S.; Zhang, F.; Youngs, W. J.; Hunstad, D. A.; Wooley, K. L. *ACS Nano* **2015**, 9 (2), 1995–2008.
- (239) Heller, A.; Feldman, B. July 2008, pp 2482–2505.
- (240) Kelley, E. G.; Albert, J. N. L.; Sullivan, M. O.; Epps III, T. H.; Epps, T. H. *Chem. Soc. Rev.* **2013**, 42 (17), 7057–7071.
- (241) Zhao, L.; Xiao, C.; Ding, J.; He, P.; Tang, Z.; Pang, X.; Zhuang, X.; Chen, X. *Acta Biomater.* **2013**, 9 (5), 6535–6543.
- (242) Lee, D.-E.; Koo, H.; Sun, I.-C.; Ryu, J. H.; Kim, K.; Kwon, I. C. *Chem. Soc. Rev.* **2012**, 41 (7), 2656–2672.
- (243) Iha, R. K.; Wooley, K. L.; Nyström, A. M.; Burke, D. J.; Kade, M. J.; Hawker, C. J. *Chem. Rev.* **2009**, 109 (11), 5620–5686.
- (244) Blum, A. P.; Kammeyer, J. K.; Rush, A. M.; Callmann, C. E.; Hahn, M. E.; Gianneschi, N. C. *J. Am. Chem. Soc.* **2015**, 137 (6), 2140–2154.
- (245) Villaraza, A. J. L.; Bumb, A.; Brechbiel, M. W. *Chem. Rev.* **2010**, 110 (5), 2921–2959.
- (246) Caravan, P. *Chem. Soc. Rev.* **2006**, 35 (6), 512–523.
- (247) Bennett, K. M.; Jo, J.; Cabral, H.; Rumiana, B.; Aoki, I. *Adv. Drug Deliv. Rev.* **2014**.
- (248) De Leon-Rodriguez, L. M.; Lubag, A. J. M.; Malloy, C. R.; Martinez, G. V.; Gillies, R. J.; Sherry, A. D. *Acc. Chem. Res.* **2009**, 42 (7), 948–957.
- (249) Davies, G.-L.; Kramberger, I.; Davis, J. J. *Chem. Commun.* **2013**.
- (250) Li, Y.; Beija, M.; Laurent, S.; Elst, L. Vander; Muller, R. N.; Duong, H. T. T.; Lowe, A. B.; Davis, T. P.; Boyer, C. *Macromolecules* **2012**, 45 (10), 4196–4204.
- (251) Li, Y.; Laurent, S.; Esser, L.; Elst, L. Vander; Muller, R. N.; Lowe, A. B.; Boyer, C.; Davis, T. P. *Polym. Chem.* **2014**, 5, 2592.

- (252) Lux, J.; Chan, M.; Elst, L. Vander; Schopf, E.; Mahmoud, E.; Laurent, S.; Almutairi, A. J. *Mater. Chem. B. Mater. Biol. Med.* **2013**, 1 (46), 6359–6364.
- (253) Perez-Baena, I.; Loinaz, I.; Padro, D.; García, I.; Grande, H. J.; Odriozola, I. *J. Mater. Chem.* **2010**, 20 (33), 6916–6922.
- (254) Laurent, S.; Forge, D.; Port, M.; Roch, A.; Robic, C.; Vander Elst, L.; Muller, R. N. *Chem. Rev.* **2008**, 108 (6), 2064–2110.
- (255) Guang Choo, E. S.; Tang, X.; Sheng, Y.; Shuter, B.; Xue, J. J. *Mater. Chem.* **2011**, 21 (7), 2310.
- (256) Gonçalves, C.; Silva, J. P.; Antunes, I. F.; Ferreira, M. F. M.; Martins, J. A.; Geraldès, C. F. G. C.; Lalatonne, Y.; Motte, L.; de Vries, E. F. J.; Gama, F. M. *Bioconjug. Chem.* **2015**, 26 (4), 699–706.
- (257) Smith, C. E.; Ernenwein, D.; Shkumatov, A.; Clay, N. E.; Lee, J.; Melhem, M.; Misra, S.; Zimmerman, S. C.; Kong, H. *Biomaterials* **2015**, 69, 184–190.
- (258) Jiang, L.; Zhou, Q.; Mu, K.; Xie, H.; Zhu, Y.; Zhu, W.; Zhao, Y.; Xu, H.; Yang, X. *Biomaterials* **2013**, 34 (30), 7418–7428.
- (259) Liu, J.; Detrembleur, C.; Debuigne, A.; De Pauw-Gillet, M.-C.; Mornet, S. S.; Vander Elst, L.; Laurent, S.; Duguet, E.; Jerome, C.; Jérôme, C. *J. Mater. Chem. B* **2014**, 2 (8), 1009–1023.
- (260) Geraldès, C. F. G. C.; Laurent, S. *Contrast Media Mol. Imaging* **2009**, 4 (1), 1–23.
- (261) Stasiuk, G. J.; Long, N. J. *Chem. Commun. (Camb)*. **2013**, 49 (27), 2732–2746.
- (262) Okada, S.; Mizukami, S.; Kikuchi, K. *Bioorg. Med. Chem.* **2012**, 20 (2), 769–774.
- (263) Santra, S.; Jativa, S. D.; Kaittanis, C.; Normand, G.; Grimm, J.; Perez, J. M. *ACS Nano* **2012**, 6 (8), 7281–7294.
- (264) Wang, X.; Niu, D.; Wu, Q.; Bao, S.; Su, T.; Liu, X.; Zhang, S.; Wang, Q. *Biomaterials* **2015**, 53, 349–357.
- (265) Oishi, M.; Sumitani, S.; Nagasaki, Y. *Bioconjug. Chem.* **2007**, 18 (5), 1379–1382.
- (266) Choo, E. S. G.; Peng, E.; Rajendran, R.; Chandrasekharan, P.; Yang, C.-T. T.; Ding, J.; Chuang, K.-H. H.; Xue, J. *Adv. Funct. Mater.* **2013**, 23 (4), 496–505.
- (267) Histed, S. N.; Lindenberg, M. L.; Mena, E.; Turkbey, B.; Choyke, P. L.; Kurdziel, K. a. *Nucl. Med. Commun.* **2012**, 33 (4), 349–361.
- (268) Kim, D.; Jeong, Y. Y.; Jon, S. *ACS Nano* **2010**, 4 (7), 3689–3696.
- (269) Nakamura, T.; Tamura, A.; Murotani, H.; Oishi, M.; Jinji, Y.; Matsuishiki, K.; Nagasaki, Y. *Nanoscale* **2010**, 2 (5), 739–746.
- (270) Lu, S.; Neoh, K. G.; Huang, C.; Shi, Z.; Kang, E.-T. T. *J. Colloid Interface Sci.* **2013**, 412, 46–55.
- (271) Jøllck, R. I.; Rydhög, J. S.; Christensen, A. N.; Hansen, A. E.; Bruun, L. M.; Schaarup-Jensen, H.; von Wenck, A. S.; Børresen, B.; Kristensen, A. T.; Clausen, M. H.; Kjaer, A.; Conradsen, K.; Larsen, R.; Af Rosenschöld, P. M.; Andresen, T. L. *Adv. Healthc. Mater.* **2015**, 4 (6), 856–863.
- (272) Naeye, B.; Deschout, H.; Caveliers, V.; Descamps, B.; Braeckmans, K.; Vanhove, C.; Demeester, J.; Lahoutte, T.; De Smedt, S. C.; Raemdonck, K. *Biomaterials* **2013**, 34 (9), 2350–2358.
- (273) Liu, Y.; Ibricevic, A.; Cohen, J. A.; Cohen, J. L.; Gunsten, S. P.; Fréchet, J. M. J.; Walter, M. J.; Welch, M. J.; Brody, S. L. *Mol. Pharm.* **2009**, 6 (6), 1891–1902.

- (274) Majmudar, M. D.; Yoo, J.; Keliher, E. J.; Truelove, J. J.; Iwamoto, Y.; Sena, B.; Dutta, P.; Borodovsky, A.; Fitzgerald, K.; Di Carli, M. F.; Libby, P.; Anderson, D. G.; Swirski, F. K.; Weissleder, R.; Nahrendorf, M. *Circ. Res.* **2013**, 112 (5), 755–761.
- (275) Lux, J.; White, A. G.; Chan, M.; Anderson, C. J.; Almutairi, A. *Theranostics* **2015**, 5 (3), 277–288.
- (276) Cao, L.; Li, X.; Wang, S.; Li, S.; Li, Y.; Yang, G. *Chem. Commun. (Camb)*. **2014**, 50 (63), 8787–8790.
- (277) Yhee, J. Y.; Kim, S. A.; Koo, H.; Son, S.; Ryu, J. H.; Youn, I.-C. C.; Choi, K.; Kwon, I. C.; Kim, K. *Theranostics* **2012**, 2 (2), 179–189.
- (278) Maya, S.; Sarmiento, B.; Nair, A.; Rejinold, N. S.; Nair, S. V.; Jayakumar, R. *Curr. Pharm. Des.* **2013**, 19 (41), 7203–7218.
- (279) Tang, F.; Ma, N.; Wang, X.; He, F.; Li, L. *J. Mater. Chem.* **2011**, 21 (42), 16943.
- (280) Wu, W.; Aiello, M.; Zhou, T.; Berliner, A.; Banerjee, P.; Zhou, S. *Biomaterials* **2010**, 31 (11), 3023–3031.
- (281) Yin, L.; He, C.; Huang, C.; Zhu, W.; Wang, X.; Xu, Y.; Qian, X. *Chem. Commun. (Camb)*. **2012**, 48 (37), 4486–4488.
- (282) Ryu, J. H.; Kim, S. A.; Koo, H.; Yhee, J. Y.; Lee, A.; Na, J. H.; Youn, I.; Choi, K.; Kwon, I. C.; Kim, B.-S.; Kim, K. *J. Mater. Chem.* **2011**, 21 (44), 17631.
- (283) Peng, H.; Stolwijk, J. A.; Sun, L.-N.; Wegener, J.; Wolfbeis, O. S. *Angew. Chemie Int. Ed.* **2010**, 49 (25), 4246–4249.
- (284) Fan, J.; Jiang, X.; Hu, Y.; Si, Y.; Ding, L.; Wu, W. *Biomater. Sci.* **2013**, 1 (4), 421.
- (285) Min, Y.; Caster, J. M.; Eblan, M. J.; Wang, A. Z. *Chem. Rev.* **2015**, 115 (19), 11147–11190.
- (286) Barenholz, Y. *J. Control. Release* **2012**, 160 (2), 117–134.
- (287) Kamaly, N.; Xiao, Z.; Valencia, P. M.; Radovic-Moreno, A. F.; Farokhzad, O. C. *Chem. Soc. Rev.* **2012**, 41 (7), 2971–3010.
- (288) Oh, J. K.; Siegwart, D. J.; Lee, H.; Sherwood, G.; Peteanu, L.; Hollinger, J. O.; Kataoka, K.; Matyjaszewski, K. *J. Am. Chem. Soc.* **2007**, 129 (18), 5939–5945.
- (289) He, C.; Zhuang, X.; Tang, Z.; Tian, H.; Chen, X. *Adv. Healthc. Mater.* **2012**, 1 (1), 48–78.
- (290) Desale, S. S.; Raja, S. M.; Kim, J. O.; Mohapatra, B.; Soni, K. S.; Luan, H.; Williams, S. H.; Bielecki, T. A.; Feng, D.; Storck, M.; Band, V.; Cohen, S. M.; Band, H.; Bronich, T. K. *J. Control. Release* **2015**, 208, 59–66.
- (291) Xing, T.; Mao, C.; Lai, B.; Yan, L. *ACS Appl. Mater. Interfaces* **2012**, 4 (10), 5662–5672.
- (292) Ding, J. X.; Zhuang, X. L.; Xiao, C. S.; Cheng, Y. L.; Zhao, L.; He, C. L.; Tang, Z. H.; Chen, X. S. *J. Mater. Chem.* **2011**, 21 (30), 11383.
- (293) Lee, E. S.; Kim, D.; Youn, Y. S.; Oh, K. T.; Bae, Y. H. *Angew. Chemie Int. Ed.* **2008**, 47 (13), 2418–2421.
- (294) Sisson, A. L.; Steinhilber, D.; Rossow, T.; Welker, P.; Licha, K.; Haag, R. *Angew. Chemie Int. Ed.* **2009**, 48 (41), 7540–7545.
- (295) Stiriba, S. E.; Kautz, H.; Frey, H. *J. Am. Chem. Soc.* **2002**, 124 (33), 9698–9699.
- (296) Zhang, X.; Achazi, K.; Haag, R. *Adv. Healthc. Mater.* **2015**, 4 (4), 585–592.

- (297) Yin, H.; Kanasty, R. L.; Eltoukhy, A. A.; Vegas, A. J.; Dorkin, J. R.; Anderson, D. G. *Nat. Rev. Genet.* **2014**, *15* (8), 541–555.
- (298) Smith, M. H.; Lyon, L. A. *Acc. Chem. Res.* **2012**, *45* (7), 985–993.
- (299) Dickerson, E. B.; Blackburn, W. H.; Smith, M. H.; Kapa, L. B.; Lyon, L. A.; McDonald, J. F. *BMC Cancer* **2010**, *10* (1), 10.
- (300) Blackburn, W. H.; Dickerson, E. B.; Smith, M. H.; McDonald, J. F.; Lyon, L. A. *Bioconjug. Chem.* **2009**, *20* (5), 960–968.
- (301) Pereira, P.; Pedrosa, S. S.; Wymant, J. M.; Sayers, E.; Correia, A.; Vilanova, M.; Jones, A. T.; Gama, F. M. *Mol. Pharm.* **2015**, *12* (6), 1970–1979.
- (302) Wang, M.; Liu, T.; Han, L.; Gao, W.; Yang, S.; Zhang, N. *Polym. Chem.* **2015**, *6* (17), 3324–3335.
- (303) Toita, S.; Sawada, S.; Akiyoshi, K. *J. Control. Release* **2011**, *155* (1), 54–59.
- (304) Raemdonck, K.; Martens, T. F.; Braeckmans, K.; Demeester, J.; De Smedt, S. C. *Adv. Drug Deliv. Rev.* **2013**, *65* (9), 1123–1147.
- (305) Tamura, A.; Oishi, M.; Nagasaki, Y. *J. Control. Release* **2010**, *146* (3), 378–387.
- (306) Dunn, S. S.; Tian, S.; Blake, S.; Wang, J.; Galloway, A. L.; Murphy, A.; Pohlhaus, P. D.; Rolland, J. P.; Napier, M. E.; DeSimone, J. M. *J. Am. Chem. Soc.* **2012**, *134* (17), 7423–7430.
- (307) Kang, H.; Trondoli, A. C.; Zhu, G.; Chen, Y.; Chang, Y.-J. J.; Liu, H.; Huang, Y.-F. F.; Zhang, X.; Tan, W. *ACS Nano* **2011**, *5* (6), 5094–5099.
- (308) Kim, D. H.; Rossi, J. J. *Nat. Rev. Genet.* **2007**, *8* (3), 173–184.
- (309) Jensen, L. B.; Griger, J.; Naeye, B.; Varkouhi, A. K.; Raemdonck, K.; Schiffelers, R.; Lammers, T.; Storm, G.; de Smedt, S. C.; Sproat, B. S.; Nielsen, H. M.; Foged, C. *Pharm. Res.* **2012**, *29* (3), 669–682.
- (310) Lu, Y.; Sun, W.; Gu, Z. *J. Control. Release* **2014**, *194*, 1–19.
- (311) Beierle, J. M.; Yoshimatsu, K.; Chou, B.; Mathews, M. A. A.; Lesel, B. K.; Shea, K. J. *Angew. Chemie Int. Ed.* **2014**, *53* (35), 9275–9279.
- (312) Zhao, M.; Hu, B.; Gu, Z.; Joo, K.-I.; Wang, P.; Tang, Y. *Nano Today* **2013**, *8* (1), 11–20.
- (313) Guo, H.; Li, H.; Gao, J.; Zhao, G.; Ling, L.; Wang, B.; Guo, Q.; Gu, Y.; Li, C. *Polym. Chem.* **2016**, *7* (18), 3189–3199.
- (314) Wu, Z.; Zhang, X.; Guo, H.; Li, C.; Yu, D. *J. Mater. Chem.* **2012**, *22* (42), 22788.
- (315) Zhao, L.; Xiao, C.; Ding, J.; Zhuang, X.; Gai, G.; Wang, L.; Chen, X. *Polym. Chem.* **2015**, *6* (20), 3807–3815.
- (316) Ferreira, S. A.; Gama, F. M.; Vilanova, M. *Nanomedicine* **2013**, *9* (2), 159–173.
- (317) Muraoka, D.; Harada, N.; Hayashi, T.; Tahara, Y.; Momose, F.; Sawada, S.; Mukai, S.; Akiyoshi, K.; Shiku, H. *ACS Nano* **2014**, *8* (9), 9209–9218.
- (318) Tahara, Y.; Akiyoshi, K. *Adv. Drug Deliv. Rev.* **2015**, *95*, 65–76.
- (319) Li, P.; Luo, Z.; Liu, P.; Gao, N.; Zhang, Y.; Pan, H.; Liu, L.; Wang, C.; Cai, L.; Ma, Y. *J. Control. Release* **2013**, *168* (3), 271–279.
- (320) Durán-Lobato, M.; Carrillo-Conde, B.; Khairandish, Y.; Peppas, N. A. *Biomacromolecules* **2014**, *15* (7), 2725–2734.

CHAPTER 3

Nanogel Formation by Inverse Nanoprecipitation of Zwitterionic Poly(amido amine)s

Antonie E. Ekkelenkamp, Johan F.J. Engbersen and Jos M.J. Paulusse

Manuscript is currently under review for the Journal of Colloid and Interface Science

Abstract

Zwitterionic poly(amido amine) nanogels have been prepared by nanoprecipitation of an aqueous polymer solution into an organic non-solvent and subsequent polymer crosslinking with ethylenediamine via *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide/*N*-hydroxysuccinimide coupling. To obtain more insight into the nanoprecipitation process, nanoprecipitation of zwitterionic poly(amido amine)s was carried out at several poly(amido amine) concentrations into six water-miscible solvents (1,2-dimethoxyethane, 1,4-dioxane, acetone, acetonitrile, ethanol and isopropyl alcohol) and the resulting nanoparticle dispersions were analyzed by dynamic light scattering. Nanoprecipitation in acetone, acetonitrile, and isopropyl alcohol resulted in stable nanoparticle dispersions. Evaluation of interaction parameters between water and the non-solvent (χ) showed that lower χ -values resulted in more polydisperse particle. Furthermore, nanoprecipitation resulted in larger nanoparticles when the poly(amido amine) concentration was increased. Stable nanogels were obtained from crosslinking poly(amido amine)s after nanoprecipitation in acetone and acetonitrile. The obtained nanogels had a lower-size limit of around 100 nm, where nanoprecipitation in acetonitrile was more stable than in acetone, allowing the use of higher polymer concentrations.

1. Introduction

Tremendous progress over the last decade in the field of nanomedicine has uncovered the importance of nanocarrier design in terms of stability, loading capacity and ability to respond to local stimuli.^{1–4} Water-soluble nano-sized crosslinked polymer networks, or nanogels, possess swelling properties that are very appealing for drug delivery and imaging applications.^{5,6} In their swollen aqueous state, nanogels may respond to changes in pH,⁷ as well as temperature⁸ by swelling or shrinking, which can trigger controlled release of therapeutic agents.⁵ Moreover, cleavable linkers, sensitive to e.g. glutathione,^{9,10} enzymes^{11,12} and pH,^{13,14} facilitate triggered release of the encapsulated payload.¹ Nanogel structures consequently offer great opportunities for responsive imaging^{15,16} and triggered drug release.^{17,18} Highly hydrophilic zwitterionic polymers,¹⁹ polymer brush surfaces²⁰ and nanogels^{21,22} proved to be highly stable when exposed to high protein concentrations, which is important for stability in biological environment and for prevention of an immune response.^{23,24}

Several methods are frequently employed to prepare nanogels. A commonly used method for preparing nanogels is by using water-in-oil emulsions, where hydrophilic monomers or polymers and crosslinkers are dissolved in the water phase.^{25–27} Nanogels are obtained after crosslinking and subsequent purification to remove surfactants, unreacted monomers and small oligomers and polymers. An alternative method to obtain nanogels is by precipitation polymerization, which requires incorporation of a monomer with lower critical solution temperature (LCST) behavior after polymerization.^{26,28–30} Finally, nanogels have also been successfully prepared by PRINT³¹ and self-assembly techniques.^{32,33}

Nanoprecipitation, or solvent displacement, is an effective method to prepare solid nanoparticles by mixing solute-containing solution, rapidly with a non-solvent. This process is referred to as the ‘Ouzo effect’, and arises from the mixing of a solute, solvent and non-solvent, and occurs within the metastable region between the binodal and spinodal curves in a ternary phase diagram.^{34–37} This metastable region is often referred to as the ‘Ouzo-region’, which is defined by the system’s components: the polymer, solvent and non-solvent.^{35,37} During solvent displacement, the solute solubility limit in the solvent/non-solvent mixture is exceeded, leading to supersaturation of the solution and creating nano-sized solute nuclei, which aggregate to form a metastable emulsion.^{34,36} The diameter of the aggregates is influenced by diffusion of solvent molecules from the aggregates into the non-solvent, causing the aggregates to shrink.³⁴ Nanoprecipitation is widely applied to prepare nanoparticles by dissolving a hydrophobic solute in an organic phase and precipitating it into water.^{35,36,38} However, it is also possible to prepare nanoparticles by precipitation of an aqueous phase into an organic phase, i.e. inverse nanoprecipitation.³⁹ Nanoprecipitation of hydrophobic

polymers, such as poly(lactic acid) and poly(lactic acid-co-glycolic acid) is often applied to prepare biocompatible and biodegradable solid polymer nanoparticles, and to encapsulate therapeutic agents inside these nanoparticles.⁴⁰ Earlier studies regarding nanoprecipitation into an aqueous solution revealed that several parameters influence the nanoprecipitation process. Influential factors on particle size during nanoprecipitation are the initial polymer concentration,^{37,41–44} the molecular weight of the polymer⁴⁵ and the solvent/non-solvent ratio.^{41,42} In a more recent study, charge interactions proved to enable the formation of very small nanoparticles (~ 30 nm).⁴⁶ The influence of the solvent/non-solvent pair has been investigated by several groups.^{37,41,44} who showed that good interaction (i.e. diffusion) between the solvent and the non-solvent correlates with the obtained particle diameter. This was explained by solvent diffusing out of the polymer phase, effectively de-swelling the particles.

Nanogels were prepared by Steinhilber et al. by inverse nanoprecipitation, by adding an aqueous dendritic polyglycerol solution to an excess of acetone, resulting in the formation of clusters in the nanometer scale.⁴⁷ After crosslinking of these clusters and acetone removal, crosslinked hydrophilic polyglycerol nanogels were obtained. Similarly, gelatin nanoparticles have been prepared by inverse nanoprecipitation, for controlled drug delivery.^{48,49} Earlier, we reported nanogel formation by inverse nanoprecipitation of zwitterionic poly(amido amine)s in acetone.²² Poly(amido amine)s are a water-soluble and peptidomimetic class of polymers, which have been extensively investigated by our group^{50–57} and others.^{58–62} Moreover, poly(amido amine)s are readily biodegradable by amide hydrolysis,⁶³ and zwitterionic poly(amido amine) nanogels exhibited minimal cell toxicity.²² Poly(amido amine) nanogel formation by inverse nanoprecipitation in acetone was optimized to result in 100 nm sized nanogels.²² Nanoparticle size and size-distribution plays an important role in biodistribution and in target tissue penetration, which makes control over these parameters desirable for in vivo applications.⁴ From this perspective, we have studied the effects of different organic solvents at varying polymer concentrations in order to gain more insight into the role of these parameters on the formation of poly(amido amine)-based nanogels by inverse nanoprecipitation.

2. Materials & Methods

2.1. Materials

1,2-dimethoxyethane (≥ 99%), 1,4-dioxane (≥ 99%), acetonitrile (anhydrous, 99.8%), N,N'-methylenebisacrylamide (99%), triethylamine (≥ 99%), sodium acetate (≥ 99%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (≥ 98%), N-hydroxysuccinimide, ethylenediamine (≥ 97%) and hydrochloric acid - 37% were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Ethylenediamine-

N,N'-diacetic acid ($\geq 98\%$), isopropyl alcohol (reagent grade), ethanol (reagent grade), methanol (reagent grade) and acetone (reagent grade) were purchased from VWR (Amsterdam, the Netherlands). Spectra/Por® dialysis tubing with a MWCO of 1 kDa was purchased from Spectrum Labs (Breda, the Netherlands) and SnakeSkin™ dialysis tubing with a MWCO of 10 kDa was purchased from Fisher (Landsmeer, the Netherlands).

2.2. General procedures

^1H NMR and ^1H COSY NMR spectra were recorded in D_2O on a Bruker Ascend 400 spectrometer; signals of the deuterated solvent were used as a reference. Size exclusion chromatography (SEC) measurements were performed on a Waters Alliance e2695 separation module equipped with a Waters 2998 PDA detector, a Waters 2914 RI detector and a Polymer Laboratories Aquagel-OH 30 column. A 0.4 M sodium acetate buffer (containing 30% *meOH*) was used as eluent at a flow rate of 0.7 mL/min at 35 °C. Poly(ethylene glycol) standards were used for calibration. DLS measurements were performed on a Malvern Zetasizer Nano ZS at a 173° scattering angle, and analyzed using the general-purpose analysis method provided in the Malvern Zetasizer software. The reference material was set to polystyrene latex and the temperature was set to 20 °C, unless stated otherwise. A standard Malvern glass cuvette was used to measure samples in organic solvents. The zeta potential was measured on a Malvern Zetasizer Nano ZS at 20 °C in milli-Q water, with polystyrene latex as a reference material and the $F(\kappa a)$ parameter set to 1.5, according to the Smoluchowski approximation.⁶⁴

2.3. Poly(amido amine) synthesis and characterization

N,N'-methylenebisacrylamide (1.00 g, 6.48 mmol), ethylenediamine-*N,N'*-diacetic acid (1.14 g, 6.48 mmol), triethylamine (3.62 mL, 25.97 mmol) and 30 vol% methanol in milli-Q water (4.87 mL) were added to a 50 mL round bottom flask, which was stirred for 7 days at room temperature. The polymer solution was then dialyzed (MWCO = 1 kDa) against demi-water ($\text{pH} = 1$) and lyophilized to obtain a white solid (834 mg, 39% yield, **p(MBA-EDDA)**). **P(MBA-EDDA)** molecular weight was determined by ^1H NMR, ^1H COSY NMR and SEC. ^1H NMR (D_2O) δ (ppm) = 2.77 (t, 4H, $\text{NHCOCH}_2\text{CH}_2\text{N}$), 3.54 (t, 4H, $\text{NHCOCH}_2\text{CH}_2\text{N}$), 3.65 (s, 4H, $\text{N}(\text{CH}_2)_2\text{N}$), 3.91 (s, 4H, NCH_2COOH), 4.08 (s, 2H, $\text{CH}_2\text{HNCH}_2\text{COOH}$), 4.24 (s, 2H, $\text{CH}_2\text{HNCH}_2\text{COOH}$), 4.58 (s, 2H, $\text{OCNHCH}_2\text{NHCO}$), 4.65 (s, 2H, $\text{NHCH}_2\text{NHCH}_2$), 5.77 (m, 1H, COCHCH_2), 6.22 (m, 2H, COCHCH_2). To determine the molecular weight, the signals at 4.08, 4.24, 5.77 and 6.22 ppm were used for end-group analysis. Residual triethylamine salt signals were found at 1.26 (t, NCH_2CH_3), and 3.27 and 3.17 (q, NCH_2CH_3) ppm. The obtained ^1H NMR spectra and ^1H COSY NMR spectrum can be found in the supporting information.

2.4. Poly(amido amine) nanoprecipitation

A stock solution was prepared by dissolving **p(MBA-EDDA)** (40 mg, 0.12 mmol rep. units) together with *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (23 mg, 0.12 mmol) and *N*-hydroxysuccinimide (14 mg, 0.12 mmol) in 8 mL milli-Q water. Triethylamine was added (67 μ L, 0.48 mmol) and the solution was diluted with milli-Q water to a 1, 2, 3, 4 and 5 mg/mL **p(MBA-EDDA)** concentration. As depicted in Table 1, 1 mL of the prepared **p(MBA-EDDA)** feed solution was added under vigorous stirring to 10 mL 1,4-dioxane or 1,2-dimethoxyethane containing 0.09 equivalents of ethylenediamine, in respect to the amount of carboxylic acid groups present in the **p(MBA-EDDA)** polymer backbone. The same procedure was carried out using acetone, acetonitrile, ethanol and isopropyl alcohol as non-solvent. After 1 day, the crosslinked solution was quenched with 4 equivalents (Table 1) of ethylenediamine for and stirred 1 day. The non-solvent was removed under reduced pressure and the remaining nanogel solution was dialyzed (MWCO = 10 kDa) against milli-Q water and afterwards lyophilized.

For the precipitation experiments at higher concentrations (6-10 mg) **p(MBA-EDDA)** in acetonitrile, a 10 mg/mL stock solution was prepared by dissolving **p(MBA-EDDA)** (50 mg, 0.15 mmol rep. units), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (29 mg, 0.15 mmol) and *N*-hydroxysuccinimide (18 mg, 0.15 mmol) in 10 mL milli-Q water, to which triethylamine was added (83 μ L, 0.60 mmol). These solutions were diluted to 6, 7, 8, 9 and 10 mg/mL **p(MBA-EDDA)** and precipitated in acetonitrile, as shown in Table 1. Quenching and purification of the nanogels was carried out as described earlier.

