Surface modifications by gas plasma control osteogenic differentiation of MC3T3-E1 cells

Ana M.C. Barradas, Kristina Lachmann, Gregor Hlawacek, Cathelijne Frielink, Roman Truckenmoller, Otto C. Boerman, Raoul van Gastel, Henk Garritsen, Michael Thomas, Lorenzo Moroni, Clemens van Blitterswijk, Jan de Boer

Abstract

Numerous studies have shown that the physicochemical properties of biomaterials can control cell activity. Cell adhesion, proliferation, differentiation as well as tissue formation in vivo can be tuned by properties such as the porosity, surface micro- and nanoscale topography and chemical composition of biomaterials. This concept is very appealing for tissue engineering since instructive properties in bioactive materials can be more economical and time efficient than traditional strategies of cell pre-differentiation in vitro prior to implantation. The biomaterial surface, which is easy to modify due to its accessibility, may provide the necessary signals to elicit a certain cellular behavior. Here, we used gas plasma technology at atmospheric pressure to modify the physicochemical properties of polylactic acid treated with tetramethyldisilane and 3-aminoethylsilane with helium as a carrier gas or a mixture of nitrogen and hydrogen. These modifications influenced protein adsorption and pre-osteoblast cell adhesion. Tetramethyldisilane and 3-aminoethylsilane increased osteogenic differentiation compared to the other surfaces. This promising surface modification could be further explored for potential development of bone graft substitutes.

1. Introduction

Bone tissue engineering has emerged as a field providing alternatives to autologous bone grafts, which are still considered the gold standard treatment for healing bone defects [1–3]. Tissue-engineering strategies focus on the development of scaffolds and/or on the combination of scaffolds with cells. Traditionally cells are pre differentiated into an osteogenic lineage through addition of growth factors or steroids, such as bone morphogenetic proteins (BMPs) or dexamethasone [4–8]. Alternatively, cell differentiation may be controlled by the physicochemical properties of the scaffold material [9–11]. This represents a more economic and expeditious approach and has the additional advantage that biologically relevant molecular signals are still transmitted to the cells through cell–surface interactions after the graft has been implanted. For example, induction of bone formation is known to be influenced by the pore size of biphasic calcium phosphate ceramic granules [12], the depth of surface concavities in hydroxyapatite ceramic discs [13] and the chemical composition of the ceramic materials [14]. This demonstrates the relevance of material properties for clinical application.

In addition to changing bulk properties of a biomaterial, one can also change the surface properties, such as topography or chemistry. For instance, it has been noted that NH2-enriched surfaces promoted osteogenesis of human bone marrow derived mesenchymal stromal cells (hMSCs), whereas chondrogenesis was favored by COOH and OH groups [15]. However, Phillips et al. could not ascribe expression of chondrogenic markers to one specific group [16]. Changes in surface chemistry are accompanied by differences in material–protein interaction, which may account for the observed

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Corresponding author. Tel.: +31 53 489 3400; fax: +31 53 489 2150.
E-mail address: j.deboer@utwente.nl (J. de Boer).
cell behavior [17,18]. For instance, adsorption of fibronectin and vitronectin to polymeric scaffolds is affected by the polymer chemical composition [10,19]. Furthermore, the conformation of fibronectin attached to silica-coated substrates depended on the size of silica sols used [20] and on the chemical groups present at the substrate surface [21]. Interestingly, when the central cell-bind- ing domain of fibronectin was blocked, the observed effects of NH2- and OH-coated surfaces on osteogenic differentiation of MC3T3 cells were no longer seen [22]. Furthermore, the authors of the study demonstrated that mineralization could be tailored by β1 and β3 integrin activity, which links cell adhesion to chemistry-dependent effects.