Table 1: Solution composition of nanoprecipitation solutions. **P(MBA-EDDA)** concentrations of 6-10 mg/mL were only used for nanoprecipitation in acetonitrile.

[p(MBA-EDDA)] ^a (mg/mL)	Non-solvent ^b crosslinker content (μ mol)	Quenching amount ethylenediamine (μ mol)
1.0	0.5	6.1
2.0	1.1	12.1
3.0	1.6	18.2
4.0	2.2	24.2
5.0	2.7	30.3
6.0	3.3	36.4
7.0	3.8	42.4
8.0	4.4	48.5
9.0	4.9	54.5
10.0	5.5	60.6

^a 1 mL of the aqueous solution was added to the non-solvent.

^b Non-solvent volume was kept constant at 10 mL.

2.5. Poly(amido amine) nanogel characterization

Lyophilized nanogels were re-dissolved in milli-Q at 0.5 mg/mL by sonication at 45 °C. All samples were filtered with a 0.45 μm syringe filter to remove any precipitated material. Conversely, DLS measurements were carried out to determine particle size and zeta potential.

3. Results & Discussion

3.1. Poly(amido amine) synthesis

Zwitterionic poly(amido amine)s were synthesized by an aza-Michael polyaddition between *N,N'*-methylenebisacrylamide and ethylenediamine-*N,N'*-diacetic acid, as shown in Scheme 1A. The synthesized poly(amido amine)s possess a high charge density and are soluble in water. After lyophilization **p(MBA-EDDA)** was isolated with a 39% yield. ^1H NMR spectroscopy confirmed the proposed poly(amido amine) structure, whereas end-group analysis showed a molecular weight of 4.9 kDa. Analysis by size exclusion chromatography (SEC) indicated a number average molecular weight of 6.0 kDa and a PDI of 1.7, relative to PEG standards (see Figure 1).

Figure 1: SEC trace of **p(MBA-EDDA)**

3.2. Nanogel formation by nanoprecipitation in different non-solvents

Poly(amido amine) nanogels were formed via nanoprecipitation and subsequent crosslinking by preparing a **p(MBA-EDDA)** solution, containing triethylamine, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) (Scheme 1B), which was nanoprecipitated in a large volume of non-solvent. This immediately leads to the formation of nano-sized poly(amido amine) clusters (Scheme 1C), which are subsequently crosslinked by ethylenediamine (Scheme 1B) present in the solution (see Table 1). After crosslinking, the non-solvent was evaporated under reduced pressure, yielding a solution of crosslinked nanogels. To remove any residual NHS esters, the crosslinking reaction was quenched with 4 equivalents of ethylenediamine, after which the nanogel solution was dialyzed and lyophilized.

Nanogel Formation by Inverse Nanoprecipitation of Zwitterionic Poly(amido amine)s

3.2.1. Nanoprecipitation in 1,2-dimethoxyethane, 1,4 dioxane and ethanol

Addition of the **p(MBA-EDDA)** solution to 1,4-dioxane resulted in the formation of a turbid solution at concentrations above 1 mg/mL and no opalescence was observed at 1 mg/mL. Furthermore, the solutions containing 3, 4 and 5 mg/mL showed precipitation rapidly after addition. This indicates non-ideal conditions for nanoprecipitation, which is also confirmed by DLS measurements after nanoprecipitation shown in Figure 2A. With increasing **p(MBA-EDDA)** concentrations, decreasing count rates were observed, as shown in Figure 2B, which is in contrast with the expected increase in count rate as a result of the increased polymer concentration. After evaporation of the dioxane phase, both particle size as well as count rate increased and after dialysis highly polydisperse particles were obtained.

Scheme 1: Poly(amido amine) synthesis (A), crosslinking reaction (B) and nanogel formation (C), where dispersion of the poly(amido amine) solution and solvent/non-solvent exchange leads to supersaturation of the non-solvent, consisting of small polymer nuclei. These nuclei aggregate until the non-solvent is no longer supersaturated.³⁶

After addition of the **p(MBA-EDDA)** solution to 1,2-dimethoxyethane, all solutions became opalescent, indicating the formation of nanoparticles. However, the intensity of the opalescence quickly increased and eventually changed to a turbid solution, indicating that the precipitated particles were unstable. Figure 2B shows that count rates of the measured particles decreased, upon increasing **p(MBA-EDDA)** concentration after nanoprecipitation. After solvent removal and dialysis, count rates were very low.

Nanoprecipitation of **p(MBA-EDDA)** in ethanol showed no opalescence after nanoprecipitation and, as shown in Figure 2B, during nanoprecipitation the observed count rates were very low at all concentrations. Furthermore, ethanol evaporation and subsequent dialysis, resulted in highly polydisperse samples with low count rates. These results show that 1,2-dimethoxyethane, 1,4-dioxane and ethanol are unsuitable non-solvents for the nanoprecipitation of **p(MBA-EDDA)**.

Figure 2: Nanoparticle size (A) and count rate (B) as obtained by DLS, during nanoprecipitation in 1,2-dimethoxyethane (■), 1,4-dioxane (●) and ethanol (▲).

3.2.2. Nanoprecipitation in isopropyl alcohol

Addition of **p(MBA-EDDA)** solutions to isopropyl alcohol, resulted in increasingly opalescent solutions upon increasing **p(MBA-EDDA)** concentration. As shown in Figure 3, excellent size distributions and increasing count rates were obtained upon increasing the **p(MBA-EDDA)** concentration. Nanoparticle size after nanoprecipitation also increased as the **p(MBA-EDDA)** concentration was increased. However, after evaporation of isopropyl alcohol and dialysis, only large particles (250 nm – 4 μm) were obtained with high polydispersity and low count rates (Figure 3B). This indicates an incomplete crosslinking reaction, most probably due to interfering reactions of isopropyl alcohol and water with the NHS ester-activated carboxylate groups, resulting in hydrolysis or transesterification of the NHS ester, respectively.

Figure 3: Particle size (A) and count rate (B) of **p(MBA-EDDA)** precipitated in isopropyl alcohol, as measured by DLS after nanoprecipitation (■), after isopropyl alcohol evaporation (●) and after dialysis (▲).

3.2.3. Nanoprecipitation in acetone

Nanoprecipitation of **p(MBA-EDDA)** in acetone resulted in opalescent solutions, which show no observable changes during the first hour, indicating the formation of stable nanoparticles. Upon increasing the polymer concentration, particle size and count rates during nanoprecipitation steadily increased, as shown in Figure 4A and 4B, respectively. After acetone evaporation, count rates steadily increased for polymer concentrations of 1, 2 and 3 mg/mL, whereas the count rate slope after acetone removal in Figure 4B decreases at polymer concentrations of 4 and 5 mg/mL. At these polymer concentrations solid material was formed on the vial walls after completing the crosslinking reaction, indicating aggregation. This shows that above a polymer concentration of 3 mg/mL the nanoprecipitated system becomes less stable.

It was also observed that particle size in the resulting aqueous solution was increased due to swelling of the crosslinked nanogels (Figure 4A). Furthermore, the increased pH caused by addition of ethylenediamine to quench the crosslinking reaction, leads to deprotonation of the polymer backbone due to the increased pH, inducing even more swelling.²² After dialysis, excessive swelling of the nanogels prepared at 1 mg/mL **p(MBA-EDDA)** was observed, which was probably caused by insufficient crosslinking due to the low polymer concentration. In contrast, stable nanogels were prepared at polymer concentrations above 1 mg/mL, showing shrinkage after dialysis due to the neutral pH of the obtained nanogel solutions.

Figure 4: Particle size (A) and count rate (B) of **p(MBA-EDDA)** precipitated in acetone, as measured by DLS after nanoprecipitation (■), after acetone evaporation (●) and after dialysis (▲).

3.2.4. Nanoprecipitation in acetonitrile

Upon increasing the **p(MBA-EDDA)** concentration during nanoprecipitation in acetonitrile, the resulting nanoparticle size steadily increased, as shown in Figure 5A. When compared to acetone, nanoprecipitation in acetonitrile initially results in the formation of

slightly larger nanoparticles. However, when the **p(MBA-EDDA)** concentration increases, the size of the formed particles increases more gradually, when compared to acetone. After crosslinking, no precipitate was formed on the walls of the glass vials with **p(MBA-EDDA)** concentrations up to 5 mg/mL. We therefore also examined if nanoprecipitation to obtain well-defined nanoparticles was possible at **p(MBA-EDDA)** concentrations of 6, 7, 8 9 and 10 mg/mL. Nanoprecipitation at **p(MBA-EDDA)** concentrations between 3 and 10 mg/mL resulted in opalescent nanoparticle dispersions, where nanoparticle diameter increased upon increasing the polymer concentration (Figure 5A). Count rates during nanoprecipitation also steadily increased under increasing polymer concentrations, as shown in Figure 5B. However, after acetonitrile evaporation, count rates did not increase for **p(MBA-EDDA)** concentrations above 6 mg/mL. Furthermore, solid material was visible on the vial walls after crosslinking at polymer concentrations above 8 mg/mL. This shows that at these higher polymer concentrations aggregation occurs, reducing the obtained amount of nanogels. As discussed before, non-solvent removal resulted in swelling, which was further increased due to an increased pH, caused by quenching with ethylenediamine. Dialysis of nanogel solutions prepared at polymer concentrations between 2 and 10 mg/mL, resulted in stable nanogel dispersions, which showed shrinkage due to pH neutralization (Figure 5A). Similar to acetone, nanogels prepared at 1 mg/mL **p(MBA-EDDA)** show excessive swelling upon dialysis. Furthermore, considerable swelling after dialysis was also observed for nanogels prepared at 2 mg/mL, as shown in Figure 5A.

Compared to acetone, nanoprecipitation of **p(MBA-EDDA)** is more stable in acetonitrile, allowing nanogel preparation at higher polymer concentrations of 6-7 mg/mL, increasing nanogel yields. Furthermore, Figures 4A and 5A show that **p(MBA-EDDA)** nanogel size of nanogels can be controlled by the **p(MBA-EDDA)** concentration employed during nanoprecipitation.

Figure 5: Particle size (A) and count rate (B) of **p(MBA-EDDA)** precipitated in acetonitrile, as measured by DLS after nanoprecipitation (■), after acetonitrile evaporation (●) and after dialysis (▲).

3.3. Influence of solubility parameters on particle size and size distribution

Nanoparticle formation by nanoprecipitation is achieved by displacing the polymer's solvent by a non-solvent. In this respect, the choice of solvent and non-solvent pair greatly influences nanoparticle characteristics. Solubility parameters (δ) estimate the interaction between solvent molecules, based on their chemical structure.⁶⁵ Similar interaction values (small $\Delta\delta$) indicate good solubility between compounds.⁴³ Solubility parameters can also be expressed by an interaction parameter χ , which can be considered for water with the non-solvent (χ_{w-NS} Equation 1).

$$\chi_{w-NS} = \frac{V_{m,w}}{RT} (\delta_w - \delta_{ns})^2 \quad \text{Equation 1}$$

Where V_m , R and T denote the molar volume, gas constant and absolute temperature, respectively.⁶⁶ The subscripts NS and w denote the non-solvent and water (the solvent), respectively. Solvents with high interaction display small interaction parameters. For nanoprecipitation, it was found that strong interaction between solvent and non-solvent causes swelling of the polymer phase, by either transfer of non-solvent into the polymer phase or retention of solvent in the polymer phase.^{37,41,43,44} It has to be noted that ethylenediamine, EDC and NHS are present in the system, which influence is not taken into account in these experiments. Estimation of the interaction parameter of the polymer can be a useful tool to optimize the nanoprecipitation process.³⁵ However, since estimation⁶⁷ of the solubility factor (δ) of **p(MBA-EDDA)** seemed unreliable (see supporting information), attention will therefore be directed towards the miscibility of the solvent and the non-solvent.

Solubility factors and interaction parameters of the used non-solvents for nanoprecipitation were calculated and are listed in Table 2. The solubility factors (δ) of 1,2 dimethoxyethane and 1,4 dioxane – compared to water – are small, resulting in large values for $\Delta\delta$ and χ_{w-NS} . Nanoprecipitation in 1,2 dimethoxyethane and 1,4 dioxane was not successful and quickly led to aggregation and precipitation, which was indicated by an increasing turbidity of the solutions, eventually resulting in material adhering to the glass vial wall. Although 1,4-dioxane has a similar χ_{w-NS} value as acetone, no nanoparticles were formed, which might be attributed to the non-polarity of the solvent. The high χ_{w-NS} and $\Delta\delta$ values for 1,2-dimethoxyethane (6.6 and 37.6, respectively), combined with the observed de-stabilization of the nanoprecipitation, suggests that there is less interaction between water and the non-solvent – compared to solvents with lower χ_{w-NS} values. This may result in a more swollen polymer phase, and the formation of large and unstable particles during nanoprecipitation.⁴⁴

Table 2: Solubility factors and derived parameters for the used non-solvents and water.

Solvent	δ^a (MPa ^{0.5})	$\Delta\delta^b$ (MPa ^{0.5})	χ_{w-NS}^c
1,2-dimethoxyethane	17.7	37.6	6.6
1,4-dioxane	20.5	37.8	5.4
Acetone	19.9	35.7	5.7
Isopropyl alcohol	23.6	27.7	4.3
Acetonitrile	24.4	36.3	4.0
Ethanol	26.5	24.0	3.3
Water	47.8	0	0

^asolubility factor⁶⁵ $\delta^2 = \delta_H^2 - \delta_D^2 - \delta_P^2$

^bsolubility difference⁴³ $\Delta\delta^2 = (\delta_{H,w} - \delta_{H,NS})^2 + (\delta_{D,w} - \delta_{D,NS})^2 + (\delta_{P,w} - \delta_{P,NS})^2$

^ccalculated from Equation 1

Particle formation during nanoprecipitation was not observed in ethanol, in spite of its low χ and $\Delta\delta$ values. Ethanol might therefore be acting as a solvent for **p(MBA-EDDA)**, since the count rates in Figure 3A remain low, independent of the used **p(MBA-EDDA)** concentration. Due to more similar solvent characteristics (small values for $\Delta\delta$ and χ_{w-NS}), **p(MBA-EDDA)** may dissolve into the ethanol phase, prohibiting the formation of **p(MBA-EDDA)** nuclei.

Nanoprecipitation and crosslinking in acetone and acetonitrile successfully resulted in the formation of nanogels, as described before, whereas nanoprecipitation in isopropyl alcohol was successful, but no crosslinked nanogels were obtained. For acetone, isopropyl alcohol and acetonitrile, the interaction parameters χ_{w-NS} (5.7, 4.3 and 4.0, respectively) are grouped together, where ethanol and 1,2-dimethoxyethane show more and less interaction with water, respectively. Furthermore, these are all polar solvents, which distinguishes them from 1,4-dioxane. The characteristics of acetone, isopropyl alcohol and acetonitrile indicate that good interaction with water, and polarity and hydrogen bond formation, play a role in nanoprecipitation.

Nanoprecipitation of 3, 4 and 5 mg/mL **p(MBA-EDDA)** solutions in acetone results in larger particles (83.7, 103.4 and 127.5 nm) than in acetonitrile (84.0, 95.8 and 96.1 nm) and isopropyl alcohol (69.7, 86.1 and 99.8 nm), as shown in Figure 6A. When comparing the $\Delta\delta$, χ_{w-NS} values in Table 2 of these solvents; acetone, isopropyl alcohol and acetonitrile display increasingly smaller χ_{w-NS} values, while $\Delta\delta$ shows the reverse trend for these solvents. For nanoprecipitation of hydrophobic polymers smaller χ values were demonstrated to correlate to smaller particles formed during nanoprecipitation.^{37,41,44,68} This trend can also be observed in inverse nanoprecipitation, with acetone and acetonitrile as a non-solvent, at **p(MBA-EDDA)** concentration above 3 mg/mL, as shown in Figure 6. However, nanoprecipitation in isopropanol did not follow this trend and led to smaller or similar-sized nanoparticles, as compared to acetonitrile between 3 and 5 mg/mL **p(MBA-EDDA)**.

Polydispersity indices of **p(MBA-EDDA)** in acetone, isopropyl alcohol and acetonitrile, as shown in Figure 6B, demonstrate that dispersity of the formed nanoparticles is affected by the non-solvent choice. A general trend can be observed, where acetone, isopropyl alcohol and acetonitrile show increasingly broader particle size distributions upon increasing polymer concentration. This is in agreement with decreasing interaction parameters (χ_{w-NS}) between water and non-solvent. Galindo-Rodriguez et al. showed that

Figure 6: Concentration dependent size (A) and PDI (B) as measured by DLS during nanoprecipitation in acetone (χ_{w-NS} = 5.7), isopropyl alcohol (χ_{w-NS} = 4.3) and acetonitrile (χ_{w-NS} = 4) at **p(MBA-EDDA)** concentrations of 1 (■), 2 (●), 3 (▲), 4 (▼) and 5 (◆) mg/mL.

the dispersity of nanoparticles increases when χ of the solvent decreases, nanoparticles with broader size distributions were obtained,⁴³ which is related to stronger inter-phase mixing due to the solvents' more similar characteristics.

3.4. Nanogel characterization

After lyophilization, nanogels prepared from either acetone or acetonitrile were obtained as white fibrous materials, except for nanogels prepared at 1 mg/mL in acetone, and at 1 and 2 mg/mL in acetonitrile, which were obtained as a non-soluble solid material. Furthermore, re-dissolving by sonication resulted in stable nanogel dispersions. In Table 3, the Z-average diameter, polydispersity and zeta potential of the nanogels prepared in acetone and acetonitrile are listed. The zeta potential values in of all nanogels show some variation, but no trend related to polymer concentration or non-solvent can be observed. In Chapter 3 and 4, the reported nanogels showed a zeta potential of -26.5 mV,²² which is within the same range as the zeta potential reported here. Table 3 also shows that the final size of nanogels prepared in acetone and acetonitrile is determined by the polymer concentration during nanoprecipitation. Nanogels prepared in acetone are smaller than nanogels prepared in acetonitrile, which is contrary to the results found

after dialysis. Nanogels prepared at e.g. a polymer concentration of 3 mg/mL showed diameters of 112.5 and 130.2 nm after dialysis for acetonitrile and acetone, respectively, whereas after re-dispersion the diameters were 126.9 and 102 nm for acetonitrile and acetone, respectively.

Table 3: Average size, polydispersity index and zeta potential of nanogels, prepared by nanoprecipitation in acetone and acetonitrile.

PAA concentration (mg/mL)	Acetone			Acetonitrile		
	Z-average size (nm)	Poly-dispersity	Zeta potential (mV)	Z-average size (nm)	Poly-dispersity	Zeta potential (mV)
1	n.d	n.d	n.d	n.d	n.d	n.d
2	97	0.24	-25.9	n.d	n.d	n.d
3	102	0.12	-26.5	114	0.24	-26.9
4	118	0.11	-25.8	144	0.26	-31.2
5	119	0.16	-20.3	127	0.18	-23.8
6	-	-	-	139	0.11	-28.1
7	-	-	-	151	0.12	-23.5
8	-	-	-	176	0.13	-30.8
9	-	-	-	188	0.16	-37.9
10	-	-	-	188	0.23	-26.7

n.d – lyophilized material could not be re-dissolved.

4. Conclusions

Zwitterionic poly(amido amine) nanogels were successfully prepared via inverse nanoprecipitation. Six water-miscible solvents were tested for their suitability for poly(amido amine) nanogel preparation. Nanoprecipitation of **p(MBA-EDDA)** in acetone, acetonitrile, and isopropyl alcohol led to stable nanoparticle dispersions, whereas 1,2-dimethoxyethane, 1,4 dioxane and ethanol were not suitable for this purpose. The subsequent crosslinking of the nanoparticles by EDC/NHS coupling, of a fraction of the carboxylic acids with ethylenediamine, yielded stable nanogels in case of acetone and acetonitrile non-solvent mixtures but not in isopropanol due to incomplete crosslinking. Nanogel preparation with acetonitrile as non-solvent gave the best results, since nanogels could be prepared at higher concentrations than in acetonitrile, as described in Chapter 4.²²

The relation between particle-size and the interaction parameter of employed solvents with the non-solvent has been investigated earlier for nanoprecipitation of hydrophobic polymers.^{37,41–44} This relation could not be demonstrated conclusively for the systems studied here. However, it was shown that the particle size distribution is clearly

influenced by the non-solvent, as a lower χ_{w-NS} value results in broader size distributions. Furthermore, the presented results show that χ_{w-NS} and $\Delta\delta$ values can provide valuable information for non-solvent selection.

The poly(amido amine) concentration during nanoprecipitation in isopropyl alcohol, acetone and acetonitrile, was shown to be a crucial parameter for the final nanogel size. This concentration-dependent relation was also seen after dialysis and re-dissolution of the nanogels prepared in acetone and acetonitrile. However, it was not possible to extrapolate this relationship to concentrations lower than 2 mg/mL to obtain nanogels with a diameter below 100 nm. Consequently, 100 nm is the lower-size limit that can be obtained for nanogels prepared via this method.

Acknowledgements: The funding of this work by the provinces of Overijssel and Gelderland, as well as the project consortium by the Center for Medical Imaging – North East Netherlands (CMI-NEN) is gratefully acknowledged.

5. References

- (1) Zhang, X.; Malhotra, S.; Molina, M.; Haag, R. *Chem. Soc. Rev.* **2015**, *44* (7), 1948–1973.
- (2) Elsabahy, M.; Wooley, K. L. *Chem. Soc. Rev.* **2012**, *41* (7), 2545.
- (3) Kim, B. Y. S.; Rutka, J. T.; Chan, W. C. W. *N. Engl. J. Med.* **2010**, *363* (25), 2434–2443.
- (4) Albanese, A.; Tang, P. S.; Chan, W. C. W. *Annu. Rev. Biomed. Eng.* **2012**, *14*, 1–16.
- (5) Kabanov, A. V.; Vinogradov, S. V. *Angew. Chemie Int. Ed.* **2009**, *48* (30), 5418–5429.
- (6) Smith, M. H.; Lyon, L. A. *Acc. Chem. Res.* **2012**, *45* (7), 985–993.
- (7) Ju, C.; Mo, R.; Xue, J.; Zhang, L.; Zhao, Z.; Xue, L.; Ping, Q.; Zhang, C. *Angew. Chemie Int. Ed.* **2014**.
- (8) Peng, J.; Qi, T.; Liao, J.; Fan, M.; Luo, F.; Li, H.; Qian, Z. *Nanoscale* **2012**, *4* (8), 2694–2704.
- (9) Li, D.; Kordalivand, N.; Fransen, M. F.; Ossendorp, F.; Raemdonck, K.; Vermonden, T.; Hennink, W. E.; van Nostrum, C. F. *Adv. Funct. Mater.* **2015**, *25* (20), 2993–3003.
- (10) Averick, S. E.; Paredes, E.; Irastorza, A.; Shrivats, A. R.; Srinivasan, A.; Siegwart, D. J.; Magenau, A. J.; Cho, H. Y.; Hsu, E.; Averick, A. A.; Kim, J.; Liu, S.; Hollinger, J. O.; Das, S. R.; Matyjaszewski, K. *Biomacromolecules* **2012**, *13* (11), 3445–3449.
- (11) Thornton, P. D.; Mart, R. J.; Webb, S. J.; Ulijn, R. V. *Soft Matter* **2008**, *4* (4), 821.
- (12) Xiong, M.-H. H.; Li, Y.-J. J.; Bao, Y.; Yang, X.-Z. Z.; Hu, B.; Wang, J. *Adv. Mater.* **2012**, *24* (46), 6175–6180.
- (13) Lu, C.; Li, B.; Liu, N.; Wu, G.; Gao, H.; Ma, J. *RSC Adv.* **2014**, *4* (92), 50301–50311.
- (14) Lou, S.; Gao, S.; Wang, W.; Zhang, M.; Zhang, J.; Wang, C.; Li, C.; Kong, D.; Zhao, Q. *Nanoscale* **2015**, *7* (7), 3137–3146.
- (15) Santra, S.; Jativa, S. D.; Kaittanis, C.; Normand, G.; Grimm, J.; Perez, J. M. *ACS Nano* **2012**, *6* (8), 7281–7294.
- (16) Oishi, M.; Tamura, A.; Nakamura, T.; Nagasaki, Y. *Adv. Funct. Mater.* **2009**, *19* (6), 827–834.
- (17) Wu, W.; Mitra, N.; Yan, E. C. Y.; Zhou, S. *ACS Nano* **2010**, *4* (8), 4831–4839.
- (18) Molina, M.; Asadian-Birjand, M.; Balach, J.; Bergueiro, J.; Miceli, E.; Calderón, M.; Calderon, M. *Chem. Soc. Rev.* **2015**, *44* (17), 6161–6186.
- (19) Zhang, L.; Cao, Z.; Bai, T.; Carr, L.; Ella-Menye, J.-R.; Irvin, C.; Ratner, B. D.; Jiang, S. *Nat. Biotechnol.* **2013**, *31* (6), 553–556.
- (20) Nguyen, A. T.; Baggerman, J.; Paulusse, J. M. J.; Zuillhof, H.; van Rijn, C. J. M. *Langmuir* **2012**, *28* (1), 604–610.
- (21) Cheng, G.; Mi, L.; Cao, Z.; Xue, H.; Yu, Q.; Carr, L.; Jiang, S. *Langmuir* **2010**, *26* (10), 6883–6886.
- (22) Ekkelenkamp, A. E.; Jansman, M. M. T.; Roelofs, K.; Engbersen, J. F. J.; Paulusse, J. M. J. *Acta Biomater.* **2016**, *30*, 126–134.
- (23) Moore, T. L.; Rodriguez-Lorenzo, L.; Hirsch, V.; Balog, S.; Urban, D.; Jud, C.; Rothen-Rutishauser, B.; Lattuada, M.; Petri-Fink, A. *Chem. Soc. Rev.* **2015**, *44*, 6287–6305.
- (24) Knop, K.; Hoogenboom, R.; Fischer, D.; Schubert, U. S. *Angew. Chemie Int. Ed.* **2010**, *49* (36), 6288–6308.

- (25) Oh, J. K.; Drumright, R.; Siegwart, D. J.; Matyjaszewski, K. *Prog. Polym. Sci.* **2008**, 33 (4), 448–477.
- (26) Arshady, R. *Colloid Polym. Sci.* **1992**, 270 (8), 717–732.
- (27) Oh, J. K.; Siegwart, D. J.; Lee, H.; Sherwood, G.; Peteanu, L.; Hollinger, J. O.; Kataoka, K.; Matyjaszewski, K. *J. Am. Chem. Soc.* **2007**, 129 (18), 5939–5945.
- (28) Dickerson, E. B.; Blackburn, W. H.; Smith, M. H.; Kapa, L. B.; Lyon, L. A.; McDonald, J. F. *BMC Cancer* **2010**, 10 (1), 10.
- (29) An, Z.; Shi, Q.; Tang, W.; Tsung, C.-K.; Hawker, C. J.; Stucky, G. D. *J. Am. Chem. Soc.* **2007**, 129 (46), 14493–14499.
- (30) Williams, M.; Penfold, N. J. W.; Armes, S. P. *Polym. Chem.* **2016**, 7 (2), 384–393.
- (31) Rolland, J. P.; Maynor, B. W.; Euliss, L. E.; Exner, A. E.; Denison, G. M.; DeSimone, J. M. *J. Am. Chem. Soc.* **2005**, 127 (28), 10096–10100.
- (32) Ryu, J.-H.; Chacko, R. T.; Jiwanpanich, S.; Bickerton, S.; Babu, R. P.; Thayumanavan, S. *J. Am. Chem. Soc.* **2010**, 132 (48), 17227–17235.
- (33) Chen, W.; Zheng, M.; Meng, F.; Cheng, R.; Deng, C.; Feijen, J.; Zhong, Z. *Biomacromolecules* **2013**, 14 (4), 1214–1222.
- (34) Ganachaud, F.; Katz, J. L. *ChemPhysChem* **2005**, 6 (2), 209–216.
- (35) Schubert, S.; Delaney, Jr, J. T.; Schubert, U. S. *Soft Matter* **2011**, 7 (5), 1581–1588.
- (36) Lepeltier, E.; Bourgaux, C.; Couvreur, P. *Adv. Drug Deliv. Rev.* **2014**, 71, 86–97.
- (37) Beck-Broichsitter, M.; Nicolas, J.; Couvreur, P. *Nanoscale* **2015**, 7, 9215–9221.
- (38) Anton, N.; Benoit, J.-P.; Saulnier, P. *J. Control. Release* **2008**, 128 (3), 185–199.
- (39) Nyambura, B. K.; Kellaway, I. W.; Taylor, K. M. G. *Int. J. Pharm.* **2009**, 372 (1), 140–146.
- (40) Hrkach, J.; Von Hoff, D.; Mukkaram Ali, M.; Andrianova, E.; Auer, J.; Campbell, T.; De Witt, D.; Figa, M.; Figueiredo, M.; Horhota, A.; Low, S.; McDonnell, K.; Peeke, E.; Retnarajan, B.; Sabnis, A.; Schnipper, E.; Song, J. J.; Song, Y. H.; Summa, J.; Tompsett, D.; Troiano, G.; Van Geen Hoven, T.; Wright, J.; LoRusso, P.; Kantoff, P. W.; Bander, N. H.; Sweeney, C.; Farokhzad, O. C.; Langer, R.; Zale, S. *Sci. Transl. Med.* **2012**, 4 (128), 128ra39.
- (41) de Oliveira, A. M.; Jäger, E.; Jäger, A.; Stepánek, P.; Giacomelli, F. C. *Colloids Surfaces A Physicochem. Eng. Asp.* **2013**, 436, 1092–1102.
- (42) Perevyazko, I. Y.; Delaney, J. T.; Vollrath, A.; Pavlov, G. M.; Schubert, S.; Schubert, U. S. *Soft Matter* **2011**, 7 (10), 5030.
- (43) Galindo-rodriguez, S.; Alle, E.; Fessi, H.; Doelker, E.; Allémann, E.; Fessi, H.; Doelker, E. *Pharm. Res.* **2004**, 21 (8), 1428–1439.
- (44) Bilati, U.; Allémann, E.; Doelker, E. *Eur. J. Pharm. Sci.* **2005**, 24 (1), 67–75.
- (45) Perevyazko, I. Y.; Vollrath, A.; Pietsch, C.; Schubert, S.; Pavlov, G. M.; Schubert, U. S. *J. Polym. Sci. Part A Polym. Chem.* **2012**, 50 (14), 2906–2913.
- (46) Reisch, A.; Runser, A.; Arntz, Y.; Mély, Y.; Klymchenko, A. S.; Reisch, A.; Runser, A.; Arntz, Y.; Me, Y. *ACS Nano* **2015**, 9 (5), 5104–5116.

- (47) Steinhilber, D.; Witting, M.; Zhang, X.; Staegemann, M.; Paulus, F.; Friess, W.; Küchler, S.; Haag, R. *J. Control. Release* **2013**, 169 (3), 289–295.
- (48) Khan, S. A.; Schneider, M. *Macromol. Biosci.* **2013**, 13 (4), 455–463.
- (49) Kuo, W.-T.; Huang, J.-Y.; Chen, M.-H.; Chen, C.-Y.; Shyong, Y.-J.; Yen, K.-C.; Sun, Y.-J.; Ke, C.-J.; Cheng, Y.-H.; Lin, F.-H. *J. Mater. Chem. B* **2016**, 4 (14), 2444–2454.
- (50) Martello, F.; Piest, M.; Engbersen, J. F. J.; Ferruti, P.; Gonçalves, C. M. B.; Tomé, L. C.; Garcia, H.; Brandão, L.; Mendes, A. M.; Marrucho, I. M. *J. Control. release* **2012**, 164 (3), 372–379.
- (51) Piest, M.; Engbersen, J. F. J. *J. Control. release* **2011**, 155 (2), 331–340.
- (52) Piest, M.; Lin, C.; Mateos-Timoneda, M. a; Lok, M. C.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. *J. J. Control. release* **2008**, 130 (1), 38–45.
- (53) Vader, P.; van der Aa, L. J.; Engbersen, J. F. J.; Storm, G.; Schiffelers, R. M. *Pharm. Res.* **2011**, 28 (5), 1013–1022.
- (54) van der Aa, L. J.; Vader, P.; Storm, G.; Schiffelers, R. M.; Engbersen, J. F. J. *J. Control. Release* **2014**.
- (55) Vercauteren, D.; Piest, M.; van der Aa, L. J.; Al Soraj, M.; Jones, A. T.; Engbersen, J. F. J.; De Smedt, S. C.; Braeckmans, K. *Biomaterials* **2011**, 32 (11), 3072–3084.
- (56) Lin, C.; Zhong, Z.; Lok, M. C.; Jiang, X.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J. *Bioconjug. Chem.* **2007**, 18 (1), 138–145.
- (57) Coué, G.; Engbersen, J. F. J. *J. Control. Release* **2011**, 152 (1), 90–98.
- (58) Chen, J.; Shi, M.; Liu, P.; Ko, A.; Zhong, W.; Liao, W.; Xing, M. M. Q. *Biomaterials* **2014**, 35 (4), 1240–1248.
- (59) Ping, Y.; Wu, D.; Kumar, J. N.; Cheng, W.; Lay, C. L.; Liu, Y. *Biomacromolecules* **2013**, 14 (6), 2083–2094.
- (60) Richardson, S. C. W.; Patrick, N. G.; Lavignac, N.; Ferruti, P.; Duncan, R. J. *J. Control. Release* **2010**, 142 (1), 78–88.
- (61) Ferruti, P. J. *Polym. Sci. Part A Polym. Chem.* **2013**, 51 (11), 2319–2353.
- (62) Parmar, R. G.; Busuek, M.; Walsh, E. S.; Leander, K. R.; Howell, B. J.; Sepp-Lorenzino, L.; Kemp, E.; Crocker, L. S.; Leone, A.; Kochansky, C. J.; Carr, B. a; Garbaccio, R. M.; Colletti, S. L.; Wang, W. *Bioconjug. Chem.* **2013**, 24 (4), 640–647.
- (63) Ferruti, P.; Ranucci, E.; Bignotti, F.; Sartore, L.; Bianciardi, P.; Marchisio, M. A. *J. Biomater. Sci. Polym. Ed.* **1995**, 6 (9), 833–844.
- (64) Doane, T. L.; Chuang, C.-H.; Hill, R. J.; Burda, C. *Acc. Chem. Res.* **2012**, 45 (3), 317–326.
- (65) Hansen, C. In *Hansen Solubility Parameters*; CRC Press, 2007; pp 385–483.
- (66) Hansen, C. M. In *Hansen Solubility Parameters*; CRC Press, 1999; pp 25–42.
- (67) Van Krevelen, D. W.; Te Nijenhuis, K. In *Properties of Polymers, 4th Edition*; 2009; Vol. i, pp 189–227.
- (68) Mora-Huertas, C. E. E.; Fessi, H.; Elaissari, A. *Adv. Colloid Interface Sci.* **2011**, 163 (2), 90–122.

6. Supporting Information

The solubility parameter of **p(MBA-EDDA)** was estimated at $22.9 \text{ MPa}^{0.5}$ via Hoy's group contribution method.⁶⁷ However, this approach seems inaccurate, since there is a large difference between the solubility parameters of **p(MBA-EDDA)** ($\delta = 22.9 \text{ MPa}^{0.5}$) and water ($\delta = 47.8 \text{ MPa}^{0.5}$), which is to our knowledge the only good solvent for this polymer. This may be because **p(MBA-EDDA)** is an ampholyte with a high charge density, i.e. protonation and deprotonation of the polymer play a major role in its solvation.

Figure S1: Obtained **p(MBA-EDDA)** ^1H NMR spectrum.

Figure S2: Obtained p(MBA-EDDA) ^1H COSY NMR spectrum.

CHAPTER 4

Functionalization of Poly(amido amine) Nanogels

Abstract

The use of nanoparticles in therapeutic applications, such as targeted drug delivery or medical imaging, demands efficient and effective functionalization strategies for conjugation of targeting or imaging groups. In this work, the site-specific functionalization of poly(amido amine) nanogels containing carboxylic acids and primary amines is described. Carboxylic acids were conjugated with *N,N*-dimethylethylenediamine via *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide/*N*-hydroxysuccinimide coupling chemistry to modify the nanogel surface charge. The nanogels were fluorescently labelled via isothiocyanate coupling for nanogel visualization. Furthermore, azide groups were introduced onto the nanogel via a diazo donor, followed by attachment of a dibenzocyclooctyne-functionalized carboxyrhodamine fluorophore, via a copper-free click reaction. These functionalization strategies were carried out successfully, while maintaining the nanogel characteristics.

1. Introduction

The application of nanotechnology in medicine demands for highly versatile nanoparticle systems for specialized applications,¹ such as therapeutic delivery,^{2,3} nanoparticle detection,⁴ and molecular^{5,6} and cellular recognition capabilities.⁷⁻⁹ These characteristics show tremendous potential to improve the efficacy of curative and diagnostic medicine,¹⁰ as demonstrated in the controlled delivery of therapeutics^{2,11} and targeted imaging.^{12,13} Precise control over the chemical composition of nanoparticles by introducing specific functional groups onto nanoparticles is therefore highly desirable, yet remaining far from trivial.^{14,15} In this respect, polymers and polymer nanostructures offer a large degree of freedom in terms of chemical design,¹⁶⁻¹⁸ where specific functional groups can be incorporated into the polymer or polymer nanoparticle.^{16,18}

Functionalization of nanoparticles relies on a wide range of conjugation reactions, which meet specific requirements for biomedical applications, such as high conversion, high selectivity or benign reaction conditions in aqueous media.¹⁴⁻¹⁸ Over the past decades, a diverse toolbox of chemical conjugation techniques has been developed for specific and effective functionalization.¹⁹ In particular the rise of 'click' chemistry has been able to meet the need for highly specific coupling strategies.²⁰⁻²² Click reactions have been defined as orthogonal reactions, which can be completed with high yields without the generation of difficult to remove byproducts. Furthermore, these reactions can be carried out under benign conditions with facile product isolation.^{20,23} Combinations of multiple orthogonal click reactions uncovers an enormous degree of control over polymer functionality, allowing efficient functional group-specific functionalization.¹⁷ In this respect, the copper (I) catalyzed Huisgen azide-alkyne cycloaddition has received considerable attention, since it allows rapid and selective conjugation under mild and aqueous conditions.²⁴ Primary amines can be conveniently converted into azide moieties by using a diazo donor, such as trifluoromethanesulfonyl azide (TfN_3) in a copper(II) catalyzed reaction, as reported by Cavender and Shiner.²⁵ TfN_3 is, however, notoriously unstable and explosive and hard to remove in the presence of polar molecules. Therefore imidazole-1-sulfonylazide·HCl has been developed as a relatively stable diazo transfer agent,^{26,27} which is suitable for azide transfer under aqueous conditions, e.g. for protein functionalization.²⁸ Despite the bio-orthogonal nature of the Huisgen azide-alkyne cycloaddition, the (cyto-) toxicity of the copper(I) catalyst requires removal of the catalyst before application in a biological environment. An azide-alkyne cycloaddition based on strained cyclooctynes enables selective and efficient conjugation without any unwanted by-products (including copper catalyst) and has been demonstrated to be suitable for conjugations, even inside living biological systems,^{29,30} as well as for conjugation of short-lived radioisotopes.³¹⁻³³

Application of nanoparticles in imaging and sensing applications, such as optical, magnetic resonance and nuclear imaging, requires nanoparticle functionalization with specific moieties, respective to the imaging modality.³⁴ For example, magnetic resonance imaging (MRI) contrast can be achieved by relatively high concentrations of water-accessible chelated gadolinium.³⁵ Conjugation of MRI contrast agents to polymer nanoparticles promises optimal MRI contrast generation,^{13,36–38} since physicochemical properties can be altered to achieve optimal blood circulation properties.³⁹ The use of nanoparticles in optical imaging is widespread and fluorophores for bio-conjugation are widely available,⁴⁰ enabling nanoparticle tracking⁴¹ and (bio)-chemical sensing.⁴² Furthermore, nanoparticle functionalization with responsive fluorophores equips nanoparticles for molecular sensing.⁶ In addition, fluorescence imaging techniques such as Förster resonance energy transfer (FRET) enable the visualization and quantification of interactions of nanoparticles with other molecules.⁴³ Functionalization of polymer nanoparticles with specific radioactive isotopes enables detailed monitoring of particle distribution via positron emission tomography (PET) and single photon emission tomography (SPECT).⁴⁴ However, labeling of nanomaterials with radioisotopes puts different demands on the involved conjugation reactions, such as short reaction times and high yields are essential, making the use of click reactions desirable.^{31–33}

Targeting of nanoparticles plays a central role in nanoparticle-mediated drug delivery and imaging, which requires controlling the nanoparticle's physicochemical properties, as well as functionalizing its surface with ligands for cellular recognition.⁸ The enhanced permeability and retention (EPR) effect,⁴⁵ caused by the different permeability of tumor tissue, enables passive targeting of nanoparticles to such tissues, which is further enhanced by prolonged circulation in the bloodstream.⁴⁶ Nanoparticle functionalization with hydrophilic polymers, such as poly(ethylene glycol) (PEG), zwitterionic polymers^{47–49} and poly(glycidol) architectures,^{50–52} increases nanoparticle stability and prolongs a particle's blood retention.^{53,54} Active targeting on the other hand requires promotion of the interactions between nanoparticles and cells or molecules by molecular recognition. This may be achieved by attachment of targeting moieties to these nanoparticles. These moieties include antibodies, antibody fragments, peptide sequences, sugars and small molecules, each containing specific functional groups and requiring specific conjugation strategies.⁸ Moreover, conjugation of targeting moieties requires specific control over the conjugation process, especially in the case of antibodies and antibody fragments, where ligand orientation influences targeting efficacy.⁴⁵

Nanogels are water-soluble polymer networks with a diameter on the nanoscale,⁵⁵ which possess highly interesting properties for biomedical applications, such as high water content, drug-loading capacity, stability, responsive properties and biocompatibility.^{56,57} Nanogel formation offers a large degree of control over their

mechanical properties, in addition to the ability to incorporate highly hydrophilic zwitterionic polymers, greatly enhancing their blood circulation properties.^{58,59} These properties make nanogels suitable for application in controlled drug^{2,60,61} and gene^{62,63} delivery, and medical imaging^{12,13,64} and sensing⁵ applications. Poly(amido amine)s are water-soluble peptidomimetic polymers, which are obtained by an aza-Michael addition and which have been predominantly applied in gene delivery applications.^{65,66} As described in Chapter 4, zwitterionic poly(amido amine) nanogels proved to be highly stable when exposed to high protein concentrations.⁶⁷ These nanogels contain carboxylic acid and primary amine groups, which can be used for functionalization purposes. In this work, we demonstrate functionalization of these nanogels by surface charge modification and effective conjugation of fluorophores by *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide/*N*-hydroxysuccinimide (EDC/NHS) and isothiocyanate coupling, respectively. Surface charge modification alters the interactions between the nanogels and cells⁶⁸ and functionalization with a fluorophore allows monitoring of nanogels by fluorescence microscopy in for instance, *in-vitro* studies. Furthermore, azide moieties were introduced onto the nanogels and functionalized with a fluorophore via a copper-free click reaction, demonstrating the versatility of these nanogels for future applications.

2. Materials & Methods

2.1. Materials

N,N'-methylenebisacrylamide (99%), triethylamine ($\geq 99\%$), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride ($\geq 98\%$), *N*-hydroxysuccinimide ($\geq 97\%$), ethylenediamine ($\geq 97\%$), hydrochloric acid (37%), *N,N'*-dimethylethylenediamine ($\geq 99\%$), sodium azide ($\geq 99.5\%$), imidazole ($\geq 99\%$), sulfurylchloride ($\geq 97\%$), sodium chloride ($\geq 99.5\%$), sulfuric acid (95-98%), 0.1 M PBS – monosodium phosphate monohydrate (3.9 mM, $\geq 99\%$) disodium phosphate dihydrate (6.1 mM, 99.5%), sodium chloride (137 mM, $\geq 99.5\%$), potassium chloride (2.7 mM, $\geq 99\%$) – fluorescein isothiocyanate (FITC, $\geq 90\%$) and rhodamine B (mixed isomers) were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Ethylenediamine-*N,N'*-diacetic acid ($\geq 98\%$), methanol (reagent grade), acetone (reagent grade), ethyl acetate (reagent grade) and DMSO (reagent grade) were purchased from VWR (Amsterdam, the Netherlands). DBCO-PEG₄-5/6-Carboxyrhodamine 110 ($> 90\%$) was purchased from Jena Biosciences (Jena, Germany). Spectra/Por® dialysis tubing with a MWCO of 1 kDa was purchased from Spectrum Labs (Breda, the Netherlands) and SnakeSkin™ dialysis tubing with a MWCO of 10 kDa was purchased from Fisher (Landsmeer, the Netherlands). Milli-Q water was obtained from a Millipore Advantage A10 Ultrapure Water Purification System.

2.2. General procedures

^1H NMR and ^1H COSY NMR spectra were recorded in D_2O on a Bruker Ascend 400 spectrometer; signals of the deuterated solvent were used as a reference. FTIR was measured on a Perkin Elmer Spectrum Two IR spectrometer. DLS measurements were performed on a Malvern Zetasizer Nano ZS at a 173° scattering angle, and analyzed using the general-purpose analysis method provided in the Malvern Zetasizer software. The reference material was set to polystyrene latex and the temperature was set to 25°C , unless stated otherwise. A standard Malvern glass cuvette was used to measure samples in acetone. The zeta potential was measured on a Malvern Zetasizer Nano ZS at 25°C in milli-Q water, with polystyrene latex as a reference material and the $F(\kappa a)$ parameter set to 1.5, according to the Smoluchowski approximation.⁶⁹ Fluorescein, rhodamine B and sulforhodamine B concentrations were determined by UV-VIS ($\lambda = 490\text{ nm}, 565\text{ nm}$ and 501 nm , respectively) on an Agilent Cary 300 UV-VIS spectrophotometer. Determination of solution pH was carried out with a Schott CG 842 laboratory pH meter fitted with a BlueLine 18 pH glass electrode, containing a temperature probe.

2.3. Poly(amido amine) synthesis and characterization

Zwitterionic poly(amido-amine)s (**p(MBA-EDDA)**) were synthesized as described in Chapter 2.⁶⁷ Briefly, *N,N'*-methylenebisacrylamide (2.00 g, 14.07 mmol) and ethylenediamine-*N,N'*-diacetic acid (2.30 g, 13.07 mmol) were dissolved in 30 vol% methanol in milli-Q water (7.30 mL) and triethylamine (7.30 mL, 52.28 mmol) was added. After 6 days reacting at room temperature, the polymer mixture was dialyzed against pH=1 demi-water (MWCO 1 kDa) and subsequently lyophilized. The polymer was obtained as a white solid and the structure of the polymer was confirmed by ^1H NMR and ^1H COSY NMR spectroscopy: $\delta(\text{ppm}) = 2.77$ (t, 4H, 2(NHCOCH₂CH₂N)), 3.53 (t, 4H 2(NHCOCH₂CH₂N)), 3.63 (s, 4H, N(CH₂)₂N), 3.86 (s, 4H, 2(NCH₂COOH)), 4.07 (s, 2H, NCH₂CH₂NHCH₂COOH), 4.19 (s, 2H, NCH₂CH₂NHCH₂COOH), 4.58 (s, 2H, OCNHCH₂NHCO), 5.78 (m, 1H, OCCHCH₂), 6.22 (m, 2H, OCCHCH₂), 8.66 (t, 2H, OCNHCH₂NHCO). The signals at 4.07, 4.19, 5.78 and 6.22 ppm were used to determine the molecular weight by end-group analysis.

2.4. Poly(amido amine) nanogel formation and characterization

Zwitterionic poly(amido amine) nanogels (**PAANG**) were prepared as described in Chapter 2 by dissolving **p(MBA-EDDA)** *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (121 mg, 0.63 mmol) and *N*-hydroxysuccinimide (73 mg, 0.63 mmol) in milli-Q water (70 mL), to which triethylamine (350 μL) was added. Nanoprecipitation and subsequent overnight crosslinking was performed by adding the polymer solution to 700 mL of acetone, containing ethylenediamine (7.70 μL , 0.11 mmol)

under vigorous stirring. After crosslinking the reaction mixture was placed on ice and ethylenediamine (5.0 mL, 92.5 mmol) was added and left stirring for 1 day. Acetone was subsequently evaporated, the nanogel mixture was filtered and then dialyzed (MWCO 10 kDa) against demi-water and subsequently lyophilized to obtain a white fibrous material (205 mg, 97% yield).

2.5. Nanogel functionalization and characterization

2.5.1. Cationic nanogel modification

Cationically modified nanogels (**DMEDA-PAANG**) were prepared by conjugation of *N,N*-dimethylethylenediamine to **PAANG**. The conjugation was performed by addition of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (623 mg, 3.25 mmol) and *N*-hydroxysuccinimide (374 mg, 3.25 mmol) to a solution of **DMEDA-PAANG** (50 mL, 2.00 mg/mL). After 10 minutes, *N,N*-dimethylethylenediamine from a 2.85 M neutralized aqueous solution (2.28 mL, 6.50 mmol) was added and the reaction was left to proceed overnight. The solution was dialyzed afterwards against demi-water (MWCO 10 kDa) and lyophilized. **DMEDA-PAANG** was obtained as a white fibrous material (89 mg, 89% yield) and the material was re-suspended in milli-Q water or PBS by sonication. **DMEDA-PAANG** was characterized by DLS and zeta potential measurements.

2.5.2. Functionalization by isothiocyanate coupling

Lyophilized **PAANG** and **DMEDA-PAANG** (10.9 mg and 10.8 mg, respectively) coupled with FITC by re-suspending both nanogels at 2.0 mg/mL in milli-Q water, the pH of **PAANG** and **DMEDA-PAANG** solutions were corrected to 10 with a NaOH solution. An FITC solution (3.9 μ L, 0.50 g/mL in DMSO) was added to both solutions. The solutions were stirred overnight in the dark and the resulting solution was dialyzed (MWCO 10 kDa) in the dark, consecutively against 1:1 methanol/demi-water and demi-water. The obtained nanogel solution was finally lyophilized, resulting in a yellow fibrous material (9.7 mg **FITC-PAANG**, 9.2 mg **FITC-DMEDA-PAANG**, 89% and 85% yield, respectively). The functionalisation degree was determined with the Lambert-Beer law. The UV-VIS absorbance was measured at 490 nm and the FITC concentration was calculated using its molar extinction coefficient of $\epsilon = 84,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$.⁷⁰

PAANG was functionalized with Rhodamine B by dissolving 25 mg poly(amido amine) nanogels in 5 mL milli-Q water by placing it in an ultrasonic bath at 40 °C. Then 5.86 μ L Rhodamine B isothiocyanate (from a 0.5 g/mL stock solution in DMSO) was added to the nanogel solution and stirred overnight at room temperature and dialyzed (MWCO = 10 kDa) against 2 L 1/1 meOH/demi water, which was changed to 2 L demi water after 2 hours. After overnight dialysis the dialysis medium was changed again and the solution was dialyzed again for 2 hours. Afterwards the rhodamine conjugated

nanogels (**Rh-PAANG**)s were freeze-dried and obtained as a dark pink powder (10.8 mg, 43% yield), after re-dissolution in PBS the nanogels were analyzed by DLS and UV-VIS ($\epsilon = 10.7 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$).⁷¹

2.5.3. Functionalization by click chemistry

Imidazole-1-sulfonylazide-HCl was synthesized as described earlier,^{26,28} by carefully adding sulfonylchloride in equimolar amounts to an anhydrous 1 M NaN_3 solution in acetonitrile at 0 °C, to which, after 2.5 hours stirring at room temperature, 2 equivalents of imidazole were added carefully at 0 °C. After 2.5 hours stirring at room temperature, ethyl acetate was added and the mixture was washed twice with milli-Q water and once with a saturated NaCO_3 solution. The ethyl acetate phase was dried with MgSO_4 and imidazole-1-sulfonylazide-HCl was obtained, by precipitation in 4M HCl in ethyl acetate. HCl-containing ethyl acetate was prepared by slowly adding sulfuric acid to sodium chloride and leading the in-situ generated HCl gas through ethyl acetate until the solution was saturated. The structure of imidazole-1-sulfonyl chloride was confirmed with FTIR and $^1\text{H-NMR}$ (D_2O) $\delta(\text{ppm}) = 7.61$ (dd, (N_3O_2) S-NCH=CH), 8.01 (dd, (N_3O_2)S-NCH=CH) and 9.39 (dd, (N_3O_2)S-NCH=N). Although imidazole-1-sulfonylazide-HCl a relatively safe azide-transfer agent, its stability is not guaranteed.²⁷ Therefore, caution is advised during synthesis and usage of the transfer-agent.

To transfer an azide to the nanogel, 10.6 mg of nanogel was dissolved at 1 mg/mL in milli-Q water, to which imidazole-1-sulfonylazide-HCl (63 mg, 0.3 mmol) and K_2CO_3 buffer (300 μL , 0.1 M) were added. The pH was corrected to 9, Cu(II) acetate (6.1 mg, 0.03 mmol) was added and the mixture was stirred overnight. Then, ethylenediaminetetraacetic acid (500 mg) and tris(hydroxymethyl)aminoethane (585 mg, 4.8 mmol) were added to the reaction mixture. After overnight stirring, the reaction mixture was dialyzed extensively against demineralized water (MWCO = 10,000 Da) and subsequently freeze-dried. Azide-functionalized nanogels (**N₃-PAANG**) were obtained as a white powder (3.6 mg, 34% yield) and the presence of the azide groups on the nanogel was confirmed by FTIR. Then 3.6 mg $\text{N}_3\text{-NG}$ was weighed and dissolved in 3.6 mL milli-Q water by placing it in an ultrasonic bath for 1 hour at 40 °C. Then 21.5 μL of a 20 mg/mL DBCO-DBCO-PEG₄-5/6-Carboxyrhodamine 110 solution in DMSO was added. After overnight stirring the reaction mixture was dialyzed (MWCO = 10,000) extensively in milli-Q water, until no rhodamine was visible in the dialysis medium and subsequently freeze-dried. The product (**Rh-DBCO-PAANG**) was obtained as a purple powder (0.8 mg, 22% yield), which was re-dissolved at 0.25 mg/mL in milli-Q water by placing the sample in an ultrasonic bath. Particle size was determined by DLS and the amount of carboxyrhodamine was quantified by UV-VIS, ($\epsilon = 71.0 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

3. Results & Discussion

3.1. Poly(amido amine) nanogel synthesis and characterization

Zwitterionic poly(amido amine)s were prepared as described in Chapter 2.⁶⁷ In short, *N,N'*-methylenebisacrylamide and ethylenediamine-*N,N'*-diacetic acid were dissolved in 30 vol% methanol in milli-Q water and triethylamine was added and reacted for 6 days. Subsequently, the polymer solution was purified by dialysis in demi-water (pH = 1). ¹H NMR spectroscopy confirmed the structure of **p(MBA-EDDA)**, while the molecular weight of the polymer was determined to be 4.8 kDa by end-group analysis. To prepare zwitterionic poly(amido amine) nanogels, **p(MBA-EDDA)** was dissolved together with EDC/NHS and a small amount of triethylamine in water, which was subsequently added to a large volume of acetone, containing a small amount of ethylenediamine for crosslinking. **PAANG** were obtained after acetone evaporation, quenching with ethylenediamine and dialysis. After lyophilization **PAANG** obtained (yield = 97%) were dissolved in milli-Q water, after which an opalescent nanogel solution was obtained. Analysis by DLS revealed an average diameter around 100 nm and a low polydispersity (Table 1).

3.2. Nanogel functionalization

PAANG contains carboxylic acid groups, originating from the polymer backbone, as well as primary amine groups, which originate from quenching with ethylenediamine and mono-functionalized crosslinks. Carboxylic acids can be conjugated to primary amines via EDC/NHS mediated coupling. Primary amines can be functionalized under aqueous conditions with isothiocyanates. Furthermore, conversion of primary amines to an azide moiety allows for further functionalization via bio-orthogonal alkyne-azide click chemistry. These functionalizations are discussed below.

3.2.1. Charge modification by EDC/NHS coupling:

Surface charge of nanoparticles has been shown to be an important factor for nanoparticle bio-distribution and cellular uptake.⁶⁸ As such, modification of a nanoparticle's surface charge can be an excellent way to control its physicochemical properties. **PAANG** was functionalized with *N,N*-dimethylethylenediamine and subsequently dialyzed to obtain **DMEDA-PAANG**, in order to obtain the particles with a more positive surface charge. The sizes of both **PAANG** and **DMEDA-PAANG** were approximately 100 nm before and after functionalization. Table 1 depicts the sizes and zeta potentials of both nanogels in milli-Q water and 100 mM PBS. These results show that the zeta potential of **DMEDA-PAANG** increased upon functionalization with *N,N*-dimethylethylenediamine, indicating successful conjugation, without affecting nanogel size and dispersity.

Functionalization of Poly(amido amine) Nanogels

Table 1: Nanogel size and PDI measured in 0.1 M PBS (pH = 7.4) and zeta potential measured in milli-Q water.

	Milli-Q water			0.1 M PBS (pH=7.4)		
	Diameter (nm)	Poly-dispersity	Zeta potential (mV)	Diameter (nm)	Poly-dispersity	Zeta potential (mV)
PAANG	98.4 ± 1.1	0.08	-26.5 ± 2.5	97.8 ± 1.0	0.12	-11.6 ± 0.3
DMEDA-PAANG	95.4 ± 0.8	0.09	11.4 ± 1.2	101.0 ± 1.6	0.23	0.31 ± 0.6
FITC-PAANG	97.9	0.16	n.d	n.d	n.d	n.d
FITC-DMEDA-PAANG	106.9	0.20	n.d	n.d	n.d	n.d
Rh-PAANG	n.d	n.d	n.d	134.2 ± 6.8	0.24	-11.0 ± 0.6

n.d. - not determined

3.2.2. Functionalization by isothiocyanate coupling

Visualization of nanoparticles is a convenient strategy for evaluation of nanoparticles in a biological environment and is often achieved by conjugation of fluorescent labels to the nanoparticles. Functionalization of a small portion of the present functional groups retains the nanogels' zwitterionic properties, while making them suitable for fluorescence imaging. Therefore, **PAANG** was labelled with fluorescein isothiocyanate and rhodamine isothiocyanate, and **DMEDA-PAANG** was labelled with fluorescein isothiocyanate. **PAANG** contains primary amine groups, as a result of quenching the activated carboxylic acids with ethylenediamine. Theoretically, approximately 30% of the carboxylic acid groups may be functionalized with primary amines at 100% crosslinking efficiency. However, since the quenching with ethylenediamine was carried out 1 day after crosslinking in basic environment, it is likely that only a small portion of the carboxylic acid groups of **PAANG** was functionalized with ethylenediamine.

Fluorescein isothiocyanate-conjugated **PAANG** and **DMEDA-PAANG** were obtained as yellow solutions after dialysis. UV-VIS measurements showed that **PAANG** and **DMEDA-PAANG** contained 34 and 149 µmol fluorescein per mg nanogel, respectively. The estimated degree of functionalization of the carboxylic acid groups is therefore 0.6% and 2.5%, respectively. Importantly, size and size distribution of the nanoparticles were only minimally affected (Table 1). After conjugation of rhodamine B isothiocyanate to **PAANG** and subsequent dialysis, a purple solution was obtained. The rhodamine concentration on the nanogels was determined by UV-VIS to be 23 µmol/mg **PAANG**. Therefore, an estimated 0.4% of the carboxylic acids was functionalized. The size of **Rh-PAANG** was slightly increased (6%), as compared to **PAANG** (Table 1).

FITC-DMEDA-PAANG was functionalized to a higher degree (2.5%) than **FITC-PAANG** (0.6%). This can be explained by electrostatic interactions between the anionic fluorescent label and both nanogels (zeta potentials: -28.5 and 11.4 mV **PAANG** and **DMEDA-PAANG**, respectively), where anionic repulsion between fluorescein and **PAANG** explain the lower **FITC-PAANG** functionalization-degree.

3.2.3. Functionalization by click chemistry

Application of the Huisgen azide-alkyne cycloaddition has become an effective strategy for functionalization of nanomaterials. To introduce functional azide groups onto **PAANG**, an azide-transfer agent (imidazole-1-sulfonylazide-HCl) was synthesized as described by Goddard-Borger et al.²⁶ Imidazole-1-sulfonylazide-HCl is a relatively safe diazo-transfer agent to convert primary amines in azide moieties.²⁷ FTIR and ¹H-NMR spectra were in good agreement with previously reported syntheses of this azido-transfer-agent, where the azide peak of the transfer agent can clearly be distinguished in the FTIR spectrum at 2171 cm⁻¹ in Figure 1.^{28,72} **N₃-PAANG** was obtained with a yield of 34%. Furthermore, azide-functionalized **N₃-PAANG** could be confirmed by FTIR, which showed a small peak at 2110 cm⁻¹ after azido-transfer, as shown in Figure 1. This result corresponds with other reported functionalization reactions.⁷²

Figure 1: FTIR spectra of Imidazole-1-sulfonylazide (—), **PAANG** (—) and **N₃-PAANG** (—). The inset between 2400 and 1800 cm⁻¹ shows the appearance of the azide functionalization on the nanogel.

After functionalization of **N₃-PAANG** with DBCO-PEG₄-5/6-Carboxyrhodamine 110 and subsequent dialysis and freeze-drying (Scheme 1, yield 22%), a 0.25 mg/mL **Rh-DBCO-PAANG** solution was prepared and measured by DLS and UV-VIS. DLS (Figure 2A) shows a z-average diameter of 118.6 nm with a narrow size distribution. The UV-VIS absorption spectrum in Figure 2B, shows the successful conjugation of carboxyrhodamine to the nanogels. From the Lambert-Beer law it was determined that the nanogels contained 47 μ mol carboxyrhodamine per mg nanogel, which corresponds to a degree of functionalization of 0.8% of the available functional groups.

Scheme 1: Reaction scheme for azide functionalization of **PAANG** and subsequent copper-free click functionalization with carboxyrhodamine. DBCO-PEG₄-5/6-Carboxyrhodamine 110 contains a mixture of R1 and R2.

Figure 2: DLS (A) and UV-VIS (B) spectra of **Rh-DBCO-NGs**.

4. Conclusions

Functionalization of nanoparticles is essential for their application in targeted drug delivery and medical imaging. Here, zwitterionic nanogels, containing carboxylic acid as well as primary amine moieties, were functionalized via three strategies. The carboxylic acid groups present on the nanogel surface were successfully functionalized by conjugation with *N,N*-dimethylethylenediamine via *N'*-ethylcarbodiimide and *N*-hydroxysuccinimide mediated coupling, as a result the zeta potential of the

functionalized nanogels was increased. Rhodamine B and fluorescein were successfully conjugated to the primary amine groups present on the nanoparticle surface via direct isothiocyanate coupling. The primary amine groups present on the nanogel were also indirectly functionalized by transferring an azide group to the primary amine group by employing a relatively stable azide transfer agent, which is suitable for aqueous reaction conditions. The obtained azide-functional nanogels were further functionalized with dibenzocyclooctyne-functionalized carboxyrhodamine via copper-free click chemistry. All functionalizations did not influence nanogel size and stability under aqueous conditions. Therefore, functionalization of these nanogels can easily be achieved.

Acknowledgements: The funding of this work by the provinces of Overijssel and Gelderland, as well as the project consortium by the Center for Medical Imaging – North East Netherlands (CMI-NEN) is gratefully acknowledged.

5. References

- (1) Kim, B. Y. S.; Rutka, J. T.; Chan, W. C. W. *N. Engl. J. Med.* **2010**, *363* (25), 2434–2443.
- (2) Ju, C.; Mo, R.; Xue, J.; Zhang, L.; Zhao, Z.; Xue, L.; Ping, Q.; Zhang, C. *Angew. Chemie Int. Ed.* **2014**.
- (3) Farokhzad, O. C.; Langer, R. *ACS Nano* **2009**, *3* (1), 16–20.
- (4) Lee, D.-E.; Koo, H.; Sun, I.-C.; Ryu, J. H.; Kim, K.; Kwon, I. C. *Chem. Soc. Rev.* **2012**, *41* (7), 2656–2672.
- (5) Wu, W.; Mitra, N.; Yan, E. C. Y.; Zhou, S. *ACS Nano* **2010**, *4* (8), 4831–4839.
- (6) Yhee, J. Y.; Kim, S. A.; Koo, H.; Son, S.; Ryu, J. H.; Youn, I.-C. C.; Choi, K.; Kwon, I. C.; Kim, K. *Theranostics* **2012**, *2* (2), 179–189.
- (7) Zhang, J.; Yuan, Z.-F.; Wang, Y.; Chen, W.-H.; Luo, G.-F.; Cheng, S.-X.; Zhuo, R.-X.; Zhang, X.-Z. *J. Am. Chem. Soc.* **2013**, *135* (13), 5068–5073.
- (8) Kamaly, N.; Xiao, Z.; Valencia, P. M.; Radovic-Moreno, A. F.; Farokhzad, O. C. *Chem. Soc. Rev.* **2012**, *41* (7), 2971–3010.
- (9) Hrkach, J.; Von Hoff, D.; Mukkaram Ali, M.; Andrianova, E.; Auer, J.; Campbell, T.; De Witt, D.; Figa, M.; Figueiredo, M.; Horhota, A.; Low, S.; McDonnell, K.; Peeke, E.; Retnarajan, B.; Sabnis, A.; Schnipper, E.; Song, J. J.; Song, Y. H.; Summa, J.; Tompsett, D.; Troiano, G.; Van Geen Hoven, T.; Wright, J.; LoRusso, P.; Kantoff, P. W.; Bander, N. H.; Sweeney, C.; Farokhzad, O. C.; Langer, R.; Zale, S. *Sci. Transl. Med.* **2012**, *4* (128), 128ra39.
- (10) Masters, G. A.; Krilov, L.; Bailey, H. H.; Brose, M. S.; Burstein, H.; Diller, L. R.; Dizon, D. S.; Fine, H. A.; Kalemkerian, G. P.; Moasser, M.; Neuss, M. N.; O'Day, S. J.; Odenike, O.; Ryan, C. J.; Schilsky, R. L.; Schwartz, G. K.; Venook, A. P.; Wong, S. L.; Patel, J. D. *J. Clin. Oncol.* **2015**, *33* (7), 786–809.
- (11) Nochi, T.; Yuki, Y.; Takahashi, H.; Sawada, S.; Mejima, M.; Kohda, T.; Harada, N.; Kong, I. G.; Sato, A.; Kataoka, N.; Tokuhara, D.; Kurokawa, S.; Takahashi, Y.; Tsukada, H.; Kozaki, S.; Akiyoshi, K.; Kiyono, H. *Nat. Mater.* **2010**, *9* (7), 572–578.
- (12) Lux, J.; White, A. G.; Chan, M.; Anderson, C. J.; Almutairi, A. *Theranostics* **2015**, *5* (3), 277–288.
- (13) Li, Y.; Duong, H. T. T.; Laurent, S.; MacMillan, A.; Whan, R. M.; Elst, L. Vander; Muller, R. N.; Hu, J.; Lowe, A.; Boyer, C.; Davis, T. P. *Adv. Healthc. Mater.* **2015**, *4* (1), 148–156.
- (14) Krumpfer, J. W.; Schuster, T.; Klapper, M.; Müllen, K. *Nano Today* **2013**, *8* (4), 417–438.
- (15) Thanh, N. T. K.; Green, L. A. W. *Nano Today* **2010**, *5* (3), 213–230.
- (16) Elsabahy, M.; Wooley, K. L. *Chem. Soc. Rev.* **2012**, *41* (7), 2545.
- (17) Campos, L. M.; Killops, K. L.; Sakai, R.; Paulusse, J. M. J.; Damiron, D.; Drockenmuller, E.; Messmore, B. W.; Hawker, C. J. *Macromolecules* **2008**, *41* (19), 7063–7070.
- (18) Nicolas, J.; Mura, S.; Brambilla, D.; Mackiewicz, N.; Couvreur, P. *Chem. Soc. Rev.* **2013**, *42* (3), 1147–1235.
- (19) Hermanson, G. *Bioconjugate Techniques*; Elsevier: London, 2013.
- (20) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chemie Int. Ed.* **2001**, *40* (11), 2004–2021.

- (21) Iha, R. K.; Wooley, K. L.; Nyström, A. M.; Burke, D. J.; Kade, M. J.; Hawker, C. J. *Chem. Rev.* **2009**, 109 (11), 5620–5686.
- (22) Jiang, Y.; Chen, J.; Deng, C.; Suuronen, E. J.; Zhong, Z. *Biomaterials* **2014**, 35 (18), 4969–4985.
- (23) Espeel, P.; Du Prez, F. E. *Macromolecules* **2015**, 48 (1), 2–14.
- (24) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chemie Int. Ed.* **2002**, 41 (14), 2596–2599.
- (25) Cavender, C. J.; Shiner, V. J. *J. Org. Chem.* **1972**, 37 (22), 3567–3569.
- (26) Goddard-Borger, E. D.; Stick, R. V. *Org. Lett.* **2007**, 9 (19), 3797–3800.
- (27) Fischer, N.; Goddard-Borger, E. D.; Greiner, R.; Klapötke, T. M.; Skelton, B. W.; Stierstorfer, J. J. *Org. Chem.* **2012**, 77 (4), 1760–1764.
- (28) van Dongen, S. F. M.; Teeuwen, R. L. M.; Nallani, M.; Van Berkel, S. S.; Cornelissen, J. J. L. M.; Nolte, R. J. M.; van Hest, J. C. M. *Bioconjug. Chem.* **2009**, 20 (1), 20–23.
- (29) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, 126 (46), 15046–15047.
- (30) Evans, H. L.; Slade, R. L.; Carroll, L.; Smith, G.; Nguyen, Q. De; Iddon, L.; Kamaly, N.; Stöckmann, H.; Leeper, F. J.; Aboagye, E. O.; Spivey, A. C. **2012**, 48 (7), 991–993.
- (31) Lee, S.; Kang, S.-W.; Ryu, J. H.; Na, J. H.; Lee, D.-E.; Han, S. J.; Kang, C. M.; Choe, Y. S.; Lee, K. C.; Leary, J. F.; Choi, K.; Lee, K.-H.; Kim, K. *Bioconjug. Chem.* **2014**.
- (32) Xing, Y.; Zhao, J.; Conti, P. S.; Chen, K. *Theranostics* **2014**, 4 (3), 290–306.
- (33) Di Mauro, P. P.; Gómez-Vallejo, V.; Baz Maldonado, Z.; Llop Roig, J.; Borrós, S. *Bioconjug. Chem.* **2015**, 26 (3), 582–592.
- (34) Elsabahy, M.; Heo, G. S.; Lim, S.-M.; Sun, G.; Wooley, K. L. *Chem. Rev.* **2015**, 115 (19), 10967–11011.
- (35) De Leon-Rodriguez, L. M.; Lubag, A. J. M.; Malloy, C. R.; Martinez, G. V.; Gillies, R. J.; Sherry, A. D. *Acc. Chem. Res.* **2009**, 42 (7), 948–957.
- (36) Bryson, J. M.; Fichter, K. M.; Chu, W.-J.; Lee, J.-H.; Li, J.; Madsen, L. a; McLendon, P. M.; Reineke, T. M. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, 106 (40), 16913–16918.
- (37) Perez-Baena, I.; Loinaz, I.; Padro, D.; García, I.; Grande, H. J.; Odriozola, I. *J. Mater. Chem.* **2010**, 20 (33), 6916–6922.
- (38) Chan, M.; Almutairi, A. *Mater. Horiz.* **2016**, 3 (1), 21–40.
- (39) Li, Y.; Laurent, S.; Esser, L.; Elst, L. Vander; Muller, R. N.; Lowe, A. B.; Boyer, C.; Davis, T. P. *Polym. Chem.* **2014**, 5, 2592.
- (40) Ueno, T.; Nagano, T. *Nat. Methods* **2011**, 8 (8), 642–645.
- (41) Reisch, A.; Runser, A.; Arntz, Y.; Mély, Y.; Klymchenko, A. S.; Reisch, A.; Runser, A.; Arntz, Y.; Me, Y. *ACS Nano* **2015**, 9 (5), 5104–5116.
- (42) Cao, L.; Li, X.; Wang, S.; Li, S.; Li, Y.; Yang, G. *Chem. Commun. (Camb)*. **2014**, 50 (63), 8787–8790.
- (43) Ryu, J.-H.; Chacko, R. T.; Jiwanpanich, S.; Bickerton, S.; Babu, R. P.; Thayumanavan, S. *J. Am. Chem. Soc.* **2010**, 132 (48), 17227–17235.
- (44) Thorp-Greenwood, F. L.; Coogan, M. P. *Dalt. Trans.* **2011**, 40 (23), 6129.
- (45) Bertrand, N.; Wu, J.; Xu, X.; Kamaly, N.; Farokhzad, O. C. *Adv. Drug Deliv. Rev.* **2014**, 66, 2–25.

- (46) Alexis, F.; Pridgen, E.; Molnar, L. K.; Farokhzad, O. C. *Mol. Pharm.* **2008**, 5 (4), 505–515.
- (47) Nguyen, A. T.; Baggerman, J.; Paulusse, J. M. J.; van Rijn, C. J. M.; Zuilhof, H. *Langmuir* **2011**, 27 (6), 2587–2594.
- (48) Zhang, L.; Cao, Z.; Li, Y.; Ella-Menye, J.-R.; Bai, T.; Jiang, S. *ACS Nano* **2012**, 6 (8), 6681–6686.
- (49) Zhang, P.; Jain, P.; Tsao, C.; Sinclair, A.; Sun, F.; Hung, H.-C.; Bai, T.; Wu, K.; Jiang, S. J. *Control. Release* **2016**, 230, 73–78.
- (50) Sisson, A. L.; Steinhilber, D.; Rossow, T.; Welker, P.; Licha, K.; Haag, R. *Angew. Chemie Int. Ed.* **2009**, 48 (41), 7540–7545.
- (51) Steinhilber, D.; Witting, M.; Zhang, X.; Staegemann, M.; Paulus, F.; Friess, W.; Küchler, S.; Haag, R. J. *Control. Release* **2013**, 169 (3), 289–295.
- (52) Spears, B. R.; Marin, M. A.; Montenegro-Burke, J. R.; Evans, B. C.; McLean, J.; Harth, E. *Macromolecules* **2016**, 49 (6), 2022–2027.
- (53) Moore, T. L.; Rodriguez-Lorenzo, L.; Hirsch, V.; Balog, S.; Urban, D.; Jud, C.; Rothen-Rutishauser, B.; Lattuada, M.; Petri-Fink, A. *Chem. Soc. Rev.* **2015**, 44, 6287–6305.
- (54) Knop, K.; Hoogenboom, R.; Fischer, D.; Schubert, U. S. *Angew. Chemie Int. Ed.* **2010**, 49 (36), 6288–6308.
- (55) Kim, J. O.; Oberoi, H. S.; Desale, S.; Kabanov, A. V.; Bronich, T. K. J. *Drug Target.* **2013**, 21 (10), 981–993.
- (56) Zhang, X.; Malhotra, S.; Molina, M.; Haag, R. *Chem. Soc. Rev.* **2015**, 44 (7), 1948–1973.
- (57) Kabanov, A. V.; Vinogradov, S. V. *Angew. Chemie Int. Ed.* **2009**, 48 (30), 5418–5429.
- (58) Anselmo, A. C.; Zhang, M.; Kumar, S.; Vogus, D. R.; Menegatti, S.; Helgeson, M. E.; Mitragotri, S. *ACS Nano* **2015**, 9 (3), 3169–3177.
- (59) Zhang, L.; Xue, H.; Cao, Z.; Keefe, A.; Wang, J.; Jiang, S. *Biomaterials* **2011**, 32 (20), 4604–4608.
- (60) Zhao, L.; Xiao, C.; Ding, J.; He, P.; Tang, Z.; Pang, X.; Zhuang, X.; Chen, X. *Acta Biomater.* **2013**, 9 (5), 6535–6543.
- (61) Xiong, M.-H. H.; Li, Y.-J. J.; Bao, Y.; Yang, X.-Z. Z.; Hu, B.; Wang, J. *Adv. Mater.* **2012**, 24 (46), 6175–6180.
- (62) Averick, S. E.; Paredes, E.; Irastorza, A.; Shrivats, A. R.; Srinivasan, A.; Siegwart, D. J.; Magenau, A. J.; Cho, H. Y.; Hsu, E.; Averick, A. A.; Kim, J.; Liu, S.; Hollinger, J. O.; Das, S. R.; Matyjaszewski, K. *Biomacromolecules* **2012**, 13 (11), 3445–3449.
- (63) Knipe, J. M.; Strong, L. E.; Peppas, N. A. *Biomacromolecules* **2016**, 17 (3), 788–797.
- (64) Majmudar, M. D.; Yoo, J.; Keliher, E. J.; Truelove, J. J.; Iwamoto, Y.; Sena, B.; Dutta, P.; Borodovsky, A.; Fitzgerald, K.; Di Carli, M. F.; Libby, P.; Anderson, D. G.; Swirski, F. K.; Weissleder, R.; Nahrendorf, M. *Circ. Res.* **2013**, 112 (5), 755–761.
- (65) van der Aa, L. J.; Vader, P.; Storm, G.; Schiffelers, R. M.; Engbersen, J. F. J. *J. Control. Release* **2014**.
- (66) Lin, C.; Zhong, Z.; Lok, M. C.; Jiang, X.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J. *Bioconjug. Chem.* **2007**, 18 (1), 138–145.

- (67) Ekkelenkamp, A. E.; Jansman, M. M. T.; Roelofs, K.; Engbersen, J. F. J.; Paulusse, J. M. J. *Acta Biomater.* **2016**, *30*, 126–134.
- (68) He, C.; Hu, Y.; Yin, L.; Tang, C.; Yin, C. *Biomaterials* **2010**, *31* (13), 3657–3666.
- (69) Doane, T. L.; Chuang, C.-H.; Hill, R. J.; Burda, C. *Acc. Chem. Res.* **2012**, *45* (3), 317–326.
- (70) Xu, J.; Sun, L.; Li, J.; Liang, J.; Zhang, H.; Yang, W. *Nanoscale Res. Lett.* **2011**, *6* (1), 561.
- (71) D’Alpino, P. H. P.; Pereira, J. C. J. C.; Svizero, N. R. N. R.; Rueggeberg, F. A.; Pashley, D. H. J. *Dent.* **2006**, *34* (9), 623–634.
- (72) McCarthy, S. a; Davies, G.-L.; Gun’ko, Y. K. *Nat. Protoc.* **2012**, *7* (9), 1677–1693.

CHAPTER 5

Surfactant-free Preparation of Highly Stable Zwitterionic Poly(amido amine) Nanogels with Minimal Cytotoxicity

Antonie E. Ekkelenkamp, Michelle M.T. Jansman, Karin Roelofs,
Johan F.J. Engbersen and Jos M.J. Paulusse

This work has been published in *Acta Biomaterialia*, 2016, 30, 126–134

Abstract

Narrowly dispersed zwitterionic poly(amido amine) (PAA) nanogels with a diameter of approximately 100 nm were prepared by a high-yielding and surfactant-free, inverse nanoprecipitation of PAA polymers. The resulting, negatively charged, nanogels (**PAANG**) were functionalized with N,N-dimethylethylenediamine via EDC/NHS coupling chemistry. This resulted in nanogels with a positive surface charge (**DMEDA-PAANG**). Both types of nanogels were fluorescently labelled via isothiocyanate coupling. **PAANG** displays high colloidal stability both in PBS and Fetal Bovine Serum solution. Moreover, both nanogels exhibit a distinct zwitterionic swelling profile in response to pH changes. Cellular uptake of FITC-labelled nanogels with RAW 264.7, PC-3 and COS-7 cells was evaluated by fluorescence microscopy. These studies showed that nanogel surface charge greatly influences nanogel-cell interactions. The PAA polymer and **PAANG** showed minimal cell toxicity as was evaluated by MTT assays. The findings reported here demonstrate that PAA nanogels possess interesting properties for future studies in both drug delivery and imaging.

1. Introduction

Nanoparticle size and surface chemistry are of key importance to their successful application in targeted drug delivery and imaging. In biomedical applications, nanoparticles will be surrounded by complex media, containing proteins and cells.^{1,2} In this environment, the adsorption of proteins onto nanoparticles requires careful consideration, as it may lead to accelerated removal of the particle by the immune system and consequently nanoparticle accumulation in the liver and spleen.^{1,3,4} To improve the half-life and efficacy of a nanoparticle, and consequently its ability to reach the targeted site of interest, reduction of protein adsorption onto a nanoparticle's surface can be achieved by engineering the nanoparticle's surface chemistry.^{1,4,5} Since polymer nanoparticle surface chemistry, particle size, biodegradability and responsiveness are readily adapted, they have accordingly been at the center of attention in the biomedical field.^{1,6–12} Polymer nanoparticles also show tremendous variability in particle structures and show circulation properties dependent on their physiochemical properties, such as particle size, surface charge and hydrophilicity.^{2,4} Therefore, they are applicable in a wide variety of applications ranging from controlled release to targeted imaging of tissues.

A layer of polyethylene glycol (PEG) is commonly used to introduce a hydrophilic layer and to increase steric hindrance around the nanoparticle to prevent protein adsorption and thus delaying an immunological response.^{1,2} Zwitterionic polymers^{13–17} have shown extremely low protein adsorption and excellent biocompatibility.¹⁸ However, the carbon-based backbone of these zwitterionic polymers is not prone to degradation. The incorporation of degradable groups along the polymer backbone^{19,20} or coupling of oligomers via degradable linkages^{21,22} improves the polymer's biodegradation properties, but still prevents degradation of the polymers down to single monomeric units. Polymers, which resemble peptides, are interesting materials for biomedical applications, because of the gradually degradable amide motif in the polymer backbone. Furthermore, the chemical resemblance to proteins would grant unique properties to these polymers in terms of interaction with biological materials.^{23,24} From this perspective, we^{25–32} and others^{33–37} have extensively studied poly(amido amine)s (PAAs) as versatile polymers for gene and drug delivery. PAAs are water-soluble peptidomimetic polymers, which are readily obtained via a Michael-type poly-addition between bisacrylamides and bifunctional amines.³⁶ PAAs showed minimal cytotoxicity on multiple occasions: such as in gene delivery,^{25,29,34,35} drug delivery,^{38,39} imaging,⁴⁰ theranostics³³ and tissue engineering.⁴¹ Introduction of disulfide moieties in the PAA backbone renders these polymers bioreducible.³¹ As such, bioreducible PAA polyplexes have successfully been applied as carriers for plasmid DNA, siRNA and proteins, with excellent transfection efficacies.^{29–32} Regarding these properties, crosslinked PAA

nanoparticles are potentially interesting materials for application in drug delivery and molecular imaging. Furthermore, incorporation of a monomer such as ethylenediamine-*N,N'*-diacetic acid introduces zwitterionic properties to the PAA.^{42,43}

Dispersed nano-sized hydrophilic polymer networks, i.e. nanogels, are nanostructures consisting of a water-soluble matrix and are promising vehicles for the delivery of a variety of different therapeutic agents.^{44–48} Swelling and shrinkage of nanogels can be induced by changes in pH or temperature,^{3,49–51} which has been demonstrated to be a useful tool for triggered release^{8,45,52,53} and stimuli-sensitive imaging.^{54–57} Nanogels are commonly prepared by polymer crosslinking in an emulsified system.³ However, the use of surfactants and emulsifiers complicates the purification of the prepared particles. Furthermore, to prepare a stable emulsion with the desired droplet size typically requires high amounts of energy, which may damage a possible payload, for example through denaturation of proteins.^{46,58} A frequently used method to prepare solid polymeric nanoparticles is nanoprecipitation, also known as solvent displacement, where at a specific solvent/non-solvent ratio the polymer will aggregate into nanoparticles.^{59–61} Nanoprecipitation may also occur by adding an aqueous polymer solution to a water-miscible organic non-solvent. Subsequent crosslinking of the nanoprecipitated polymer leads to the formation of stable, narrowly dispersed nanogels.^{9,46} Moreover, Haag and coworkers have reported the formation of polyglycerol nanogels for protein encapsulation by inverse nanoprecipitation.^{46,62} This inverse nanoprecipitation method^{10,46,62} was chosen as a facile high-yielding method to form PAA nanoparticles without the use of surfactants.

Here we report the formation of stable nanogels composed of crosslinked zwitterionic PAAs by inverse nanoprecipitation. These zwitterionic nanogels are designed to display less non-specific interactions with proteins and cells, which is beneficial for particle's application in targeted imaging and drug delivery.² After crosslinking, the nanogel surface charge was increased by conjugation with cationic moieties. The PAA nanogels were characterized with ¹H NMR spectroscopy, dynamic light scattering, and scanning electron microscopy. Their pH-responsiveness was studied, as well as their effects on cell viability of RAW 264.7, PC-3 and COS-7 cells. Finally, cell uptake properties were studied by fluorescence microscopy.

2. Materials & methods

2.1. Materials

N,N'-methylenebisacrylamide (99%), triethylamine (≥ 99%), sodium acetate (≥ 99%), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (≥ 98%), *N*-hydroxysuccinimide, ethylenediamine (≥ 97%), hydrochloric acid - 37%, *N,N'*-dimethylethylenediamine (≥ 99%), ethylenediamine dihydrochloride (98%), sodium

hydroxide ($\geq 98\%$), 0.1 M PBS – monosodium phosphate monohydrate (3.9 mM, $\geq 99\%$) disodium phosphate dihydrate (6.1 mM, 99.5%), sodium chloride (137 mM, $\geq 99.5\%$), potassium chloride (2.7 mM, $\geq 99\%$) – Rifampicin ($\geq 97\%$) and FITC ($\geq 90\%$) were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Ethylenediamine-*N,N'*-diacetic acid ($\geq 98\%$), methanol (reagent grade), acetone (reagent grade) and DMSO (reagent grade) were purchased from VWR (Amsterdam, the Netherlands). Phosphotungstic acid hydrate (Acros), was purchased from Fisher (Landsmeer, the Netherlands). Spectra/Por® dialysis tubing with a MWCO of 1 kDa was purchased from Spectrum Labs (Breda, the Netherlands) and SnakeSkin™ dialysis tubing with a MWCO of 10 kDa was purchased from Fisher (Landsmeer, the Netherlands). MTT, RPMI medium with phenol-red, RPMI medium without phenol-red, DMEM/F-12 medium without phenol red, RPMI-1640 medium (Gibco), DMEM (Gibco) and Ham's F12K medium (Gibco), PBS, Saponin-O and Greiner 48-wells cell culture plates were purchased from Invitrogen (Bleiswijk, the Netherlands). RAW 264.7 (RAW) cells were obtained from the American Type Culture Collection (ATCC catalogue no. TIB-71), COS-7 cells were obtained from the European Catalog of Animal Cultures (ECACC Catalogue No. 87021302) and PC-3 cells were obtained from Sigma Aldrich. Milli-Q water was obtained from a Millipore Advantage A10 Ultrapure Water Purification System and holey carbon TEM grids (Electron Microscopy Sciences, mesh size: 200) were purchased from Aurion (Wageningen, the Netherlands).

2.2. General procedures

^1H NMR and ^1H COSY-NMR spectra were recorded in D_2O on a Bruker Ascend 400 spectrometer; signals of the deuterated solvent was used as a reference.

Size exclusion chromatography (SEC) measurements were performed on a Waters Alliance e2695 separation module equipped with a Waters 2998 PDA detector, a Waters 2914 RI detector and a Polymer Laboratories Aquagel-OH 30 column. A 0.4 M sodium acetate buffer (containing 30% *meOH*) was used as eluent at a flow rate of 0.7 mL/min at 35 °C. Poly(ethylene glycol) standards were used for calibration.

Determination of solution pH was carried out with a Schott CG 842 laboratory pH meter fitted with a BlueLine 18 pH glass electrode, containing a temperature probe.

DLS measurements were performed on a Malvern Zetasizer Nano ZS at a 173° scattering angle, and analyzed using the general-purpose analysis method provided in the Malvern Zetasizer software. The reference material was set to polystyrene latex and the temperature was set to 25 °C, unless stated otherwise. A standard Malvern glass cuvette was used to measure samples in acetone. The zeta potential was measured on a Malvern Zetasizer Nano ZS at 25 °C in milli-Q water, with polystyrene latex as a reference material and the $F(\kappa a)$ parameter set to 1.5, according to the Smoluchowski approximation.

Electron microscopy samples were prepared by depositing a 5 μ L droplet of a 0.1 mg/mL nanogel solution onto a 200 Mesh size holey carbon TEM grid (Electron Microscopy Sciences). Samples were stained after the nanogel solution had dried, by placing a 5 μ L droplet of a 2% (w/v) phosphotungstic acid hydrate solution (pH=7) on top of the TEM grid and removing it after 10 seconds with filter paper. SEM images were recorded on a Zeiss Merlin EQ-90 SEM.

Rifampicin concentration was determined by UV-VIS (λ = 344 nm) on an Agilent Cary 300 UV-VIS spectrophotometer.

Cell studies were performed on RAW-264.7, COS-7 and PC-3 cells, which were cultured in RPMI-1640 medium (Gibco), DMEM (Gibco) and Ham's F12K medium (Gibco), respectively. All culture media were supplemented with 10% Fetal Bovine Serum (FBS). Cell viability was determined through an MTT assay. Formazan concentration was determined using a Magellan Tecan Infinite 200 PRO plate reader at a wavelength of 540 nm. Cell cultures were imaged by an EVOS XL cell imaging system equipped with an excitation/emission filter of 470/520 nm for fluorescence imaging. Cell cultures were imaged at 10x and 40x magnifications.

2.3. Poly(amido amine) synthesis and characterization.

In a typical synthesis, *N,N'*-methylenediacrylamide (2.00 g, 13.07 mmol) and ethylenediamine-*N,N'*-diacetic acid (2.30 g, 13.07 mmol) were added to a 50 mL round bottom flask and 30 vol% methanol in milli-Q water (9.80 mL) and triethylamine (7.30 mL) were subsequently added. The mixture was stirred during 6 days at room temperature. Afterwards, the obtained polymer solution was dialyzed against pH=1 demi-water (MWCO 1 kDa). After lyophilization, the PAA polymer was isolated as a white solid (2.43 g, 56% yield, **p(MBA-EDDA)**). Finally, the molecular weight of **p(MBA-EDDA)** was determined by ^1H NMR, COSY-NMR and SEC. ^1H -NMR (D_2O) $\delta(\text{ppm})$ = 2.78 (t, 4H, 2($\text{NHCOCH}_2\text{CH}_2\text{N}$)), 3.54 (t, 4H 2($\text{NHCOCH}_2\text{CH}_2\text{N}$)), 3.65 (s, 4H, $\text{N}(\text{CH}_2)_2\text{N}$), 3.88 (s, 4H, 2(NCH_2COOH)), 4.08 (s, 2H, $\text{NCH}_2\text{CH}_2\text{NHCH}_2\text{COOH}$), 4.21 (s, 2H, $\text{NCH}_2\text{CH}_2\text{NHCH}_2\text{COOH}$), 4.59 (s, 2H, $\text{OCNHCH}_2\text{NHCO}$), 5.79 (m, 1H, OCCHCH_2), 6.22 (m, 2H, OCCHCH_2), 8.66 (t, 2H, $\text{OCNHCH}_2\text{NHCO}$). The signals at 4.08, 4.21, 5.79, 6.22 ppm were used to determine the molecular weight by end-group analysis.

2.4. Nanogel formation

For the synthesis of **PAANG**, **p(MBA-EDDA)** (210 mg), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (121 mg, 0.63 mmol) and *N*-hydroxysuccinimide (73 mg, 0.63 mmol) were dissolved in milli-Q water (70 mL) and triethylamine (350 μ L) was added and simultaneously acted as base and emulsifier. This solution was added to a mixture of acetone (700 mL) and ethylenediamine

(7.70 μL , 0.11 mmol) under vigorous stirring. After overnight stirring, acetone was removed under reduced pressure. Subsequently, the reaction was quenched with 20 mL ethylenediamine, and left stirring for 2 days. The obtained nanogel solution was dialyzed against demi-water (MWCO 10 kDa) and filtered to remove any precipitates. After lyophilization **PAANG** was obtained as a white fibrous material (188 mg, 90% yield). The obtained zwitterionic nanogels were re-suspended in milli-Q water by sonication.

2.5. Nanogel surface charge modification

Cationically modified nanogels (**DMEDA-PAANG**) were prepared by conjugation of *N,N*-dimethylethylenediamine to **PAANG**, as described in Chapter 3. Briefly, by *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (623 mg, 3.25 mmol) and *N*-hydroxysuccinimide (374 mg, 3.25 mmol) was added to a solution of **PAANG** (50 mL, 2.00 mg/mL and after 10 minutes a 2.85 M *N,N*-dimethylethylenediamine solution (2.28 mL, 6.50 mmol) was added and reacted overnight. **DMEDA-PAANG** was obtained after dialysis (MWCO 10 kDa) and lyophilization as a white fibrous material (89 mg, 89% yield), which was re-suspended in milli-Q water by sonication.

2.6. Fluorescein coupling

PAANG and **DMEDA-PAANG** were fluorescently labelled as described in Chapter 3. In short, **PAANG** and **DMEDA-PAANG** (10.9 mg and 10.8 mg, respectively) were re-suspended at 2.0 mg/mL and the pH was corrected to 10. FITC (3.9 μL , 0.50 g/mL in DMSO) was added and reacted overnight, subsequently dialyzed consecutively against 1:1 methanol/demi-water and demi-water, and lyophilized to obtain 9.7 mg **FITC-PAANG** and 9.2 mg **FITC-DMEDA-PAANG**, (89% and 85% yield, respectively). Functionalization degree was determined by UV-VIS, measured at 490 nm and the FITC concentration was calculated using its molar extinction coefficient of $\epsilon = 84,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$.⁶³

2.7. Particle size, zeta potential and stability

After lyophilization, the **PAANG** and **DMEDA-PAANG** were dissolved in milli-Q water (1.0 mg/mL) by sonication, and particle size and zeta potential were determined by DLS. Particle stability was evaluated by dissolving the nanogels at 1.0 mg/mL in 100 mM PBS (pH = 7.4). Particle size was again determined by DLS and the stability of the particles in solution was determined by DLS every 10 minutes during 24 hours at 37 °C. Hemocompatibility was assessed on a solution consisting of 500 μL nanogel solution (1.0 mg/mL in milli-Q) and 500 μL FBS. DLS measurements were carried out every 10 minutes during 24 hours at 37 °C.

2.8. pH titration

Ionic compositions of **p(MBA-EDDA)**, **PAANG** and **DMEDA-PAANG** were determined by pH titrations. The pH of **p(MBA-EDDA)**, **PAANG** and **DMEDA-PAANG** solutions (3.00 mg/mL, 20.0 mL) was adjusted to pH = 2 and all solutions were titrated with a 0.1 M sodium hydroxide solution with steps of 100 μ L. During titration the pH of the solution was continuously monitored under continuous stirring. In the meanwhile, zeta potential and particle size were measured in the pH range 2-12.

2.9. Drug loading and release

Rifampicin-loaded nanogels (**RIF-PAANG**) were prepared via co-precipitation in the presence of rifampicin during the nanogel formation process. Rifampicin (70 mg, 0.09 mmol), **P(MBA-EDDA)** (210 mg), ethylcarbodiimide hydrochloride (121 mg, 0.63 mmol), N-hydroxysuccinimide (73 mg, 0.63 mmol) and triethylamine (350 μ L) were dissolved in milli-Q water (70 mL). This solution was added to 700 mL acetone, containing ethylenediamine dihydrochloride (15 mg, 0.11 mmol) under vigorous stirring. After overnight stirring, the acetone was evaporated and the reaction was quenched with 20 g ethylenediamine dihydrochloride for 2 days.

To evaluate drug release, 10 mL milli-Q water was added to the obtained **RIF-PAANG** solution after which the solution was divided over a number of dialysis bags (MWCO 10 kDa) in portions of 4 mL. These were dialyzed in 5 L of demi-water and after 2, 4, 6, 8, 12, and 24 hours, 3 bags were removed and measured with UV-VIS to determine the rifampicin concentration. The dialysis solution was changed after 2, 4, 6, and 8 hours. In order to evaluate the influence of incorporation into the nanogels, rifampicin (70 mg, 0.09 mmol) was dissolved in 80 mL milli-Q water with 350 μ L triethylamine. This solution was dialyzed in the same manner as described above.

2.10. MTT cell viability assay and imaging

Cytotoxicities of **p(MBA-EDDA)**, **PAANG** and **DMEDA-PAANG** on RAW, COS-7 and PC-3 cells were evaluated with MTT assays. All cells were cultured in 48-well plates at 15,000 cells per well. To each well culture medium (300 μ L), supplemented with 10% FBS, was added. After 1 day the medium was aspirated and 300 μ L of a 2/3 (v/v) mixture of culture medium and PBS containing 2.5, 5.0, 10, 25, 50 or 100 μ g/mL **p(MBA-EDDA)**, **PAANG** or **DMEDA-PAANG**, was added to the wells. The mixture added to the wells for the positive and negative controls did not contain any polymer or nanogel and all experimental conditions were evaluated in triplicate. After 24 hours incubation 1% Saponin (10.0 μ L) was added to the negative controls and the cells were subsequently incubated for another 10 minutes. Then the medium was aspirated, indicator-free RPMI medium (RAW cells) or indicator-free DMEM/F-12 (COS-7 and PC-3 cells) (300 μ L, containing 0.5 mg/mL MTT) was

added to each well and the cells were incubated for 4 hours. Medium in each well was then aspirated, DMSO (300 μ L) was added to every well and the well-plates were incubated for 10 minutes, after which UV-VIS absorbance was recorded.

FITC-PAANG and **FITC-DMEDA-PAANG** were imaged by fluorescence microscopy in cell cultures: cells were plated on a 48-well plate at 15,000 cells per well in culture medium (300 μ L) – containing 10% FBS – and were incubated for 24 hours. The medium was aspirated, replaced with fresh culture medium (200 μ L) and sterile PBS and FITC-labelled **NG1** and **FITC-DMEDA-PAANG** solutions (100 μ L) were added to obtain final concentrations of 0, 10, 25 and 50 μ g/mL of **FITC-PAANG** or **FITC-DMEDA-PAANG**. All experimental conditions were performed in triplicate and the cells were incubated for 24 hours. The medium was aspirated, and culture medium (300 μ L) was added to each well and fluorescence images were recorded.

2.11. Statistical analysis

All data points, except for the measurement of the nanogel stability in FBS solution and the titration curves, are expressed as a mean $\pm$ standard deviation (SD). Statistical significance of the cytotoxicity data was determined with a one-way ANOVA with a Tukey post-hoc analysis using IBM SPSS statistics version 21.0.0.

3. Results & Discussion

3.1. Poly(amido amine) synthesis.

Zwitterionic properties were incorporated in a PAA by a Michael-type polyaddition between *N,N*-methylenebisacrylamide and ethylenediamine-*N,N'*-diacetic acid, as shown in Scheme 1. This reaction yielded a biodegradable polymer with a high zwitterionic charge density within the main polymer chain – **p(MBA-EDDA)**. After dialysis and lyophilization, the polymer was isolated with a yield of 56%. The polymer structure was confirmed by ^1H NMR spectroscopy and ^1H NMR end-group analysis and size exclusion chromatography (SEC, PEG-standards) showed that molecular weights of 4.5 and 5.2 kDa were achieved, respectively. The PDI, as determined by SEC, was 1.4 and the SEC-trace is shown in Figure S1.

3.2. Nanogel preparation.

Nanogels with functional carboxylic acid groups were prepared by inverse nanoprecipitation, as shown in Scheme 1. Therefore, an aqueous *N*-hydroxysuccinimide-activated polymer solution was added to a 10-fold volume of acetone. Immediately, the mixture turned opalescent, indicating the presence of nanoparticles. The water/acetone ratio, the amount of PAA used and the amount of triethylamine added, all influenced

Scheme 1: Synthesis of zwitterionic **p(MBA-EDDA)** polymer (A) and **PAANG** nanogel (B); schematic overview of the inverse nanoprecipitation process for the formation of **PAANG** (C).

the particle size and were therefore carefully chosen. DLS measurements showed that narrowly dispersed particles of 85 nm had formed (Figure S2). During crosslinking with ethylenediamine, the particle size of nanoprecipitated **p(MBA-EDDA)** solution increased to 95 nm. The **PAANG** solution remained stable after acetone evaporation and during quenching with ethylenediamine. After crosslinking, **PAANG** was dialyzed, lyophilized and isolated as a white solid. After re-suspension, by sonication with an ultrasonic bath at 40 °C, DLS analysis on **PAANG** revealed a similar diameter as prior to lyophilization. **PAANG** was functionalized with *N,N*-dimethylethylenediamine and subsequently dialyzed to obtain **DMEDA-PAANG**. The sizes of both **PAANG** and **DMEDA-PAANG** were approximately 100 nm after re-suspending. Table 1 shows the sizes and zeta potentials of both nanogels in milli-Q water and 100 mM PBS.

Table 1: Nanogel size and PDI measured in 0.1 M PBS (pH = 7.4) and zeta potential measured in milli-Q water.

	Milli-Q water			0.1 M PBS (pH=7.4)		
	Diameter (nm)	Polydispersity index	Zeta potential (mV)	Diameter (nm)	Polydispersity index	Zeta potential (mV)
PAANG	98.4 ± 1.1	0.08	-26.5 ± 2.5	97.8 ± 1.0	0.12	-11.6 ± 0.3
DMEDA-PAANG	95.4 ± 0.8	0.09	11.4 ± 1.2	101 ± 1.6	0.23	0.31 ± 0.6

The size distribution of **PAANG** in milli-Q water as measured by DLS and the SEM image of **PAANG** are shown in Figures 1A and 1B, respectively. The SEM image shows clusters of spherical particles with diameters ranging from ~60 nm up to ~150 nm. This is in good agreement with the DLS obtained results in Table 1, where it is taken into account that the nanogels in solution are in their swollen state, while the SEM image shows the nanogels in a dried state.

3.3. Nanogel ionic composition.

The zwitterionic **p(MBA-EDDA)** polymer contains a stoichiometric ratio of amines and carboxylic acids. During crosslinking of **PAANG**, some of the carboxylic acids are coupled to ethylenediamine to form amide bonds. In addition, conjugation of *N,N*-dimethylethylenediamine in the case of **DMEDA-PAANG**, results in conversion of carboxylic acids into amides. Therefore, the titration curves of **p(MBA-EDDA)**, **PAANG** and **DMEDA-PAANG** are expected to display a decreasing amine/carboxylic acid ratio. To confirm crosslinking and functionalization of the nanogels, the ionic compositions of both **p(MBA-EDDA)**, as well as **PAANG** and **DMEDA-PAANG** were assessed by titration with aqueous NaOH. To protonate all amine and carboxylate groups, the pH of all solutions was adjusted to 2 prior to pH titration.

The obtained titration curve of **p(MBA-EDDA)** in Figure 2 shows full deprotonation of the carboxylate groups in the pH range between 5 and 7, which required approximately 0.3 mmol of NaOH. The ammonium groups are fully deprotonated at pH = 12, requiring approximately 0.6 mmol NaOH. This confirms that the stoichiometric ratio of carboxylic acid and amine groups in **p(MBA-EDDA)**.

After crosslinking, primary amines have been introduced into the nanogel because of incomplete crosslinking reactions. **PAANG** therefore shows an increased buffering capacity below pH 5. Furthermore, due to the higher amine content more NaOH is required to bring the pH of the **PAANG** solution to pH 12. In Figure 2 it can be observed that between pH 4-7 **DMEDA-PAANG** shows an increased buffering capacity in comparison to **PAANG**. This confirms that **DMEDA-PAANG** has an increased amine content in comparison to **PAANG**. Moreover, the buffering capacity of **DMEDA-PAANG** between pH 4-7 can be attributed to the conjugated *N,N*-dimethylethylenediamine, confirming the successful conjugation. Furthermore, the buffering capacity in the **DMEDA-PAANG** titration curves below pH 4, indicates that **DMEDA-PAANG** still contains a substantial amount of carboxylic acid groups.

3.4. Nanogel pH response

Nanogel shrinkage and swelling is caused by ionic attractions and repulsions, which are dictated by the ionic composition of the nanogel. The unique rapid swelling

Figure 2: Titration curves of **p(MBA-EDDA)** (○), **PAANG** (△) and **DMEDA-PAANG** (□). For each titrant, a 20 mL (3.0 mg/mL) solution was titrated with an aqueous 0.1 M NaOH solution.

and shrinkage exhibited by nanogels are unique tools for drug loading and release, and stimuli-responsive imaging.³ DLS measurements showed that the size of **PAANG** is influenced by the pH. At pH 2 both the ammonium and carboxylic acid groups of **PAANG** are protonated, causing electrostatic repulsion between the ammonium groups, which leaves **PAANG** in a swollen state. Increasing the pH from 2-4 removes protons from the carboxylic acid groups, which induces electrostatic attraction between the carboxylate and ammonium groups. The resulting shrinkage of **PAANG** can be observed in Figure 3A. Further increase of the pH from 6-10 causes deprotonation of the ammonium groups and causes electrostatic repulsion between the carboxylates. This is observed in Figure 3A by swelling of **PAANG** at pH 6-10. Furthermore, **PAANG** also displays a zwitterionic zeta potential profile, where the zeta potential decreases from 16.6 mV to -27.3 mV upon increasing the pH. The isoelectric point of **PAANG** lies approximately at pH = 4.9, which coincides with the nanogel transition point between shrinkage and swelling. At pH = 7 the nanogel is negatively charged, which is caused by full deprotonation of all carboxylic acid groups and deprotonation of a portion of the ammonium groups. Under physiological conditions the nanogel will therefore be mostly negatively charged, and is likely to display better circulation properties than cationic nanoparticles.⁶⁴ As discussed previously, Figure 2 shows that **DMEDA-PAANG** exhibits some buffering capacity below pH 4, indicating the presence of carboxylic acid groups. Therefore, it can be concluded that, **DMEDA-PAANG** still contains carboxylic acid groups, but has a substantially higher amine-content than **PAANG**. Due to the higher amine content, the contribution of deprotonation of the carboxylic acids on nanogel shrinkage is less pronounced. The initial shrinkage in Figure 3B between pH 2-4 is smaller than observed in Figure 3A and between pH 4-8, Figure 3B shows only a small decrease in particle size caused by ammonium deprotonation. However, in this pH range the amount of ammonium groups is larger than the amount of

carboxylates. Furthermore, no swelling similar to the swelling Figure 3A is observed below pH = 8. Above pH = 8 the amount of carboxylates is larger than the amount of ammonium groups and causes swelling, as observed in Figure 3B. Likewise, the estimated isoelectric point of **DMEDA-PAANG** increases to approximately pH 7.8. The overall surface charge of the cationic nanogel will therefore be positive below physiological pH.

Figure 3: Particle diameter as measured by DLS (●) and zeta potential (□) of **PAANG** (A) and **DMEDA-PAANG** (B) as function of the pH of the solution.

3.5. Drug loading and release

Controlled release of drugs has become a major research topic in the biomedical field.⁶⁵ In order to assess the potential for drug delivery of the poly(amido amine) nanogels, these were loaded with a model drug. Rifampicin is an antibiotic, which interacts with bacterial RNA polymerase. Despite its efficacy, it suffers from instability and delivery issues and as a result of this very high doses are typically administered. Therefore efficient delivery would signify a major improvement in fighting infectious diseases.⁶⁶ Nanogels were loaded by carrying out the nanoprecipitation in the presence of rifampicin. **RIF-PAANGs** were prepared and the rifampicin content of the nanogel solution was monitored during a dialysis experiment. This revealed that approximately 20% of the initial concentration of rifampicin was contained in the nanogels after 24 hours, whereas free rifampicin was fully removed after only 12 hours of dialysis (See Figure S4).

3.6. Nanogel stability in buffer and serum.

Intravenously administered nanoparticles should remain stable under physiological conditions in the presence of high concentrations of proteins in order to prevent particle-protein aggregation. To evaluate the stability of the formed nanogels, the particle sizes of **PAANG** and **DMEDA-PAANG** were measured by DLS during 24 hours at 37 °C, both in PBS and 50% fetal bovine serum (FBS). The results are displayed in Figure 4. Particle sizes of **PAANG** and **DMEDA-PAANG** were measured in 100 mM PBS by DLS, which revealed

average mean particle diameters of $117 \text{ nm} \pm 1$ and $127 \text{ nm} \pm 3$, respectively. PDIs and photon count rates also remained stable during the stability measurement, confirming that both types of nanogels do not display significant aggregation or degradation under these conditions. To evaluate their serum stability the nanogels were also measured in a 50% FBS solution by DLS. This revealed average mean particle diameters of $110 \text{ nm} \pm 11$ and $134 \text{ nm} \pm 27$ were measured for **PAANG** and **DMEDA-PAANG**, respectively. The PDI and photon count rate of **PAANG** remained stable during the 24-hour measurement (Figure S3), which confirmed that the nanogels were not noticeably affected by the presence of proteins. In contrast, as shown in Figure 4B the particle size of **DMEDA-PAANG** fluctuated between 50 and 200 nm throughout the 24-hour measurement. Furthermore, **DMEDA-PAANG** did not show a stable PDI and photon count rate in 50% FBS. Both the PDI and photon count rate of **DMEDA-PAANG** were stable during the first 10 hours of the experiment (Figure S4). However, after approximately 10 hours the PDI started fluctuating and the photon count rate increased steadily. The latter indicates the formation of larger particles during the measurement and that the nanogels form aggregates with proteins present in the solution. Moreover, **DMEDA-PAANG** was already demonstrated to be stable in PBS. Therefore, **DMEDA-PAANG** is not as stable as **PAANG** in the presence of FBS.

Figure 4: Nanogel stability of nanogel 1 (A) and nanogel 2 (B). Stabilities were measured in 100 mM PBS ($\triangle$) and 50% FBS ($\blacksquare$)

3.7. Cell viability.

Introduction of new biomaterials requires detailed knowledge about the interaction of cells and tissues with the biomaterial. Cell toxicities of **PAANG** and **DMEDA-PAANG** were evaluated by MTT assays in murine macrophages (RAW), African green monkey kidney cells (COS-7) and human prostate cancer (PC-3) cells. These cell lines were chosen for their phagocytic (RAW) and non-phagocytic (COS-7 and PC-3) nature, since surface charge is an important factor in cellular uptake of nanoparticles. Phagocytic cell-lines have been shown to ingest negatively charged nanoparticles to a greater extent than non-phagocytic cell-lines.^{5,67}

Figures 5A, B and C show cell toxicities of **p(MBA-EDDA)**, **PAANG** and **DMEDA-PAANG** in RAW, PC-3 and COS-7 cells, respectively. **P(MBA-EDDA)** did not decrease the cell viability of PC-3 and COS-7 cells, while cell viability of RAW cells was decreased at concentrations higher than 50 µg/mL after 24 hours of incubation. Similarly, Figures 5B and C show that 24 hours of incubation with **PAANG** did only minimally affect cell viabilities (Cell viability above 80%) of PC-3 and COS-7 cells. Figure 5A shows that **PAANG** caused a decrease in RAW cell viability. In contrast, **DMEDA-PAANG** did not influence the cell viability of the RAW cells, while Figure 5B shows that incubation with **DMEDA-PAANG** caused a decrease in PC-3 cell viability at **DMEDA-PAANG** concentrations above 10 µg/mL. Furthermore, Figure 5C shows that **DMEDA-PAANG** induces reduced cell viability towards COS-7 cells to levels below 80% at all measured concentrations.

3.8. Nanogel-cell interactions.

Interactions between nanoparticles and cells depend upon several factors, such as size, lipophilicity and surface charge.^{64,67,68} It has been established that changes in a particle's zeta potential can enhance or decrease the cellular uptake of the nanoparticle.⁶⁷ Furthermore, cellular uptake and interactions with the cell membrane determine the cell toxicity of a nanoparticle. In order to evaluate cellular interactions with the developed nanogels, both **PAANG** and **DMEDA-PAANG** were labelled with FITC, exploiting the residual primary amines of the ethylenediamine crosslinker. The FITC degrees of functionalization of **PAANG** and the **DMEDA-PAANG** were 0.5% and 1.2%, respectively, as measured by UV-VIS.

To determine whether **FITC-PAANG** and **FITC-DMEDA-PAANG** interact with the previously mentioned cell types, RAW, PC-3 and COS-7 cells were incubated with the **FITC-PAANG** and **FITC-DMEDA-PAANG**. After 24 hours incubation with RAW, PC-3 or COS-7 cells, the medium was aspirated and fresh medium was added. No fluorescence was observed in the PC-3 and COS-7 cultures exposed to **FITC-PAANG**, while in RAW cultures minimal fluorescence was observed after exposure to **FITC-PAANG**. Fluorescence was observed in all cell types exposed to **DMEDA-PAANG** (Figure 6). Cells exposed to **FITC-PAANG** show no, or only minimal fluorescence in Figure 6.

Figure 5: RAW (A), PC-3 (B) and (C) COS-7 cell viability after 24 hours exposure to p(MBA-EDDA) (■), PAANG (■) and DMEDA-PAANG (■). Cell viabilities were determined relative to the respective untreated cell cultures. All samples were measured in triplicate and *, ** and *** denote significant statistical difference for $P \leq 0.05$, 0.01 and 0.001, respectively.

Figure 6: Fluorescence microscopy images of RAW, PC-3 and COS-7 cells after 24 hours of culturing under normal conditions and in the presence of **FITC-PAANG** or **FITC-DMEDA-PAANG**. Both nanogels were added to a final concentration of 25 $\mu\text{g/mL}$. Scale bars represent 100 μm .

In combination with the stability shown in Figure 4A, it can be concluded that **FITC-PAANG** shows little to no interaction with cells or serum, which limits undesired cell uptake or removal from the bloodstream. This makes **FITC-PAANG** promising as a long-circulating nanoparticle. Enhanced circulation can be beneficial to reduce particle accumulation in untarged tissues and improve targeting efficacy.^{13,69} The decreased cell viability of RAW cells exposed to high concentrations of **FITC-PAANG** (Figure 5A) suggests that **FITC-PAANG** shows some interaction with RAW cells, while Figure 6 does not show any fluorescence. Since FITC is a pH-sensitive fluorophore, it is possible that the endocytosed particles might be in a different pH-environment. In addition, it has been established that macrophages display enhanced uptake of negatively charged particles,^{64,67,68} which might also explain the increased cell toxicity in Figure 5A at 50 and 100 $\mu\text{g/mL}$. It can be concluded that **FITC-DMEDA-PAANG** at least has an interaction with cell surfaces and is likely also able to enter cells. Figure 6 shows that all cell-types exhibit fluorescence after exposure to **FITC-DMEDA-PAANG**. The fluorescence in Figure 6 indicates cell

entry or cell membrane interaction in RAW cells and PC-3 cells. The uptake of **FITC-DMEDA-PAANG** is more likely, since the removal of the cell medium should remove loosely bound particles. The amount of fluorescence in Figure 6 in COS-7 cultures exposed to **FITC-DMEDA-PAANG**, clearly indicate cellular uptake. Furthermore, the amount of fluorescence observed in Figure 6 for the PC-3 cells is small for both **FITC-PAANG** and **FITC-PAANG**, which is reflected by the cell toxicity data in Figure 5B. In contrast, the amount of fluorescence in RAW cells of **FITC-DMEDA-PAANG** is not reflected by Figure 5A in terms of cell toxicity.

4. Conclusions

Zwitterionic poly(amido amine)s (PAAs) were successfully used in the preparation of PAA nanogels with low dispersity and negative surface charge (**PAANG**) via inverse nanoprecipitation and subsequent covalent crosslinking. After purification and lyophilization, the nanogel formation process had a 90% yield. The nanogels display narrow size distributions in DLS measurements, which is further reflected in SEM images. Nanogels with a slightly positive surface charge (**DMEDA-PAANG**) were successfully prepared as well, by conjugating *N,N*-dimethylethylenediamine to carboxylic acid groups present on the nanogel. Both nanogels were functionalized with FITC by isothiocyanate coupling onto the free primary amines, enabling their visualization in cell experiments. These functionalization methods demonstrate that the nanogels contain both primary amine and carboxylic acid functional groups, which are readily accessible for further conjugation. Furthermore, it was shown that a model drug (rifampicin) could be incorporated into the nanogels by nanoprecipitation in the presence of the drug. However, further evaluation in a controlled release model is required to assess the possibilities for controlled drug delivery.

PAANG displays a distinct zwitterionic swelling profile and zeta potential in response to pH changes, arising from deprotonation of carboxylic acid and amine groups. **DMEDA-PAANG** exhibited a similar swelling profile, with a diminished influence of carboxylic acid deprotonation. The isoelectric point of nanogel **DMEDA-PAANG** increased in comparison with its negatively charged counterpart. This shows that the pH responsive properties of the nanogel can be adjusted to a pH range of interest.

Cell viability assays in RAW, PC-3 and COS-7 cells showed that nanogel interaction with cells is surface charge dependent and varies between RAW, PC-3 and COS-7 cells. However, a clear correlation between particle-cell interactions could not yet be established. The negatively charged **FITC-PAANG** showed minimal toxicity for all three cell types. Although negatively charged nanoparticles have been shown to enter (murine) macrophages by endocytosis, they still show better biodistribution results *in-vivo*, in comparison to their cationic counterparts.⁶⁴ Therefore, further research to evaluate the potential for targeted imaging and drug delivery capabilities of these nanogels is required.

Acknowledgements: The funding of this work by the provinces of Overijssel and Gelderland, as well as the project consortium by the Center for Medical Imaging – North East Netherlands (CMI-NEN) is gratefully acknowledged.

5. References

- (1) Elsabahy, M.; Wooley, K. L. *Chem. Soc. Rev.* **2012**, 41 (7), 2545.
- (2) Zhang, X.-Q.; Xu, X.; Bertrand, N.; Pridgen, E.; Swami, A.; Farokhzad, O. C. *Adv. Drug Deliv. Rev.* **2012**, 64 (13), 1363–1384.
- (3) Kabanov, A. V.; Vinogradov, S. V. *Angew. Chemie Int. Ed.* **2009**, 48 (30), 5418–5429.
- (4) Alexis, F.; Pridgen, E.; Molnar, L. K.; Farokhzad, O. C. *Mol. Pharm.* **2008**, 5 (4), 505–515.
- (5) Albanese, A.; Tang, P. S.; Chan, W. C. W. *Annu. Rev. Biomed. Eng.* **2012**, 14, 1–16.
- (6) Nicolas, J.; Mura, S.; Brambilla, D.; Mackiewicz, N.; Couvreur, P. *Chem. Soc. Rev.* **2013**, 42 (3), 1147–1235.
- (7) van der Ende, A. E.; Kravitz, E. J.; Harth, E. J. *Am. Chem. Soc.* **2008**, 130 (27), 8706–8713.
- (8) Ryu, J.-H.; Chacko, R. T.; Jiwanpanich, S.; Bickerton, S.; Babu, R. P.; Thayumanavan, S. J. *Am. Chem. Soc.* **2010**, 132 (48), 17227–17235.
- (9) Urakami, H.; Hentschel, J.; Seetho, K.; Zeng, H.; Chawla, K.; Guan, Z. *Biomacromolecules* **2013**, 14 (10), 3682–3688.
- (10) Sisson, A. L.; Steinhilber, D.; Rossow, T.; Welker, P.; Licha, K.; Haag, R. *Angew. Chemie Int. Ed.* **2009**, 48 (41), 7540–7545.
- (11) Nochi, T.; Yuki, Y.; Takahashi, H.; Sawada, S.; Mejima, M.; Kohda, T.; Harada, N.; Kong, I. G.; Sato, A.; Kataoka, N.; Tokuhara, D.; Kurokawa, S.; Takahashi, Y.; Tsukada, H.; Kozaki, S.; Akiyoshi, K.; Kiyono, H. *Nat. Mater.* **2010**, 9 (7), 572–578.
- (12) Hrkach, J.; Von Hoff, D.; Mukkaram Ali, M.; Andrianova, E.; Auer, J.; Campbell, T.; De Witt, D.; Figa, M.; Figueiredo, M.; Horhota, A.; Low, S.; McDonnell, K.; Peeke, E.; Retnarajan, B.; Sabnis, A.; Schnipper, E.; Song, J. J.; Song, Y. H.; Summa, J.; Tompsett, D.; Troiano, G.; Van Geen Hoven, T.; Wright, J.; LoRusso, P.; Kantoff, P. W.; Bander, N. H.; Sweeney, C.; Farokhzad, O. C.; Langer, R.; Zale, S. *Sci. Transl. Med.* **2012**, 4 (128), 128ra39.
- (13) Zhang, L.; Cao, Z.; Li, Y.; Ella-Menye, J.-R.; Bai, T.; Jiang, S. *ACS Nano* **2012**, 6 (8), 6681–6686.
- (14) Cheng, G.; Mi, L.; Cao, Z.; Xue, H.; Yu, Q.; Carr, L.; Jiang, S. *Langmuir* **2010**, 26 (10), 6883–6886.
- (15) Nguyen, A. T.; Baggerman, J.; Paulusse, J. M. J.; Zuilhof, H.; van Rijn, C. J. M. *Langmuir* **2012**, 28 (1), 604–610.
- (16) Nguyen, A. T.; Baggerman, J.; Paulusse, J. M. J.; van Rijn, C. J. M.; Zuilhof, H. *Langmuir* **2011**, 27 (6), 2587–2594.
- (17) Yamada, H.; Hasegawa, Y.; Imai, H.; Takayama, Y.; Sugihara, F.; Matsuda, T.; Tochio, H.; Shirakawa, M.; Sando, S.; Kimura, Y.; Toshimitsu, A.; Aoyama, Y.; Kondo, T. *J. Am. Chem. Soc.* **2015**.
- (18) Zhang, L.; Cao, Z.; Bai, T.; Carr, L.; Ella-Menye, J.-R.; Irvin, C.; Ratner, B. D.; Jiang, S. *Nat. Biotechnol.* **2013**, 31 (6), 553–556.
- (19) Hedir, G. G.; Bell, C. A.; Jeong, N. S.; Chapman, E.; Collins, I. R.; O'Reilly, R. K.; Dove, A. P. *Macromolecules* **2014**, 47 (9), 2847–2852.
- (20) Paulusse, J. M. J.; Amir, R. J.; Evans, R. A.; Hawker, C. J. J. *Am. Chem. Soc.* **2009**, 131 (28), 9805–9812.

- (21) You, Y.-Z.; Manickam, D. S.; Zhou, Q.-H.; Oupický, D. J. *Control. Release* **2007**, 122 (3), 217–225.
- (22) Whittaker, M. R.; Goh, Y.-K.; Gemici, H.; Legge, T. M.; Perrier, S.; Monteiro, M. J. *Macromolecules* **2006**, 39 (26), 9028–9034.
- (23) Sun, H.; Meng, F.; Dias, A. A.; Hendriks, M.; Feijen, J.; Zhong, Z. *Biomacromolecules* **2011**, 12 (6), 1937–1955.
- (24) Zhang, D.; Lahasky, S. H.; Guo, L.; Lee, C.-U.; Lavan, M. *Macromolecules* **2012**, 45 (15), 5833–5841.
- (25) Martello, F.; Piest, M.; Engbersen, J. F. J.; Ferruti, P.; Gonçalves, C. M. B.; Tomé, L. C.; Garcia, H.; Brandão, L.; Mendes, A. M.; Marrucho, I. M. J. *Control. release* **2012**, 164 (3), 372–379.
- (26) Piest, M.; Engbersen, J. F. J. *Control. release* **2011**, 155 (2), 331–340.
- (27) Piest, M.; Lin, C.; Mateos-Timoneda, M. a; Lok, M. C.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J. *Control. release* **2008**, 130 (1), 38–45.
- (28) Vader, P.; van der Aa, L. J.; Engbersen, J. F. J.; Storm, G.; Schiffelers, R. M. *Pharm. Res.* **2011**, 28 (5), 1013–1022.
- (29) van der Aa, L. J.; Vader, P.; Storm, G.; Schiffelers, R. M.; Engbersen, J. F. J. *Control. Release* **2014**.
- (30) Vercauteren, D.; Piest, M.; van der Aa, L. J.; Al Soraj, M.; Jones, A. T.; Engbersen, J. F. J.; De Smedt, S. C.; Braeckmans, K. *Biomaterials* **2011**, 32 (11), 3072–3084.
- (31) Lin, C.; Zhong, Z.; Lok, M. C.; Jiang, X.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J. *Bioconjug. Chem.* **2007**, 18 (1), 138–145.
- (32) Coué, G.; Engbersen, J. F. J. *Control. Release* **2011**, 152 (1), 90–98.
- (33) Chen, J.; Shi, M.; Liu, P.; Ko, A.; Zhong, W.; Liao, W.; Xing, M. M. Q. *Biomaterials* **2014**, 35 (4), 1240–1248.
- (34) Ping, Y.; Wu, D.; Kumar, J. N.; Cheng, W.; Lay, C. L.; Liu, Y. *Biomacromolecules* **2013**, 14 (6), 2083–2094.
- (35) Richardson, S. C. W.; Pattrick, N. G.; Lavignac, N.; Ferruti, P.; Duncan, R. J. *Control. Release* **2010**, 142 (1), 78–88.
- (36) Ferruti, P. J. *Polym. Sci. Part A Polym. Chem.* **2013**, 51 (11), 2319–2353.
- (37) Parmar, R. G.; Busuek, M.; Walsh, E. S.; Leander, K. R.; Howell, B. J.; Sepp-Lorenzino, L.; Kemp, E.; Crocker, L. S.; Leone, A.; Kochansky, C. J.; Carr, B. a; Garbaccio, R. M.; Colletti, S. L.; Wang, W. *Bioconjug. Chem.* **2013**, 24 (4), 640–647.
- (38) Sun, Y.; Huang, Y.; Bian, S.; Liang, J.; Fan, Y.; Zhang, X. *Colloids Surf. B. Biointerfaces* **2013**, 112, 197–203.
- (39) Sun, Y.; Zou, W.; Bian, S.; Huang, Y.; Tan, Y.; Liang, J.; Fan, Y.; Zhang, X. *Biomaterials* **2013**, 34 (28), 6818–6828.
- (40) Maggioni, D.; Fenili, F.; D'Alfonso, L.; Donghi, D.; Panigati, M.; Zanoni, I.; Marzi, R.; Manfredi, A.; Ferruti, P.; D'Alfonso, G.; Ranucci, E. *Inorg. Chem.* **2012**, 51 (23), 12776–12788.
- (41) Ferruti, P.; Bianchi, S.; Ranucci, E.; Chiellini, F.; Caruso, V. *Macromol. Biosci.* **2005**, 5 (7), 613–622.
- (42) Ranucci, E.; Ferruti, P.; Lattanzio, E.; Manfredi, A.; Rossi, M.; Mussini, P. R.; Chiellini, F.; Bartoli, C. J. *Polym. Sci. Part A Polym. Chem.* **2009**, 47 (24), 6977–6991.

- (43) Donghi, D.; Maggioni, D.; D'Alfonso, G.; Amigoni, F.; Ranucci, E.; Ferruti, P.; Manfredi, A.; Fenili, F.; Bisazza, A.; Cavalli, R. *Biomacromolecules* **2009**, *10* (12), 3273–3282.
- (44) Yim, H.; Park, S.-J.; Bae, Y. H.; Na, K. *Biomaterials* **2013**, *34* (31), 7674–7682.
- (45) Nukolova, N. V.; Oberoi, H. S.; Cohen, S. M.; Kabanov, A. V.; Bronich, T. K. *Biomaterials* **2011**, *32* (23), 5417–5426.
- (46) Steinhilber, D.; Witting, M.; Zhang, X.; Staegemann, M.; Paulus, F.; Friess, W.; Küchler, S.; Haag, R. *J. Control. Release* **2013**, *169* (3), 289–295.
- (47) Lee, H.; Mok, H.; Lee, S.; Oh, Y.-K.; Park, T. G. *J. Control. Release* **2007**, *119* (2), 245–252.
- (48) Ramos, J.; Forcada, J.; Hidalgo-Alvarez, R. *Chem. Rev.* **2014**, *114* (1), 367–428.
- (49) Oh, J. K.; Drumright, R.; Siegwart, D. J.; Matyjaszewski, K. *Prog. Polym. Sci.* **2008**, *33* (4), 448–477.
- (50) Urban, M. W.; Motornov, M.; Roiter, Y.; Tokarev, I.; Minko, S. *Prog. Polym. Sci.* **2010**, *35* (1), 174–211.
- (51) Hamidi, M.; Azadi, A.; Rafiei, P. *Adv. Drug Deliv. Rev.* **2008**, *60* (15), 1638–1649.
- (52) Stuart, M. A. C.; Huck, W. T. S.; Genzer, J.; Müller, M.; Ober, C.; Stamm, M.; Sukhorukov, G. B.; Szleifer, I.; Tsukruk, V. V.; Urban, M.; Winnik, F.; Zauscher, S.; Luzinov, I.; Minko, S. *Nat. Mater.* **2010**, *9* (2), 101–113.
- (53) McAllister, K.; Sazani, P.; Adam, M.; Cho, M. J.; Rubinstein, M.; Samulski, R. J.; DeSimone, J. M. *J. Am. Chem. Soc.* **2002**, *124* (51), 15198–15207.
- (54) Okada, S.; Mizukami, S.; Kikuchi, K. *Bioorg. Med. Chem.* **2012**, *20* (2), 769–774.
- (55) Viger, M. L.; Sankaranarayanan, J.; de Gracia Lux, C.; Chan, M.; Almutairi, A. *J. Am. Chem. Soc.* **2013**, *135* (21), 7847–7850.
- (56) Wu, W.; Shen, J.; Banerjee, P.; Zhou, S. *Biomaterials* **2010**, *31* (32), 8371–8381.
- (57) Kim, J.-H.; Lee, S.; Park, K.; Nam, H. Y.; Jang, S. Y.; Youn, I.; Kim, K.; Jeon, H.; Park, R.-W.; Kim, I.-S.; Choi, K.; Kwon, I. C. *Angew. Chemie Int. Ed.* **2007**, *46* (30), 5779–5782.
- (58) Rolland, J. P.; Maynor, B. W.; Euliss, L. E.; Exner, A. E.; Denison, G. M.; DeSimone, J. M. *J. Am. Chem. Soc.* **2005**, *127* (28), 10096–10100.
- (59) Farokhzad, O. C.; Cheng, J.; Teply, B. A.; Sherifi, I.; Jon, S.; Kantoff, P. W.; Richie, J. P.; Langer, R. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (16), 6315–6320.
- (60) Lebouille, J. G. J. L. G. J. L.; Stepanyan, R.; Slot, J. J. M. J. M.; Cohen Stuart, M. A. A.; Tuinier, R. *Colloids Surfaces A Physicochem. Eng. Asp.* **2013**, *460*, 225–235.
- (61) Lepeltier, E.; Bourgaux, C.; Couvreur, P. *Adv. Drug Deliv. Rev.* **2014**, *71*, 86–97.
- (62) Steinhilber, D.; Seiffert, S.; Heyman, J. A.; Paulus, F.; Weitz, D. A.; Haag, R. *Biomaterials* **2011**, *32* (5), 1311–1316.
- (63) Xu, J.; Sun, L.; Li, J.; Liang, J.; Zhang, H.; Yang, W. *Nanoscale Res. Lett.* **2011**, *6* (1), 561.
- (64) He, C.; Hu, Y.; Yin, L.; Tang, C.; Yin, C. *Biomaterials* **2010**, *31* (13), 3657–3666.
- (65) Duncan, R.; Vicent, M. J. *Adv. Drug Deliv. Rev.* **2013**, *65* (1), 60–70.
- (66) Dube, A.; Lemmer, Y.; Hayeshi, R.; Balogun, M.; Labuschagne, P.; Swai, H.; Kalombo, L. *Expert Opin. Drug Deliv.* **2013**, *10* (12), 1725–1734.

- (67) Fröhlich, E. *Int. J. Nanomedicine* **2012**, 7, 5577–5591.
- (68) Tomita, Y.; Rikimaru-Kaneko, A.; Hashiguchi, K.; Shirotake, S. *Immunopharmacol. Immunotoxicol.* **2011**, 33 (4), 730–737.
- (69) Sano, K.; Iwamiya, Y.; Kurosaki, T.; Ogawa, M.; Magata, Y.; Sasaki, H.; Ohshima, T.; Maeda, M.; Mukai, T. *J. Control. Release* **2014**, 194C, 310–315.

6. Supporting information

Figure S1: SEC-trace of **p(MBA-EDDA)**. Mn and PDI were determined to be 5.2 kg/mol and 1.40, respectively, relative to PEG standards.

Figure S2: DLS measured during nanoprecipitation, at the beginning of the formation (black) and at the end of the particle formation (in blue). The samples were measured in acetone.

Figure S3: Derived count rates of the DLS data of **PAANG** (△) and **DMEDA-PAANG** (■) in 50% FBS. Average diameters as measured by DLS are shown in Figure 4.

Figure S4: Release of rifampicin from **RIF-PAANG** measured over 24 hours. Release of free rifampicin was monitored as control. All datapoints were measured in triplicate. Rifampicin content was determined by measuring the UV-VIS absorption at 334 nm and was normalized to the absorption at $t = 0$ h (100%). All datapoints were corrected for volume changes resulting from the dialysis. The initial burst release from the **RIF-PAANG** is attributed to the residual amount of ethylenediamine used to quench the crosslinking reaction, which leads to an increased osmotic pressure inside the dialysis bag.

CHAPTER 6

Poly(amido amine) nanogels for prostate cancer targeting

Abstract

Prostate cancer is one of the most commonly encountered cancers in the world. The prostate-specific membrane antigen (PSMA) is overexpressed by many types of prostate cancer, which is interesting for screening and targeted drug delivery. In this work, zwitterionic poly(amido amine) nanogels (**PAANG**) is prepared and functionalized with *S,S*-2-(3-(5-amino-1-carboxypentyl)-ureido)-pentanedioic acid (ACUPA), a small PSMA targeting ligand. Functionalization of the **PAANG** was successful, without significantly influencing **PAANG** size. However, the zeta potential of the ACUPA-functionalized nanogels had increased after ACUPA functionalization, possibly by conformational changes within the nanogel. The targeting ability of ACUPA-functionalized **PAANG** is evaluated in *in-vitro* PC-3 and LNCaP cell cultures, of which only LNCaP cells express PSMA. FACS and fluorescence spectroscopy showed that ACUPA-functionalized **PAANG** was taken up to a larger extent by LNCaP as well as PC-3 cells, which was attributed to an increased zeta potential of the ACUPA-functionalized **PAANG**. Therefore, selective targeting of PSMA-positive cells was not possible with these nanogels, most likely due to the increased zeta potential after ACUPA functionalization.

1. Introduction

The combination of nanotechnology and new advanced therapeutic technologies in nanomedicine has given rise to potentially numerous therapeutic tools for future clinical applications.¹ In this respect, the use of highly specialized nanocarriers for drug delivery and diagnostic purposes occupies a pivotal role.² The versatility of polymer-based nanoparticles is furthermore reflected in the large variety of polymeric nanostructures available for nanotherapeutic applications,³ providing numerous possibilities for designing a nanoparticle's structure and chemical composition.^{4,5} Nano-sized polymeric hydrogel nanoparticles, or nanogels, are elastic nanoparticles^{6,7} with high water content,⁸ making these materials interesting for medical imaging⁷ and delivery of therapeutics.⁸⁻¹⁰ The aqueous state of nanogels grants opportunities for incorporation of chemically responsive moieties,^{11,12} which enables, for instance, triggered drug release¹³ and charge conversion.^{14,15} Moreover, nanogels with a high elasticity modulus, show extended circulation half-lives, as compared to nanogels with lower elasticity modulus,⁷ which is a favorable property for passive as well as active targeting of nanoparticles towards diseased areas.¹⁶

Nanomedicines ideally exhibit so-called 'magic bullet' characteristics, as coined by Paul Ehrlich in the 19th century, in which medicines are specifically targeted to diseases without harming the patient.¹⁷ Medical practice is starting to benefit from the progress made in the fields of targeted medicine and controlled drug delivery,^{2,18} which have conceived, for example, the introduction of antibody-drug conjugates¹⁹ and liposomal drug formulations,²⁰ respectively. Targeting of nanomedicines can be achieved by passive and active targeting. Passive targeting is achieved by controlling a nanoparticle's physical properties, such as size, surface charge and shape,^{16,21} resulting in accumulation of nanoparticles in certain tissues and cells. Size-dependent nanoparticle accumulation in cancerous and inflamed tissues is caused by the enhanced permeation and retention (EPR) effect,²¹ implying that the local tissue vasculature is more permeable, while lymphatic drainage is insufficient, or even absent. Targeting by controlling nanoparticle surface charge relies on the increased cellular uptake of cationic nanoparticles, compared to nanoparticles with a negative surface charge.^{16,22,23} This is for instance achieved by incorporation of charge-conversion functionalities onto nanoparticles.¹⁵ Additionally, nanoparticle shape, linked with size, also determines cellular uptake, where for instance elongated nanoparticles show different cellular uptake than spherical nanoparticles.¹⁶ Active targeting involves functionalization of molecules and particles with moieties, which selectively bind to specific cell types.^{16,21} Monoclonal antibodies have opened the possibility for specifically targeting epitopes, presented by diseased cells,²⁴ allowing selective targeting of specific cell types, which is especially important for cancer therapeutics.²⁵ However, monoclonal antibody-mediated targeted medicine is far from cost-effective, due to the required extensive purification and conjugation processes.^{24,26} Conjugation

of antibodies also locks in their orientation, which can markedly influence targeting efficacy.²¹ In addition, production of antibody-conjugated targeted nanomedicines could potentially far exceed the costs of antibody-drug conjugates,²⁶ which on itself already caused societal controversy. Peptide sequences,^{27–29} aptamers³⁰ and small molecules^{29,31} for cellular targeting are therefore promising alternatives in targeted nanomedicine,^{21,32} owing to the ease of their preparation and modification, compared to antibodies.^{5,26} Moreover, conjugation of these targeting moieties is generally more straightforward than antibody conjugation.²¹

Prostate cancer is one the most prevalent types of cancer among men. Globally, an estimated 1.1 million men were diagnosed with prostate cancer in 2012 and it is the fifth cause of cancer-related death worldwide.³³ The prostate-specific membrane antigen (PSMA) is a glycoprotein, which is overexpressed in many types of prostate cancer and therefore targeted in screening for prostate cancer diagnosis.^{34,35} PSMA has been demonstrated to be a promising target for prostate cancer treatment and PSMA-targeted nanomedicines have been reported with antibodies,³⁶ aptamers^{37–39} and small molecules.^{35,40–43} A particularly attractive targeting ligand is *S,S*-2-(3-(5-amino-1-carboxypentyl)-ureido)-pentanedioic acid (ACUPA), a small molecule that inhibits PSMA's enzymatic activity towards *N*-acetyl-*L*-aspartyl-*L*-glutamate.⁴² ACUPA is easily conjugated onto polymer nanospheres for targeted drug delivery,^{40,41} which are currently under investigation in clinical trials.^{1,40} To our knowledge, nanogels for small-molecule prostate cancer targeting have not been investigated. Nanogels offer a variety of possibilities for specific design¹¹ and also have shown good retention within the blood circulation, which are interesting properties for targeted imaging and drug delivery.^{6,7}

In Chapter 4, we described the formation of zwitterionic poly(amido amine) nanogels.⁴⁴ Zwitterionic poly(amido amine) nanogels were shown to be highly stable under physiological conditions and showed minimal interactions when exposed to three different cell types.⁴⁴ The minimal interactions between these nanogels and biological molecules and cells, make these particular nanogels promising candidates for specific targeting, in order to detect and treat diseased tissues. In this work, a *tert*-butyl ester protected ACUPA (t-Bu-ACUPA) with a primary amine functionality, was conjugated to zwitterionic poly(amido amine) nanogels, followed by *tert*-butyl deprotection of t-Bu-ACUPA (Scheme 1), to target PSMA-expressing LNCaP cells. The degree of functionalization with targeting ligand was determined by ¹H NMR spectroscopy, in combination with an estimation of nanogel molecular weight via static light scattering measurements. Rhodamine B was conjugated onto the nanogels for visualization purposes (Scheme 1). To evaluate the targeting capabilities of the nanogel, non-PSMA expressing PC-3 cells and PSMA-expressing LNCaP cells were incubated with ACUPA-functionalized nanogels and analyzed with fluorescence microscopy and spectroscopy, and fluorescence-activated cell sorting (FACS).

Scheme 1: Reaction scheme for nanogel functionalization, where first *tert*-butylester-protected ACUPA is coupled to the bare nanogel via EDC/NHS coupling and after *tert*-butyl deprotection, a rhodamine label is added to the ACUPA-functionalized nanogel.

2. Materials and methods

2.1. Materials

N,N'-methylenebisacrylamide (99%), triethylamine ($\geq 99\%$), sodium acetate ($\geq 99\%$), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride ($\geq 98\%$), *N*-hydroxysuccinimide, ethylenediamine ($\geq 97\%$), hydrochloric acid - 37%, *N,N'*-dimethylethylenediamine ($\geq 99\%$), ethylenediamine dihydrochloride (98%), sodium hydroxide ($\geq 98\%$), 0.1 M PBS – monosodium phosphate monohydrate (3.9 mM, $\geq 99\%$) disodium phosphate dihydrate (6.1 mM, 99.5%), sodium chloride (137 mM, $\geq 99.5\%$), potassium chloride (2.7 mM, $\geq 99\%$), lysis buffer – Triton X-100 (1% w/v), sodium chloride (150 mM, $\geq 99.5\%$) and tris(hydroxymethyl)aminomethane (50 mM, pH 8, $\geq 99\%$) – and rhodamine B isothiocyanate (mixed isomers) and fluorescein isothiocyanate ($\geq 90\%$) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Ethylenediamine-*N,N'*-diacetic acid ($\geq 98\%$), methanol (reagent grade), acetone (reagent grade), toluene (reagent grade) and DMSO (reagent grade) were purchased from VWR (Amsterdam, the Netherlands). A *tert*-butyl ester protected PSMA inhibitor – di-*tert*-butyl (((*S*)-6-amino-1-(*tert*-butoxy)-1-oxohexan-2-yl) carbamoyl)-*L*-Glutamate – (99.8%) was synthesized and kindly provided by SYNCOM (Groningen, the Netherlands). Spectra/Por® dialysis tubing with a MWCO of 1 kDa was purchased from Spectrum Labs (Breda, the Netherlands) and SnakeSkin™ dialysis tubing with a MWCO of 10 kDa was purchased from Fisher (Landsmeer, the

Netherlands). MTT, RPMI medium without phenol-red, DMEM/F-12 medium without phenol red, RPMI-1640 medium (Gibco), DMEM (Gibco) and Ham's F12K medium (Gibco), PBS, Saponin-O and Greiner 48-wells cell culture plates were purchased from Invitrogen (Bleiswijk, the Netherlands). LNCaP Cells were obtained from the American Type Culture Collection (ATCC catalogue no. CRL-1740) and PC-3 cells were obtained from Sigma-Aldrich (Zwijndrecht). Milli-Q water was obtained from a Millipore Advantage A10 Ultrapure Water Purification System.

2.2. General procedures

^1H NMR and ^1H COSY-NMR spectra were recorded in D_2O on a Bruker Ascend 400 spectrometer; signals of the deuterated solvent were used as a reference. To determine the concentration of conjugated PSMA inhibitor, a small glass capillary was filled with CDCl_3 , sealed and added to an NMR tube. The resulting signal arising from residual CHCl_3 was used as an internal NMR standard. The standard was calibrated with 800 μL of a 10 mM *tert*-butylamine solution in D_2O in an NMR tube.

Size exclusion chromatography (SEC) measurements were performed on a Waters Alliance e2695 separation module equipped with a Waters 2998 PDA detector, a Waters 2914 RI detector and a Polymer Laboratories Aquagel-OH 30 column. A 0.4 M sodium acetate buffer (containing 30% meOH) was used as eluent at a flow rate of 0.7 mL/min at 35 $^\circ\text{C}$. Poly(ethylene glycol) standards were used for calibration.

Dynamic light scattering (DLS) and zeta potential measurements were performed on a Malvern Zetasizer Nano ZS. Particle size was measured at a 173° scattering angle, and analyzed using the general-purpose analysis method provided in the Malvern Zetasizer software. Zeta potential was measured in milli-Q water and the $F(\kappa a)$ parameter set to 1.5, according to the Smoluchowski approximation.⁴⁵ For both DLS and zeta potential measurements, the reference material was set to polystyrene latex and the temperature was set to 25 $^\circ\text{C}$, unless stated otherwise.

Static light scattering measurements were carried out on a Malvern Zetasizer Nano ZS. All samples were measured in a glass cuvette and toluene was used as a scattering standard. The concentration dependent refractive index (dn/dC) was determined by measuring the refractive index of nanogel solutions at several concentrations with a Krüss Optronic DR6100T digital refractometer.

Nano tracking analysis was performed at room temperature on a NanoSight NS500 equipped with an NS500 G532nm Green Laser Module. Samples were dissolved in milli-Q water at a concentration of 1 $\mu\text{g}/\text{mL}$, injected into the sample chamber and measured for 160 s in triplicate. Nanoparticle tracks were analyzed with the accompanied NanoSight NTA software (version 2.3).

Cell studies were performed on LNCaP and PC-3 cells, which were cultured

in RPMI-1640 medium (Gibco) and Ham's F12K medium (Gibco), respectively. Both culture media were supplemented with 10% Fetal Bovine Serum (FBS). Cell viabilities were determined through MTT assays. Formazan concentration was determined using a Magellan Tecan Infinite 200 PRO plate reader at a wavelength of 540 nm. Cell cultures were imaged by an EVOS XL cell imaging system equipped with an excitation/emission filter of 531/593 nm for fluorescence imaging. Cell cultures were imaged at 10x and 40x magnifications. Fluorescence-activated cell sorting (FACS) analysis was performed using a Becton Dickinson FACSCalibur, with a BD FACS Rinse solution as running buffer.

2.3. Poly(amido amine) synthesis and characterization

A zwitterionic poly(amido amine) was prepared as described Chapter 2.⁴⁴ Briefly, *N,N'*-methylenebisacrylamide (1.00 g, 6.49 mmol) and ethylenediamine-*N,N'*-diacetic acid (1.14 g, 6.49 mmol) and triethylamine (3.6 mL, 25.96 mmol) were dissolved in a 30 vol% methanol solution (3.6 mL) and polymerized for 7 days at room temperature. The polymer mixture was subsequently dialyzed (MWCO 1 kDa) against milli-Q water with a pH of 1 and finally lyophilized to obtain the polymer as a white solid (580 mg, 27% yield, **p(MBA-EDDA)**). Finally, the molecular weight of **p(MBA-EDDA)** was determined by ¹H NMR and SEC. ¹H NMR (D₂O) δ(ppm) = 2.77 (t, 4H, 2(NHCOCH₂CH₂N)), 3.53 (t, 4H, 2(NHCOCH₂CH₂N)), 3.64 (s, 4H, N(CH₂)₂N), 3.87 (s, 4H, 2(NCH₂COOH)), 4.07 (s, 2H, NCH₂CH₂NHCH₂COOH), 4.21 (s, 2H, NCH₂CH₂NHCH₂COOH), 4.58 (s, 2H, OCNHCH₂NHCO), 5.78 (m, 1H, OCCHCH₂), 6.21 (m, 2H, OCCHCH₂), 8.66 (t, 2H, OCNHCH₂NHCO). The signals at 4.07, 4.21, 5.78, 6.21 ppm were used to determine the molecular weight by end-group analysis (5 kDa).

2.4. Nanogel formation

Nanogels were prepared by dissolving **p(MBA-EDDA)** (210 mg), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (121 mg, 0.63 mmol) and *N*-hydroxysuccinimide (73 mg, 0.63 mmol) in milli-Q water (70 mL) and triethylamine (350 μL) was added. This solution was added under vigorous stirring to acetone (700 mL), which contained a small amount ethylenediamine (7.70 μL, 0.11 mmol) for crosslinking. After overnight stirring, acetone was removed under reduced pressure and the reaction mixture was subsequently placed on ice and the crosslinking reaction was quenched with 5 mL ethylenediamine, and left stirring overnight. The obtained nanogel solution was dialyzed against milli-Q water (MWCO 10 kDa) and filtered to remove any precipitates. After lyophilization **PAANG** was obtained as a white fibrous material (205 mg, 96% yield).

2.5. Nanogel characterization

The obtained nanogels were re-dissolved in milli-Q water and their size was determined by DLS as well as NTA. The molecular weight of the nanogels was determined by static light scattering. To determine the concentration dependent refractive index (dn/dc) of the prepared nanogels, **PAANG** was dissolved in milli-Q water at 10 mg/mL, from which solutions were prepared containing 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mg/mL **PAANG**. The refractive indices of these solutions were measured and plotted against the sample concentration in g/mL, to obtain the dn/dc value.

2.6. PSMA-ligand conjugation

Nanogels were functionalized with ACUPA and rhodamine as shown in Scheme 1. **PAANG** (50 mg) was dissolved 10 mL in milli-Q water, aided by sonication at 40 °C for 10 minutes. The amount of carboxylic acid groups was estimated at 0.30 mmol. Then N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (58 mg, 0.30 mmol) and N-hydroxysuccinimide (35 mg, 0.30 mmol) were added and 5 minutes later the protected PSMA ligand – di-*tert*-butyl (((S)-6-amino-1-(*tert*-butoxy)-1-oxohexan-2-yl)carbamoyl)-L-Glutamate – was added (7.3 mg, 15 μ mol). After overnight stirring, the obtained solution was dialyzed (MWCO 10,000 Da) against milli-Q water and subsequently lyophilized. **t-Bu-ACUPA-PAANG** was obtained as a white solid (40 mg, 80% yield) of which 29 mg was dissolved in 1.4 mL D₂O and the concentration of *tert*-butyl esters was determined via ¹H NMR spectroscopy employing a CDCl₃ external standard. The concentration of conjugated ACUPA ligand was determined to be 0.06 μ mol per mg of nanogel. The *tert*-butanol protecting group was removed from the ligand by dissolving 29 mg **t-Bu-ACUPA-PAANG** in D₂O at 20 mg/mL and 2 mL trifluoroacetic acid was added. The deprotection was monitored by ¹H NMR spectroscopy, by observing the disappearance of the *tert*-butyl peak at 1.4 ppm. After 3 hours the reaction the *tert*-butyl peak fully disappeared and the mixture was added to a dialysis tube (MWCO 10,000 Da) and dialyzed for 1 day against milli-Q water and lyophilized to obtain **ACUPA-PAANG** as a white powder (22 mg, 76% yield).

2.7. Fluorescent labelling

PAANG and **ACUPA-PAANG** were dissolved (25 mg and 19 mg, respectively) at 5 mg/mL in milli-Q and rhodamine B isothiocyanate (0.3 μ mol per mg of nanogel) was added from a stock solution (0.5 g/mL in DMSO). Both solutions were stirred overnight in the dark and subsequently dialyzed in the dark (MWCO 10,000 Da) against milli-Q water. The nanogel solutions were retrieved and subsequently lyophilized. **ACUPA-Rh-PAANG** (17.7 mg, 93% yield) and **Rh-PAANG** (10.8 mg, 43% yield) were retrieved as a purple fibrous material. The rhodamine functionalization degree was determined by UV-VIS spectrophotometry via the Lambert-Beer law with a extinction coefficient of $\epsilon = 107,000 \text{ M}^{-1} \text{ cm}^{-1}$.⁴⁶

2.8. Cell viability and cell uptake

Fluorescent uptake of **Rh-PAANG** and **ACUPA-Rh-PAANG** was quantified by seeding 10,000 PC-3 and LNCaP cells per well onto a 48 well-plates and cultured for 48 hours in 300 μ L culture medium per well. The medium was then aspirated and 200 μ L of growth medium was added to each well and supplemented with 100 μ L PBS, containing 0, 7.5, 15, 30, 75, 150 or 300 μ g/mL **Rh-PAANG** or **ACUPA-Rh-PAANG**. After 4 hours of exposure, all wells were aspirated and 300 μ L culture medium was added. After recording fluorescence microscopy images, the medium was aspirated again and replaced with 300 μ L lysis buffer, after which fluorescence was measured with a Magellan Tecan Infinite 200 PRO plate reader at excitation/emission wavelengths of 540/625 nm.

Flow cytometry and an MTT viability assay were performed by plating LNCaP and PC-3 cells onto 48 wells plates at a seeding density of 20,000 cells per well. Due to the low adherence of LNCaP cells to tissue culturing plates, the well-plates for the LNCaP cells were coated with fibronectin to improve LNCaP cell adhesion. Prior to plating, 150 μ L of an 8 μ g/mL fibronectin solution (in sterile Milli-Q water) was added to each well, as described by Liberio et al.⁴⁷ To assess the cell viability LNCaP and PC-3 cells were cultured for 48 and 24 hours in 300 μ L culture medium, respectively. The medium was then aspirated and 200 μ L of growth medium was added to each well and supplemented with 100 μ L PBS, containing 0, 7.5, 15, 30, 75, 150 or 300 μ g/mL **Rh-PAANG** or **ACUPA-Rh-PAANG**. After 4h hours incubation, the medium was removed, replaced with growth medium and incubated overnight. The next day, 50 μ L of a 4% Triton X-100 solution was added to the negative controls and the plates were incubated for 3 minutes. Then the medium was replaced by indicator-free growth medium, containing 0.5 mg/mL MTT and the plates were incubated for 4 hours. Finally, the medium was removed and 300 μ L of DMSO was added and the UV-VIS absorbance was measured at 500 nm.

For FACS analysis LNCaP and PC-3 cells were plated into 48-well plates and cultured for 48 hours in growth medium. The medium was then aspirated and 200 μ L of growth medium was added to each well and supplemented with 100 μ L PBS, containing 0, 7.5, 15, or 150 μ g/mL **Rh-PAANG** or **ACUPA-Rh-PAANG**. After 4 hours of incubation, medium was removed, the cells were then trypsinized, transferred to BD Falcon tubes and subsequently centrifuged at 600 rcf for 5 minutes. The supernatant was removed and the cells were fixated with 100 μ L of 3.7% formaldehyde (in PBS) for 30 minutes at 4 °C and further diluted with 100 μ L PBS. The cells were then centrifuged again and re-suspended in 50 μ L PBS. FACS analysis was performed by using the RFP channel (585/542 nm) for the rhodamine signal.

3. Results and discussion

3.1. Nanogel formation and functionalization

Zwitterionic poly(amido amine) polymers were prepared via an aza-Michael addition, yielding **p(MBA-EDDA)** polymer (yield, 27%) with an estimated molecular weight of 5 kDa based on ^1H NMR spectroscopy end-group analysis. SEC analysis revealed an M_n of 8 kDa with a PDI of 1.3. The obtained ^1H NMR spectroscopy and SEC results were in agreement with the results in Chapter 2 and 4.⁴⁴ Poly(amido amine) nanogels with functional primary amine and carboxylic acid groups were prepared via inverse nanoprecipitation of **p(MBA-EDDA)** and subsequent EDC/NHS mediated crosslinking with ethylenediamine. Zwitterionic Poly(amido amine) were obtained after dialysis and lyophilization in a high yield (96%).

3.2. Nanogel characterization

3.2.1. Nanogel size and molecular weight

PAANG particle size and size distributions were analyzed by NTA and DLS. NTA analysis results are depicted in Figure 1A, showing a size distribution with a mean size of 160 ± 9 nm with a standard deviation of 74 ± 4 nm. DLS measurements (Figure 1B) on the other hand showed a monodisperse size distribution with a mean diameter of 119 ± 2 nm. Both of these results show that monodisperse nanogels were formed with a similar size distribution, as shown in Figure 1A and B. The molecular weight of **PAANG** was determined by SLS, by extrapolation of the Debye plot (Figure 1C) to zero concentration, $1/M_w$ is obtained. For further detail on SLS calculations for molecular weight determination, please refer to ref. ⁴⁸. SLS measurements requires the determination of the concentration dependent refractive index (dn/dc). This was achieved by measuring the refractive index of several solutions with varying **PAANG** concentrations. Plotting the nanogel concentration against the measured refractive index resulted in a determined dn/dc of 0.294 mL/g. SLS measurements of several **PAANG** dilutions in milli-Q water (for the Debye plot, see Figure 1C). Extrapolation of the Debye plot to zero concentration and application revealed an estimated molecular weight of 5 MDa.⁴⁸

Figure 1: **PAANG** particle size distributions as measured by NTA (A) and DLS (B), and SLS Debye plot (C) for molecular weight estimation.

From the NTA results in Figure 1A, the number of particles in a sample and thus their molecular weight can also be estimated. These results showed that a 1 µg/mL nanogel sample contained $3 \cdot 10^8$ nanoparticles, resulting in an estimated molecular weight of 2 GDa, which is, compared to the SLS data, an enormous overestimation. Most likely, this is a result of the possibility that not all particles were included due to low scattering or interference with the scatter of other particles.⁴⁹

3.2.2. Nanogel functionalization degree

The availability of specific chemical moieties on nanoparticles is important for further nanoparticle functionalization with targeting ligands for selective targeting. Especially the possibility to attach multiple ligands to a single nanoparticle has shown to improve nanoparticle targeting.^{50,51} However, high ligand densities can lead to decreased blood circulation times and reduced targeting capacity, which is attributed to altered physical properties, such as increased particle hydrophobicity and receptor saturation.²¹ Therefore, the amount of conjugated targeting ligands needs to be controlled during the functionalization process.

PAANG was functionalized with ACUPA and rhodamine B isothiocyanate for the evaluation of their cell targeting properties. Functionalization with di-*tert*-butyl (((*S*)-6-amino-1-(*tert*-butoxy)-1-oxohexan-2-yl)carbamoyl)-*L*-glutamate – a *tert*-butyl protected version of ACUPA – was conjugated to **PAANG** by EDC/NHS mediated coupling. ¹H NMR spectroscopic analysis with a CHCl₃ external standard showed that during the conjugation reaction, 20% of the added ACUPA was conjugated to the nanogels. Furthermore, from the ¹H NMR spectrum, containing the CHCl₃ standard, it was calculated that the sample contained 60 nmol per mg **t-Bu-ACUPA-PAANG**. Equation 1 shows the calculation to obtain the number of ACUPA units per **PAANG**, where N_A , M_w and $[ACUPA]$ are Avogadro's number, the molecular weight of **PAANG** (in g/mol) and the ACUPA concentration (in mol/g), respectively. This results in around 300 ACUPA units per nanoparticle and a functionalization degree of 0.9%, when taking into account the estimate **PAANG** molecular weight as determined by SLS (5 MDa), which is comparable to other reported ACUPA-functionalized nanoparticles.⁴⁰

$$ACUPA \text{ per } PAANG = \frac{ACUPA \text{ per } g}{PAANG \text{ per } g} = \frac{N_A \cdot ACUPA}{N_A / M_w} = [ACUPA] \cdot M_w \quad (\text{Equation 1})$$

After conjugation, the **t-Bu-ACUPA-PAANG** was deprotected under acidic conditions, by adding trifluoroacetic acid, and subsequently functionalized with rhodamine B isothiocyanate in tandem with non-functionalized **PAANG** for visualization with fluorescence microscopy. The obtained **Rh-ACUPA-PAANG** and **Rh-PAANG** were

characterized with DLS and UV-VIS spectroscopy. These characterizations showed that particle size distributions were not affected by rhodamine B or ACUPA conjugation, nor by the deprotection of the *tert*-butyl ester, as shown in Table 1 and Figure 2A. However, the zeta potential of **ACUPA-Rh-PAANG** compared to **Rh-PAANG** (-5.7 and -11.0 mV, respectively) obtained after ACUPA conjugation and deprotection became less negative, as shown in Table 1. One explanation for the increased zeta potential might be a conformational change within the nanogel, burying carboxylic acids groups into the nanogel matrix during *tert*-butyl ester deprotection under acidic conditions. Figure 2B shows that UV-VIS spectra of **Rh-PAANG** and **ACUPA-Rh-PAANG** are overlapping and via the Lambert-Beer law it was calculated that **Rh-PAANG** and **ACUPA-Rh-PAANG** contained 22 and 23 nmol/mg rhodamine, respectively. This is in agreement with an estimated 100 rhodamine B units per nanogel and a degree of functionalization of 0.4% for both functionalized nanogels.

Table 1: Z-average diameter, particle polydispersity index and zeta potential as measured in PBS.

	Z-average diameter (nm)	Polydispersity index	Zeta potential (mV)
PAANG	130.2 ± 0.9	0.06	-13.2 ± 1.2
Rh-PAANG	134.2 ± 6.8	0.24	-11.0 ± 0.6
ACUPA-Rh-PAANG	133.7 ± 4.2	0.19	-5.7 ± 0.7

Figure 2: DLS particle size distribution (A) and UV-VIS spectra (B) of 0.3 mg/mL **Rh-PAANG** (—) and **ACUPA-Rh-PAANG** (—) solutions in PBS.

3.3. Cell viability

Application of nanomaterials in biomedical applications requires careful evaluation of their interaction with biological systems and evaluation of their potential toxicity. Therefore, we evaluated the toxicity of the prepared nanogels (**Rh-PAANG** and **ACUPA-Rh-PAANG**) in human PC-3 and LNCaP cell-lines, of which only LNCaP cells express PSMA, which is targeted by ACUPA. No cell toxicity of **Rh-PAANG** in both cell lines was detected at all

tested concentrations (Figure 3A), which is in agreement the results in Chapter 4.⁴⁴ However, after exposure to **ACUPA-Rh-PAANG** PC-3 cells showed a decreased cell viability at concentrations above 5 $\mu\text{g/mL}$, as observed in Figure 3A. The increased **ACUPA-Rh-PAANG**-induced cell toxicity can be explained by its higher zeta potential (Table 1),^{16,23} which was demonstrated with *N,N*-dimethylethylenediamine-conjugated **PAANG** in Chapter 4.⁴⁴ Evaluation of the **Rh-PAANG** and **ACUPA-Rh-PAANG**-induced toxicity in LNCaP cell resulted in a high variation in cell toxicity values, which at almost all concentrations showed rather large standard deviations (see Figure 3B). The high variation in the obtained data is most likely the result of poor cell attachment of the LNCaP cells,⁴⁷ which results in cell detachment during medium replacement. In spite of the employed fibronectin coating, cells still detached from the culture plate during cell medium replacement, resulting in fewer available cells for analysis.

Figure 3: PC-3 (A) and LNCaP (B) cell viability after 4 hours of exposure to **Rh-PAANG** (■) and **ACUPA-Rh-PAANG** (■).

3.4. Cell uptake

Specific cellular uptake is an important property for the application of targeted medicine. To assess the specific targeting ability of the **ACUPA-Rh-PAANG**, the cell uptake of **ACUPA-Rh-PAANG** was compared to **Rh-PAANG**. For this purpose, the cellular uptake was investigated by fluorescence microscopy and fluorescence spectrometry in PC-3 and LNCaP cells after 4 hours of exposure to **Rh-PAANG** and **ACUPA-Rh-PAANG**, where only LNCaP cells express the antigen targeted by ACUPA. Figure 4 shows the obtained fluorescence microscopy images. These images reveal that **Rh-PAANG** are taken up by PC-3 as well as LNCaP cells, and that the amount of uptake is increased at a **Rh-PAANG** concentration of 100 $\mu\text{g/mL}$, as compared to 2.5 $\mu\text{g/mL}$. LNCaP cells exposed to **ACUPA-Rh-PAANG** showed higher fluorescence intensity in the microscopy images in Figure 4, compared to **Rh-PAANG** for PC-3 as well as LNCaP cells. LNCaP cells exposed to **ACUPA-Rh-PAANG**,

Figure 4: Fluorescence microscopy images of PC-3 and LNCaP cells after 4 hours of exposure to Rh-PAANG and ACUPA-Rh-PAANG. Scale bars represent 100 μm .

however, showed brighter fluorescence than PC-3 cells. Moreover, LNCaP cells exposed to **Rh-PAANG** and **ACUPA-Rh-PAANG** showed a larger increase in fluorescence intensity upon increasing nanogel concentration, compared to PC-3 cells.

Although qualitative evaluation of Figure 4 appears to indicate higher fluorescence intensity for LNCaP cells after exposure to **ACUPA-Rh-PAANG**, fluorescence spectroscopy measurements in Figure 5 show that both PC-3, as well as LNCaP cells display an increased uptake of **ACUPA-Rh-PAANG**. The fluorescence intensity in LNCaP cells in Figure 5B was lower than the fluorescence intensity in PC-3 cells (Figure 5A). This is, similar to the cell viability results in Figure 3B, possibly a result of poor LNCaP cell adherence to the culture plates, which could lead to removal of cells during medium replacement and washing steps.

Figure 5: Fluorescence uptake PC-3(A) and LNCaP cells (B) of **Rh-PAANG** (■) and **ACUPA-Rh-PAANG** (■) after 4-hour exposure.

To further quantitatively evaluate the targeting capabilities of **ACUPA-Rh-PAANG**, PC-3 and LNCaP cells were exposed to **Rh-PAANG** and **ACUPA-Rh-PAANG**. After 4 hours of incubation fluorescence images were taken and flow cytometry measurements were carried out. Minimal **Rh-PAANG** uptake is observed in the histograms depicted in Figure 6 at concentrations of 2.5 and 10 μg/mL in PC-3 and LNCaP cells, respectively. Moreover, PC-3 cells exposed to **Rh-PAANG** at a concentration of 50 μg/mL, shows increased fluorescence in the evaluated population. This is however not observed in the LNCaP cell populations. Evaluation of the obtained histograms in Figure 6 also shows that **Rh-PAANG** are taken up at a lesser extent than **ACUPA-Rh-PAANG** by PC-3 and LNCaP cells at all concentrations. This is most striking at the highest employed nanogel concentrations and is observed in both evaluated cell lines. Furthermore, this is also confirmed by statistical analysis of the measured cell populations. The mean fluorescence of the evaluated cell populations is elevated when comparing PC-3 and LNCaP cells exposed to **ACUPA-Rh-PAANG** to cells exposed to

Figure 6: Histograms obtained from the FACS RFP channel for PC-3 and LNCaP cells exposed to 2.5, 10 and 50 mg **Rh-PAANG** (—) and **ACUPA-Rh-PAANG** (—), and unexposed LNCaP cells (—), which were taken as a negative control.

Rh-PAANG, as depicted in Figure 7A and C. Furthermore, the percentage of positive cells in both PC-3 and LNCaP cell populations exposed to **ACUPA-Rh-PAANG** in Figure 7B and D, respectively, is larger than for cells exposed to **Rh-PAANG**. Moreover, Figure 7B shows that the number of positive cells in **ACUPA-Rh-PAANG** PC-3 cells is much larger than the amount of positive LNCaP cells exposed to **ACUPA-Rh-PAANG** (Figure 7D).

However, due to poor LNCaP cell attachment, cells tended to detach in clusters after trypsinization, which are not taken into account in FACS. This led to a low number of cells available for FACS, making the FACS results for LNCaP cells less reliable. Figure 6 shows that PC-3 cells, exposed to **ACUPA-Rh-PAANG**, display more fluorescence than PC-3 cells exposed to **Rh-PAANG**. Figure 7B shows that almost the entire cell population of PC-3 cells exposed to **ACUPA-Rh-PAANG** exhibits fluorescence, as opposed to 20% fluorescence-positive PC-3 cells after exposure to **Rh-PAANG**. These results show, combined with the results in Figure 5 and 6, that **ACUPA-Rh-PAANG** uptake in PC-3 cells is increased and thus show non-specific uptake of **ACUPA-Rh-PAANG**. Therefore, it is not possible to draw a conclusion on the PSMA-targeting ability of **ACUPA-Rh-PAANG**. An explanation for the increased uptake of **ACUPA-Rh-PAANG**, compared to **Rh-PAANG**, could be its increased zeta potential, which is known to enhance cellular uptake,²² as indicated in Table 1. Furthermore, the difficulties encountered during LNCaP cell culturing also hindered accurate analysis of the analyzed cell populations.

Figure 7: Mean fluorescence intensity of PC-3 cells (A) and LNCaP cells (B) and the percentage of positive PC-3 cells (C) and LNCaP cells (D) exposed to **Rh-PAANG** (■) and **ACUPA-Rh-PAANG** (■), as measured by flow cytometry. The area of 99.5% under the histogram of the unexposed cells was set as the region for negative fluorescence.

4. Conclusions

Zwitterionic poly(amido amine) nanogels were prepared by surfactant-free nanoprecipitation of poly(amido amine)s and subsequent crosslinking with ethylenediamine. **PAANG** was obtained in high yield and were easily re-dissolved in water. **PAANG** was obtained with sizes around 100 nm and narrow size distributions, as measured by NTA and DLS. Furthermore, SLS measurements estimated the **PAANG** molecular weight at 5 MDa.

The presence of functional carboxylic acid groups, as well as primary amine groups in **PAANG**, allowed further **PAANG** functionalization for PSMA targeting. For this purpose, a *tert*-butyl ester-protected PSMA ligand (t-Bu-ACUPA) with a primary amine functionality was conjugated to the **PAANG** carboxylic acid functionalities. Quantification via ^1H NMR spectroscopy revealed a functionalization-degree of 0.9%, which computes to 300 ACUPA units per nanogel. **PAANG** and **ACUPA-PAANG** were further functionalized

with rhodamine B for imaging of the targeted cells. DLS showed that both nanogels had comparable sizes. However, the zeta potential of the **ACUPA-Rh-PAANG** (-5.7 mV) had increased markedly, compared to **Rh-PAANG** (-11.0 mV).

An MTT toxicity assay showed an increased toxicity of **ACUPA-Rh-PAANG** in PC-3 and LNCaP cells, which do not express PSMA and suggests non-specific cellular uptake. Comparing the fluorescence of lysed cell cultures after exposure to **Rh-PAANG** and **ACUPA-Rh-PAANG** also showed that PC-3 and LNCaP cells exhibited more fluorescence after **ACUPA-Rh-PAANG** exposure, further indicating non-specific cellular uptake. Fluorescence microscopy images however showed bright fluorescence in LNCaP cells, whereas only a pale fluorescence was observed in PC-3 cells. FACS analysis of PC-3 and LNCaP cell cultures, exposed to **Rh-PAANG** and **ACUPA-Rh-PAANG**, showed no PSMA-specific uptake of **ACUPA-Rh-PAANG**. Moreover, analysis of the number of positive cells, showed that PC-3 cell populations exhibited significantly higher fluorescence after exposure to **ACUPA-Rh-PAANG** than after exposure to **Rh-PAANG**. Taking into account all discussed results leads to the conclusion that **ACUPA-Rh-PAANG** shows an increased non-specific cellular uptake, which makes evaluation of its PSMA-targeting capabilities impossible. The increased non-specific cellular uptake is most likely due to the increased zeta potential. This might have been caused by the severe *tert*-butyl deprotection conditions, which might have led to a conformational change of the nanogel. Positively charged nanoparticles, including cationically modified **PAANG**,⁴⁴ are generally known to be taken up by cells to a larger extent than nanoparticles with a negative zeta potential, caused by electrostatic repulsion between nanoparticles and the negatively charged cellular membrane.^{16,22,23} This stresses the importance of nanoparticle functionalization without altering other characteristics, such as size, hydrophilicity and surface charge. For future evaluation of the prostate cancer targeting abilities of **PAANG**, *tert*-butyl deprotection under less severe conditions might have less influence on the nanogel's zeta potential. Alternatively, coupling a targeting ligand containing a functional group that is readily identified (by ¹H NMR spectroscopy for example), such as benzene, can be a viable option. Furthermore, analysis of LNCaP cells requires good cell attachment and detachment for MTT assays and FACS analysis, respectively. Alternatively, PC-3 cells, which are genetically modified for PSMA expression, are another option to analyze PSMA targeting efficacy.

Acknowledgements: Dr. Roelofs from 20MED therapeutics is acknowledged for helping with the FACS measurements and analysis. Dr. Visser (SYNCOM) is gratefully acknowledged for providing the protected PSMA-targeting ligand. The funding of this work by the provinces of Overijssel and Gelderland, as well as the project consortium by the Center for Medical Imaging – North East Netherlands (CMI-NEN) is gratefully acknowledged.

5. References

- (1) Min, Y.; Caster, J. M.; Eblan, M. J.; Wang, A. Z. *Chem. Rev.* **2015**, *115* (19), 11147–11190.
- (2) Kim, B. Y. S.; Rutka, J. T.; Chan, W. C. W. *N. Engl. J. Med.* **2010**, *363* (25), 2434–2443.
- (3) Elsabahy, M.; Heo, G. S.; Lim, S.-M.; Sun, G.; Wooley, K. L. *Chem. Rev.* **2015**, *115* (19), 10967–11011.
- (4) Elsabahy, M.; Wooley, K. L. *Chem. Soc. Rev.* **2012**, *41* (7), 2545.
- (5) Nicolas, J.; Mura, S.; Brambilla, D.; Mackiewicz, N.; Couvreur, P. *Chem. Soc. Rev.* **2013**, *42* (3), 1147–1235.
- (6) Zhang, L.; Cao, Z.; Li, Y.; Ella-Menye, J.-R.; Bai, T.; Jiang, S. *ACS Nano* **2012**, *6* (8), 6681–6686.
- (7) Anselmo, A. C.; Zhang, M.; Kumar, S.; Vogus, D. R.; Menegatti, S.; Helgeson, M. E.; Mitragotri, S. *ACS Nano* **2015**, *9* (3), 3169–3177.
- (8) Oh, J. K.; Siegwart, D. J.; Lee, H.; Sherwood, G.; Peteanu, L.; Hollinger, J. O.; Kataoka, K.; Matyjaszewski, K. *J. Am. Chem. Soc.* **2007**, *129* (18), 5939–5945.
- (9) Ryu, J.-H.; Chacko, R. T.; Jiwanpanich, S.; Bickerton, S.; Babu, R. P.; Thayumanavan, S. *J. Am. Chem. Soc.* **2010**, *132* (48), 17227–17235.
- (10) Xiong, M.-H. H.; Li, Y.-J. J.; Bao, Y.; Yang, X.-Z. Z.; Hu, B.; Wang, J. *Adv. Mater.* **2012**, *24* (46), 6175–6180.
- (11) Zhang, X.; Malhotra, S.; Molina, M.; Haag, R. *Chem. Soc. Rev.* **2015**, *44* (7), 1948–1973.
- (12) Kabanov, A. V.; Vinogradov, S. V. *Angew. Chemie Int. Ed.* **2009**, *48* (30), 5418–5429.
- (13) Ju, C.; Mo, R.; Xue, J.; Zhang, L.; Zhao, Z.; Xue, L.; Ping, Q.; Zhang, C. *Angew. Chemie Int. Ed.* **2014**.
- (14) Chen, W.; Achazi, K.; Schade, B.; Haag, R. *J. Control. Release* **2015**, *205*, 15–24.
- (15) Du, J.-Z. Z.; Sun, T.-M. M.; Song, W.-J. J.; Wu, J.; Wang, J. *Angew. Chemie Int. Ed.* **2010**, *49* (21), 3621–3626.
- (16) Albanese, A.; Tang, P. S.; Chan, W. C. W. *Annu. Rev. Biomed. Eng.* **2012**, *14*, 1–16.
- (17) Strebhardt, K.; Ullrich, A. *Nat. Rev. Cancer* **2008**, *8* (6), 473–480.
- (18) Masters, G. A.; Krilov, L.; Bailey, H. H.; Brose, M. S.; Burstein, H.; Diller, L. R.; Dizon, D. S.; Fine, H. A.; Kalemkerian, G. P.; Moasser, M.; Neuss, M. N.; O'Day, S. J.; Odenike, O.; Ryan, C. J.; Schilsky, R. L.; Schwartz, G. K.; Venook, A. P.; Wong, S. L.; Patel, J. D. *J. Clin. Oncol.* **2015**, *33* (7), 786–809.
- (19) Zolot, R. S.; Basu, S.; Million, R. P. *Nat. Rev. Drug Discov.* **2013**, *12* (4), 259–260.
- (20) Barenholz, Y. *J. Control. Release* **2012**, *160* (2), 117–134.
- (21) Bertrand, N.; Wu, J.; Xu, X.; Kamaly, N.; Farokhzad, O. C. *Adv. Drug Deliv. Rev.* **2014**, *66*, 2–25.
- (22) He, C.; Hu, Y.; Yin, L.; Tang, C.; Yin, C. *Biomaterials* **2010**, *31* (13), 3657–3666.
- (23) Fröhlich, E. *Int. J. Nanomedicine* **2012**, *7*, 5577–5591.
- (24) Nelson, A. L.; Dhimolea, E.; Reichert, J. M. *Nat. Rev. Drug Discov.* **2010**, *9* (10), 767–774.
- (25) Wang, M.; Thanou, M. *Pharmacol. Res.* **2010**, *62* (2), 90–99.
- (26) Cheng, Z.; Al Zaki, A.; Hui, J. Z.; Muzykantov, V. R.; Tsourkas, A. *Science* **2012**, *338* (6109), 903–910.
- (27) Broda, E.; Mickler, F. M.; Lächelt, U.; Morys, S.; Wagner, E.; Bräuchle, C. *J. Control. Release* **2015**, *213*, 79–85.

- (28) Li, W.; Su, B.; Meng, S.; Ju, L.; Yan, L.; Ding, Y.; Song, Y.; Zhou, W.; Li, H.; Tang, L.; Zhao, Y.; Zhou, C. *Eur. J. Radiol.* **2011**, *80* (2), 598–606.
- (29) Quan, C.-Y.; Chang, C.; Wei, H.; Chen, C.-S.; Xu, X.-D.; Cheng, S.-X.; Zhang, X.-Z.; Zhuo, R.-X. *Nanotechnology* **2009**, *20* (33), 335101.
- (30) Lao, Y.-H.; Phua, K. K. L.; Leong, K. W. *ACS Nano* **2015**, *9* (3), 2235–2254.
- (31) Nukolova, N. V.; Oberoi, H. S.; Cohen, S. M.; Kabanov, A. V.; Bronich, T. K. *Biomaterials* **2011**, *32* (23), 5417–5426.
- (32) Smith, B. A.; Smith, B. D. *Bioconjug. Chem.* **2012**, *23* (10), 1989–2006.
- (33) Torre, L. A.; Bray, F.; Siegel, R. L.; Ferlay, J.; Lortet-Tieulent, J.; Jemal, A. *CA. Cancer J. Clin.* **2015**, *65* (2), 87–108.
- (34) Sardana, G.; Dowell, B.; Diamandis, E. P. *Clinical Chemistry*. American Association for Clinical Chemistry December 1, 2008, pp 1951–1960.
- (35) Maresca, K. P.; Hillier, S. M.; Femia, F. J.; Keith, D.; Barone, C.; Joyal, J. L.; Zimmerman, C. N.; Kozikowski, A. P.; Barrett, J. A.; Eckelman, W. C.; Babich, J. W. *J. Med. Chem.* **2009**, *52* (2), 347–357.
- (36) Patri, A. K.; Myc, A.; Beals, J.; Thomas, T. P.; Bander, N. H.; Baker, J. R. *Bioconjug. Chem.* **2004**, *15* (6), 1174–1181.
- (37) Kim, D.; Jeong, Y. Y.; Jon, S. *ACS Nano* **2010**, *4* (7), 3689–3696.
- (38) Farokhzad, O. C.; Cheng, J.; Teply, B. A.; Sherifi, I.; Jon, S.; Kantoff, P. W.; Richie, J. P.; Langer, R. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (16), 6315–6320.
- (39) Dhar, S.; Gu, F. X.; Langer, R.; Farokhzad, O. C.; Lippard, S. J. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (45), 17356–17361.
- (40) Hrkach, J.; Von Hoff, D.; Mukkaram Ali, M.; Andrianova, E.; Auer, J.; Campbell, T.; De Witt, D.; Figa, M.; Figueiredo, M.; Horhota, A.; Low, S.; McDonnell, K.; Peeke, E.; Retnarajan, B.; Sabnis, A.; Schnipper, E.; Song, J. J.; Song, Y. H.; Summa, J.; Tompsett, D.; Troiano, G.; Van Geen Hoven, T.; Wright, J.; LoRusso, P.; Kantoff, P. W.; Bander, N. H.; Sweeney, C.; Farokhzad, O. C.; Langer, R.; Zale, S. *Sci. Transl. Med.* **2012**, *4* (128), 128ra39.
- (41) Li, X.; Yang, W.; Zou, Y.; Meng, F.; Deng, C.; Zhong, Z. *J. Control. Release* **2015**, *220*, 704–714.
- (42) Huang, B.; Otis, J.; Joice, M.; Kotlyar, A.; Thomas, T. P. *Biomacromolecules* **2014**.
- (43) Lütje, S.; Heskamp, S.; Cornelissen, A. S.; Poeppel, T. D.; Broek, S. A. M. W. van den; Rosenbaum-Krumme, S.; Bockisch, A.; Gotthardt, M.; Rijpkema, M.; Boerman, O. C. *Theranostics* **2015**, *5* (12), 1388–1401.
- (44) Ekkelenkamp, A. E.; Jansman, M. M. T.; Roelofs, K.; Engbersen, J. F. J.; Paulusse, J. M. J. *Acta Biomater.* **2016**, *30*, 126–134.
- (45) Doane, T. L.; Chuang, C.-H.; Hill, R. J.; Burda, C. *Acc. Chem. Res.* **2012**, *45* (3), 317–326.
- (46) D’Alpino, P. H. P.; Pereira, J. C. J. C.; Svizero, N. R. N. R.; Rueggeberg, F. A.; Pashley, D. H. *J. Dent.* **2006**, *34* (9), 623–634.
- (47) Liberio, M. S.; Sadowski, M. C.; Soekmadji, C.; Davis, R. A.; Nelson, C. C. *PLoS One* **2014**, *9* (11), e112122.

- (48) Patterson, J. P.; Robin, M. P.; Chassenieux, C.; Colombani, O.; O'Reilly, R. K. *Chem. Soc. Rev.* **2014**, 43 (8), 2412.
- (49) Troiber, C.; Kasper, J. C.; Milani, S.; Scheible, M.; Martin, I.; Schaubhut, F.; Küchler, S.; Rädler, J.; Simmel, F. C.; Friess, W.; Wagner, E. *Eur. J. Pharm. Biopharm.* **2013**, 84 (2), 255–264.
- (50) Dubacheva, G. V.; Curk, T.; Mognetti, B. M.; Auzély-Velty, R.; Frenkel, D.; Richter, R. P. *J. Am. Chem. Soc.* **2014**, 136 (5), 1722–1725.
- (51) (51) Elias, D. R.; Poloukhine, A.; Popik, V.; Tsourkas, A. *Nanomedicine Nanotechnology, Biol. Med.* **2013**, 9 (2), 194–201.

SUMMARY

Nanotechnology provides the tools to influence biological processes on the cellular and molecular level, which offers great opportunities for medical applications, such as targeted imaging and drug delivery. Owing to their tremendous variability, polymeric nanoparticles can act as a versatile delivery vehicle for targeted imaging and controlled drug delivery. Hydrogel nanoparticles, or nanogels, are a class of biocompatible nanostructures, which can be used for imaging and delivery of therapeutics. Nanogels can be rendered responsive to a range of biophysical cues for triggered release and environment-responsive imaging. Moreover, zwitterionic nanogels show minimal non-specific interactions with proteins and cells, rendering them highly stable in biological environments and very suitable for targeting specific tissues and cell types. This thesis describes the formation and evaluation of peptidomimetic zwitterionic poly(amido amine) nanogels as a suitable carrier for the targeted delivery of imaging agents and therapeutics.

Chapter 1 contains a general introduction to the subject, as well as a problem statement and an outline of this thesis.

Chapter 2 is an overview of the current literature on covalently crosslinked nanogels with a focus on application of nanogels as a responsive material. Methods for nanogel preparation are discussed as well as methods to incorporate specific responsive properties into nanogels. Furthermore, an overview of existing literature is given of nanogels applied for imaging and drug delivery with special attention for their responsive properties.

Chapter 3 describes the synthesis and characterization of zwitterionic poly(amido amine)s, synthesized via Michael addition between *N,N'*-methylene(bisacrylamide) and ethylenediamine-*N,N'*-diacetic acid. Zwitterionic poly(amido amine) nanogels were prepared by inverse nanoprecipitation and subsequently crosslinked with ethylenediamine via *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide/*N*-hydroxysuccinimide (EDC/NHS) coupling. To investigate the optimal conditions to prepare zwitterionic poly(amido amine) nanogels, nanoprecipitation was carried out in six non-solvents (1,2-dimethoxyethane, 1,4-dioxane, acetone, acetonitrile, ethanol and isopropyl alcohol) and under varying poly(amido amine) concentration. Stable crosslinked nanogels were obtained from nanoprecipitation in acetone and acetonitrile. The lower-size limit of the obtained nanogels was around 100 nm, while nanogels could be prepared in acetonitrile at higher polymer concentrations than in acetone.

Chapter 4 describes functionalization strategies for poly(amido amine) nanogels by EDC/NHS coupling, isothiocyanate coupling and copper-free Huisgen azide-alkyne cycloaddition. Zwitterionic poly(amido amine) nanogels were successfully modified

with a cationic surface charge by conjugation of the nanogel's carboxylic acid groups with *N,N*-dimethylethylenediamine via EDC/NHS coupling. This resulted in an increase in zeta potential from -26.5 mV to 11.4 mV. Zwitterionic and cationically-modified nanogels were functionalized with a fluorophore (rhodamine B and fluorescein) via isothiocyanate coupling with primary amines arising from the crosslinking and subsequent quenching reaction. To introduce azide moieties to the nanogel, imidazole-1-sulfonylazide-HCl – a diazo-donor – was used. Azide groups were subsequently functionalized via a copper-free Huisgen azide-alkyne cycloaddition with a fluorophore.

Chapter 5 describes the further characterization of zwitterionic and cationically-modified poly(amido amine) nanogels. Zwitterionic poly(amido amine) nanogels exhibited excellent colloidal stability in PBS and 50% fetal bovine serum for 24 hours, contrasting to the cationically-modified nanogels. *In-vitro* evaluation of cytotoxicity, induced by both nanogels, was evaluated in RAW 264.7, PC-3 and COS-7 cells. Cationically-modified nanogels showed high cytotoxicity in PC-3 and COS-7 cells, whereas the zwitterionic nanogels showed only minimal cytotoxicity in these cell lines. Furthermore, zwitterionic nanogels showed an increased cytotoxicity in RAW cells, which was suggested to be caused by active cellular uptake of these nanogels by RAW cells. Evaluation of nanogel cell interactions was evaluated by fluorescence microscopy of RAW, PC-3 and COS-7 cultures, which were exposed to fluorescently-labelled zwitterionic and cationically-modified poly(amido amine) nanogels. Cationically-modified nanogels showed accumulation in all three cell types, whereas zwitterionic nanogel cellular uptake was not observed.

Chapter 6 evaluates zwitterionic poly(amido amine) nanogels for the selective targeting of prostate cancer-specific membrane antigen (PSMA)-expressing cells. For this purpose poly(amido amine) nanogels were functionalized with *S,S*-2-(3-(5-amino-1-carboxypentyl)-ureido)-pentanedioic acid (ACUPA) by EDC/NHS coupling. First, a *tert*-butyl-ester protected ACUPA ligand was conjugated to the nanogel and subsequently deprotected and functionalized with rhodamine B. Characterization of the ACUPA-functionalized nanogel showed no influence of the functionalization on nanogel size. However, the zeta potential of the ACUPA-functionalize nanogel was increased from -11.0 to -5.7 mV, which was possibly the result of conformational changes of the nanogel during *tert*-butyl ester deprotection. *In-vitro* evaluation of the targeting ability of ACUPA-functionalized nanogels was evaluated in PC-3 and LNCaP cell cultures, of which only LNCaP cell expressed PSMA. Evaluation by FACS and fluorescence microscopy showed uptake of the ACUPA-functionalized nanogels in both cell lines, whereas the non-functionalized nanogels showed lower cellular uptake in both cell lines. Therefore, it was not yet possible to selectively target PSMA-expressing cells, most likely due to the more positive surface charge of the ACUPA-functionalize nanogel.

SAMENVATTING

Nanotechnologie kan ingezet worden om biologische processen op een cellulair en moleculair niveau te beïnvloeden. Dit biedt grote mogelijkheden voor medische toepassingen, zoals het specifiek herkennen van cellen en weefsels voor medische beeldvorming en de gecontroleerde afgifte van medicijnen. Polymeer nanodeeltjes kunnen, vanwege hun grote variëteit en diversiteit, dienen als veelzijdige dragermaterialen voor deze toepassingen. Hydrogel nanodeeltjes, of nanogelen, zijn biocompatibele nanostructuren welke kunnen worden toegepast in medische beeldvorming en medicijnafgifte. Nanogels kunnen zo worden gemaakt dat ze reageren op biofysische signalen, bijvoorbeeld voor toepassing in responsieve beeldvorming en gecontroleerde medicijnafgifte. Daarbij komt dat zwitterionische nanogelen minimale niet-specifieke interacties vertonen met eiwitten en cellen, waardoor deze nanogelen uitermate geschikt zijn voor toepassing in het specifiek herkennen van weefsel- en celtypen. Dit proefschrift beschrijft nanogelen voor toepassing in de specifieke afgifte van medicijnen en contrastmiddelen voor medische beeldvorming.

Hoofdstuk 1 is een algemene introductie in het onderwerp, alsmede een probleemstelling en een algemene opzet van dit proefschrift.

Hoofdstuk 2 is een overzicht van de literatuur over covalent gecrosslinkte nanogelen, waarbij de nadruk ligt op de toepassing van nanogelen als responsieve materialen. Diverse productiemethoden voor nanogelen worden beschreven, evenals methoden voor het toevoegen van specifieke responsieve eigenschappen aan nanogelen. Daarbij wordt een literatuuroverzicht gegeven van de toepassing van nanogelen in medische beeldvorming en gecontroleerde medicijnafgifte, waarbij in het bijzonder aandacht wordt besteed aan de responsieve eigenschappen van de nanogelen.

Hoofdstuk 3 beschrijft de synthese en karakterisatie van zwitterionische poly(amido amine)s door synthese via een Michael-additie tussen *N,N'*-methyleen(bisacrylamide) en ethyleendiamine-*N,N'*-diazijnzuur. Zwitterionische poly(amido amine) nanogel werden geformeerd door inverse nanoprecipitatie, gevolgd door crosslinken met ethyleendiamine via een *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide/*N*-hydroxysuccinimide (EDC/NHS) koppeling. Om de optimale condities voor het maken van zwitterionische poly(amido amine) nanogel, werd de nanoprecipitatie uitgevoerd in zes verschillende non-solvents (1,2-dimethoxyethaan, 1,4-dioxaan, aceton, acetonitril, ethanol en isopropylalcohol) en bij variërende poly(amido amine) concentraties. Stabiele, gecrosslinkte nanogelen konden worden verkregen door nanoprecipitatie in aceton en acetonitril. De ondergrens voor de grootte van de verkregen nanogelen lag rond de 100 nm en nanogelen konden in acetonitril bij hogere poly(amido amine) concentraties worden gevormd dan in aceton.

Hoofdstuk 4 beschrijft functionalisatie-strategieën voor poly(amido amine) nanogelen door EDC/NHS koppeling, isothiocyanaatkoppeling en kopervrije Huisgen azide-alkyn cycloadditie. Zwitterionische nanogelen werden succesvol aangepast met een kationische oppervlakteading door conjugatie van de, op de nanogel aanwezige, carbonzuurgroepen met *N,N*-dimethylethyleendiamine via een EDC/NHS koppeling. Dit resulteerde in een verhoging van het zeta potentiaal van -26.5 mV naar 11.4 mV. Zwitterionische en kationisch-gemodificeerde nanogelen werden gefunctionaliseerd met een fluorofoor (rhodamine B en fluoresceïne) via een isothiocyanaatkoppeling met op de nanogel aanwezige primaire amine-residuen, ontstaan door de crosslinken en de daaropvolgende uitdovings-reactie. Azide groepen werden op de nanogelen geïntroduceerd door een reactie met een diazo donor (imidazole-1-sulfurylazide-HCl). Deze azide groepen werden daarna gefunctionaliseerd door een koper-vrije Huisgen azide-alkyn cycloadditie met een fluorofoor.

Hoofdstuk 5 beschrijft de verdere karakterisatie van zwitterionische en kationisch-gemodificeerde poly(amido amine) nanogelen. Zwitterionische poly(amido amine) nanogelen vertoonden, gedurende 24 uur, uitstekende colloïdale stabiliteit in PBS en 50% foetaal runderserum, in tegenstelling tot de kationische-gemodificeerde nanogelen. Evaluatie van de door beide nanogelen veroorzaakte *in-vitro* cytotoxiciteit, werd gedaan met behulp van RAW 264.7, PC-3 and COS-7 cellen. Kationisch-gemodificeerde nanogelen vertoonden hoge cytotoxiciteit in PC-3 en COS-7 cellen, terwijl de zwitterionische nanogelen slechts minimale cytotoxiciteit in deze cellijnen veroorzaakten. Daarentegen vertoonden de zwitterionische nanogelen een verhoogde celtoxiciteit in RAW cellen, wat waarschijnlijk werd veroorzaakt door een hogere opname van de nanogelen door de RAW cellen. De nanogel-cel interacties werden geëvalueerd door middel van fluorescentiemicroscopie van RAW, PC-3 en COS-7 cellen, na blootstelling aan fluorescent-gelabelde zwitterionische en kationische nanogelen. Kationisch-gemodificeerde nanogelen vertoonden accumulatie in alle geteste celtypen, terwijl bij zwitterionische nanogelen geen cel-opname werd waargenomen.

Hoofdstuk 6 evalueert zwitterionische poly(amido amine) nanogelen voor het specifiek herkennen van cellen die het prostaatkanker-membraanantigen (PSMA) tot expressie brengen. Voor dit doel werden poly(amido amine) nanogelen gefunctionaliseerd met *S,S*-2-(3-(5-amino-1-carboxypentyl)-ureido)-pentaandizuur (ACUPA) via een EDC/NHS koppeling. Een *tert*-butylester beschermd ACUPA ligand werd achtereenvolgens geconjugeerd aan de nanogel, ontschermd en gefunctionaliseerd met rhodamine B. Karakterisatie van de ACUPA-gefunctionaliseerde nanogelen liet geen invloed zien van de functionalisatie op de grootte van de nanogel. Daarentegen was het zeta potentiaal van de ACUPA-gefunctionaliseerde nanogel verhoogd van -11.0 naar -5.7 mV, wat mogelijk werd veroorzaakt door conformationele veranderingen

van de nanogel tijdens de *tert*-butylester ontscherming. *In-vitro* evaluatie van de specifieke herkenningmogelijkheden van ACUPA-gefunctionaliseerde nanogelen werd geëvalueerd in PC-3 en LNCaP celculturen, waarvan alleen LNCaP cellen PSMA tot expressie brengen. Evaluatie door middel van FACS en fluorescentiemicroscopie toonde aan dat de ACUPA-gefunctionaliseerde nanogelen werden opgenomen door beide cellijnen. Echter, de niet-gefunctionaliseerde nanogelen vertoonden een lagere opname in beide cellijnen. Daarom was het nog niet mogelijk om cellen, die PSMA tot expressie brengen, specifiek te herkennen met ACUPA-gefunctionaliseerd nanogelen, wat mogelijkerwijs werd veroorzaakt door de meer positieve oppervlaktelading van de ACUPA-gefunctionaliseerde nanogelen.

DANKWOORD

Hoewel er maar één naam op de voorkant van dit proefschrift staat, is dit boekwerk niet alleen het resultaat van mijn inspanningen. Een promotieonderzoek is misschien wel het werk van een eenling, maar gedoemd te mislukken zonder goede begeleiding, ondersteuning en de nodige afleiding. Daarvoor moet ik een flink aantal mensen erkennen en bedanken.

In de eerste plaats mijn promotor en co-promotor: Johan, bedankt voor deze mogelijkheid en je supervisie in de afgelopen jaren, ik heb veel van je geleerd de afgelopen jaren. Jos, zonder jouw ondersteuning en motivatie had dit proefschrift hier nooit kunnen liggen. Ik heb de afgelopen vier jaar ervan genoten om met je samen te werken, ook al was het hele traject absoluut niet vrij van de nodige hindernissen. Jij hebt me door de afgelopen vier jaar heen kunnen slepen en geholpen om de nodige tegenslagen te verwerken. Dank je voor het meedenken, het aandragen van ideeën en voor je ondersteuning en geduld, vooral in het afgelopen jaar. Je grondige manier van werken en je positief kritische blik zijn kwaliteiten die ik erg bewonder. Bovendien bewonder ik je inzet voor het onderwijs hier op de UT, je bent nooit te beroerd om iets uit te leggen of het nou om een PhD student gaat of een eerstejaars student. We hebben gelukkig ook een hoop lol gehad tijdens overleggen, groepsbesprekingen en congressen. Je hebt een nuchtere kijk op de dingen en dat straalt je uit, wat de groep enorm ten goede komt. Ik wens jou en je gezin alle geluk toe, ik had geen betere begeleider kunnen wensen.

I would also like to thank my office mates from over the past years: Dewi, Guoying, Pia, Rachel and Hui. Dewi, thank you for getting me started during my Master thesis and the beginning of my PhD. Guoying, I have enjoyed sharing an office with you and the occasional 'cultural exchange'. Pia, you brought a lot of experience and spirit into our small group when it was most needed. Thanks for helping me out with the GPC measurements. Also, thank you for the fun times in and outside the lab, the skiing lesson during the group trip to Bottrop and for the support in the last year, which helped to keep me going. Rachel, ook jij bedankt voor de afgelopen jaren. Voor de gezelligheid en leuke dingen op kantoor, je ondersteuning in het afgelopen jaar en dat je mijn paranimf wilde zijn. Hui, also thank you for the fun and the conversations we had in- and outside the office. Also, I had a lot of fun with you (Pia, Rachel and Hui) during the symposium in Egmond, especially during the conference dinner.

Onmisbaar voor dit proefschrift was de ondersteuning van Karin Roelofs, Hetty en Lydia in het cellab. Hetty bedankt voor de hulp bij mijn eerste cel-experimenten en het helpen bij de AFM-metingen. Karin, ik kon altijd bij jou terecht als ik vragen had of jouw hulp of mening vroeg. Dank je ook voor je hulp voor de laatste twee hoofdstukken van dit proefschrift. Lydia, ook bedankt voor je hulp in het cellab en dat je altijd beschikbaar

was voor vragen. Marc, Niels en René: ik wil jullie bedanken voor de hulp en ervaring op scheikundig gebied. Wanneer ik een praktische vraag had op het gebied van chemie kon ik bij jullie terecht. René, dank je voor je input en je hulp aan het begin van mijn promotieonderzoek. Niels, ik wil jou ook bedanken voor de hulp in de laatste fase van mijn promotieonderzoek, door jouw luchtige kijk op de zaken kon ik zelf ook dingen beter relativeren. Heel veel geluk en succes toegewenst bij je baan in Hilversum. Marc, jij was voor mij een constante factor tijdens mijn tijd op de UT. Ik herinner me nog je begeleiding bij het eerstejaarspracticum BMT waar we onze eerste polymeren maakten. Dank je voor je vrolijke kijk op het leven en alle hulp en nuttige (en minder nuttige) tips en informatie die je me hebt gegeven. Karin (Hendriks), dank je voor alle grote en kleine administratieve zaken die je voor me hebt geregeld. Je staat altijd klaar voor iedereen, ook al heb je het zelf ook enorm druk. Anita, het was prettig om af en toe een praatje te maken bij je op kantoor. Zlata, ik wil jou ook bedanken voor je hulp bij bestellingen en het lokaliseren van exotische chemicaliën.

Tijdens de afgelopen jaren heb ik een aantal studenten mogen begeleiden. Meri, you were my first student and I became your supervisor when I just started my PhD. I enjoyed working with you and I admire your drive and meticulous experimental skills. Michelle, ik heb je mogen begeleiden als Bachelor en Master-student. Ik vond het erg leuk om met je samen te werken en je te zien groeien in je onderzoek. Jouw resultaten zorgden ervoor dat hoofdstuk 4 geaccepteerd kon worden, waar ik nog steeds erg trots op ben. Manuel, I still remember your time here in the group and you showed how much you can grow in a short amount of time. I have enjoyed working with you in the lab and your humor, I hope you are well. Sjoerd, je kwam hier voor je stage vanuit de Hogeschool en je liet meteen zien hoe je ervan genoot om op het lab te staan. Jouw resultaten waren het voorwerk voor het tweede hoofdstuk van dit proefschrift. Leonie, ik heb je voor een klein gedeelte kunnen begeleiden bij je afstuderen in Groningen. Ik vond het erg leuk om met je op het lab te staan en het was heel mooi om te zien hoe je in een onderwerp kon groeien waar je niet echt bekend mee was.

During the past few years I have enjoyed the company of many colleagues. Bas, je was altijd aanwezig, zeer actief (vooral tijdens Sinterklaas) en enthousiast voor borrels of pubquizen. Erwin, Frits en Mike, bedankt voor jullie aanwezigheid en de lol tijdens de lunchpauzes. Nick, ik wil jou ook bedanken voor je humor, je nuchtere kijk op de dingen. Heel veel succes met je onderzoek in Maastricht. Gert-Jan, je was me net voor met promoveren en het was altijd leuk om even een praatje met je te maken als je weer eens in Nederland was, heel veel succes in je verdere loopbaan. Natalia, Ilaria, Odyl, Aga, Aysun, Bade, Kasia, Thijs, Zhengchao and Denys, thank you for the time here in the group, I enjoyed your company a lot. Jay, Ruchi, Karin (Binnemars), Iris, Praneeth, Dwi and Jonas, your group was new at the UT and it developed over the past years. Karin en

Iris, ik vond het leuk om met jullie te werken op het cellab (al was het maar een heel klein gedeelte). Ik wens jullie en je gezin het beste toe. Dwi and Praneeth, also all the best for you and your families. Jonas, ik heb veel lol met je gehad tijdens borrels en het congres in Egmond, heel veel succes bij de rest van je PhD. All of you thank you for the time here at the UT, you made working here a lot of fun and it brings back a lot of good memories.

Binnen een onderzoeksgroep zijn eigenlijk altijd wel Bachelor of Master studenten bezig met hun respectievelijke eindopdrachten. Ik kan nauwelijks meer nagaan wie er allemaal bij BST hun bachelor- of master thesis hebben gedaan tijdens mijn tijd hier. Een aantal wil ik in het bijzonder noemen: Arantxa, Maria, Thomas, Regina, Kostas, Eveline, Verena, Lena, Christian, Monika, Robbert, Kate, Charlotte, Alex, Roy, Muhabbat, Jonathan, Aniek, Maaïke en Annerieke. Het was altijd erg gezellig tijdens de groepsbesprekingen, het borrelen met de vakgroep op vrijdagmiddag en op activiteiten buiten het werk.

Ik wil hier ook mijn vrienden en familie bedanken voor alles wat ze (al dan niet onbewust) hebben bijgedragen. Albert, dank je voor je humor, je vriendschap en het helpen met het ontwerp van dit boekwerk. Jan, Annelies, Jonathan, Pieter, Aniek, Petra, David, Daan, Wilco, Hinke, dank jullie voor jullie gezelschap en steun in dit, voor mij, zware jaar. Pa, ma, Josine, Paul, Rien, Ans en Maarten, ik wil ook jullie bedanken voor jullie steun en gezelschap. Ik ben blij en dankbaar voor zo'n lieve familie, waar ik de afgelopen jaren steeds meer de waarde van heb leren inzien, waarvoor ik erg dankbaar voor ben.

Deze laatste alinea is voor jou, Eline, want zonder jou weet ik dat ik dit nooit had kunnen afmaken. Aan het begin van dit traject was je een goede vriendin en vervolgens mijn vriendin, mijn verloofde en uiteindelijk mijn fantastische vrouw. Jij bent voor mij het hoogtepunt van de afgelopen vier jaar, want zonder jouw ondersteuning, vrolijkheid, enthousiasme en liefde zou ik dit niet hebben kunnen afronden. Ik weet niet hoe vaak je de afgelopen twee jaar me weer hebt weten op te beuren, omdat het onderzoek en alles daar omheen ronduit teleurstellend en frustrerend was. Ja, het heeft geholpen, meer dan je misschien dacht. We hebben (gelukkig) ook veel lol gehad en samen kunnen genieten van het leven met elkaar, ik zou je voor geen goud willen missen! Eline, ik ben God ontzettend dankbaar dat we het leven met elkaar mogen delen en dat Hij jou de kracht heeft gegeven die ik niet had, je bent zo veel sterker dan ik.

Waar ik dit dankwoord mee wil afsluiten is dat ik Gods zegen heb mogen ervaren in de mensen in mijn omgeving. Ik kan me niet voorstellen hoe dit proefschrift tot stand gekomen zou zijn zonder al deze mensen.

Tony

Curriculum Vitae

Tony Ekkelenkamp werd geboren op 18 september 1986 te Zwolle. Na zijn VWO-opleiding met natuur & gezondheid profiel aan het Greijdanus College in Zwolle, begon hij aan de opleiding Biomedische Technologie. In 2012 werd de mastertrack molecular, cell & tissue engineering afgerond met een afstudeeropdracht over oppervlakte plasmon resonantie-actieve poly(amido amine) layer-by-layer multilagen bij de vakgroep Biomedical Chemistry onder supervisie van prof. dr. J.F.J. Engbersen en dr. R. Gill. Na het afronden van deze afstudeeropdracht werd begonnen aan een promotieonderzoek onder leiding van prof. dr. J.F.J. Engbersen en dr. J.M.J. Paulusse. De belangrijkste resultaten van dit onderzoek zijn opgenomen in dit proefschrift

Tony Ekkelenkamp was born on the 18th of September 1986 in Zwolle. After his pre-university education in high school, with a focus on biology & natural sciences, at the Greijdanus College in Zwolle, he started with the Biomedical Engineering education at the University of Twente. In 2012 the molecular, cell & tissue engineering track was completed with a master-assignment in the Biomedical Chemistry group on surface plasmon resonance-active poly(amido amine) multilayers, which was supervised by prof. dr. J.F.J. Engbersen and dr. R. Gill. After finishing his master-thesis he started with his PhD in the same group under supervision of prof. dr. J.F.J. Engbersen and dr. J.M.J. Paulusse. The most important results of this research are described in this thesis.

The background of the entire page is an abstract, vibrant composition. It features numerous green spheres of varying sizes, some of which are connected by thick, wavy orange lines. A network of thinner, translucent blue lines crisscrosses the entire scene, creating a complex, web-like pattern. The overall color palette is dominated by these three colors: green, orange, and blue, set against a dark, slightly textured background.

UNIVERSITY OF TWENTE.

ISBN 978-90-365-4255-5

9

789036

542555