Thus, we consider chemical modification of surfaces as an efficacious strategy to control cell behavior. Gas plasma treatment offers an efficient method to chemically modify surfaces. Gas plasma is a state of matter in which molecules of a gas are ionized by an electric discharge, increasing the probability of interaction with surrounding molecules. Charged molecules as well as radicals are formed after applying a high voltage. These reactive species interact with material surfaces and lead to the incorporation of functional groups. Depending on the process parameters used (pressure, gas mixture, addition of film-forming agent, treatment time, applied power) different effects on the surface are observed. Gas plasma treatments applied to polymeric biomaterials modify not only their surface chemical composition but also roughness and wettability, which, as expected, can affect cell behavior as well [23–30]. For instance, adhesion of human umbilical vein endothelial cells (HUVECs) to poly(lactic acid) (PLA) was improved by plasma treatment with oxygen, argon or nitrogen [31]. Proliferation of fibroblasts was enhanced by treating polyetheretherketone (PEEK) with a plasma mixture of ammonia and argon, compared to the non-treated scaffold [27]. Similarly, treating poly(ethylene glycol)-terephthalate–poly(butylene terephthalate) (PEGT/PBT) block co-polymer with argon plasma increased the numbers of chondrocytes [32]. Whereas adhesion and proliferation are often markedly affected by plasma treatments, effects on cellular differentiation have been reported less often. For instance, expression of alkaline phosphatase (ALP) in osteoblast precursors cultured in PLA was not affected compared to cultures in PLA treated with gas plasma [27]. Similarly, expression of ALP and collagen type I by fibroblasts cultured on plasma-treated PEEK did not change compared to cultures on the non-treated film. Conversely, plasma treatment enhanced collagen type II expression in chondrocytes to levels similar to those observed in a stained film. Conversely, plasma treatment enhanced collagen type II expression in chondrocytes to levels similar to those observed in a stained film. Conversely, plasma treatment enhanced collagen type II expression in chondrocytes to levels similar to those observed in a stained film. Conversely, plasma treatment enhanced collagen type II expression in chondrocytes to levels similar to those observed in a stained film. Consequently, the authors of the study demonstrated that mineralization could be tailored by β1 and β3 integrin activity, which links cell adhesion to chemistry-dependent effects.

2. Materials and methods

2.1. Gas plasma treatment of PLA samples

FDA-approved poly-L,D-lactic acid (PLA) transparent foils, 150 μm thick, were a kind gift from Folienwerk Wolfen GmbH (PLA-type 2002D). Disc-shaped samples of approximately 10 cm2 were punched out of the PLA foil (PLAd) and placed in a grounded substrate carrier facing the high-voltage electrode (HVE) in a dielectric barrier discharge configuration [37,38]. In all cases, only one side of the PLAd was treated. An electric discharge was produced in the space between the HVE and the grounded substrate, charging the atmospheric molecules present in that space (gas plasma). PLAd were treated by adding the monomers 3-aminopropyl-trime-thoxysilane (APTMMS, >97%, Sigma-Aldrich) or tetramethylsilane (TMS, >99, 9%, abcr GmbH)) to the gas phase (helium, 5.0 Linde) or treated with a gas mixture of nitrogen and 3.4% hydrogen (N2/H2). An overview of the different treatments is given in Table 1.

2.2. PLAd disinfection and sterilization

PLAd were disinfected in 70% v/v ethanol in demineralized water (dH2O) for 15 min, followed by 15 min in phosphate-buffered saline (PBS, Gibco). This procedure was performed twice. Ethanol solutions were filtered with a 0.22 μm pore size filter to remove any particles in suspension that could adhere to the polymeric surfaces. Gamma-irradiation (GI) was performed at Membrana GmbH (D-42289 Wuppertal) with an irradiation dose of 30.5 kGy.

2.3. Water contact angle

After removal of samples from the different solutions in which they had been incubated, samples were flushed in a stream of dry N2. Afterwards, advancing contact angle measurements were performed on an OCA 20 L system (Dataphysics Instruments GmbH) with double-distilled water as test liquid and a dose rate of 0.06 μl s−1. For each sample at least three drops were measured and approximately 150 points (4 values s−1) were taken from each measurement.

2.4. Fourier transform infrared spectroscopy–attenuated total reflectance

Film composition was determined by Fourier transform infrared spectroscopy–attenuated total reflectance (FTIR-ATR) spectroscopy on a Nicolet 5700 FTIR spectrometer (Thermo Scientific Inc.) equipped with a mercury cadmium telluride detector and a DuraSampIR single-reflection 45° diamond crystal. The spectra were taken with non-polarized light at a spectral resolution of 4 cm−1 and 64 scans were made. To identify characteristic absorption bands, spectra were achieved by subtracting the PLAd spectrum from the spectrum of PPTMS, PAPTMMS or PN2H2.

Coating thickness was roughly calculated based on the FTIR-ATR spectra and refractive index of the foil and coating. For this the coating was deposited on a polyethylene terephthalate foil (Mylar). The thickness was estimated by the attenuation of the characteristic C=O absorption band from the substrate, which is reduced by the deposited film. To estimate the film thickness the following formula was applied:

\[ d = \frac{dp}{2} \ln \left( \frac{a_{sub}(0)}{a_{sub}(d)} \right), \]

where \( a_{sub}(0) \) is the area of the absorption band at 1720 cm−1 from an uncoated substrate, \( a_{sub}(d) \) the area of the absorption band at 1720 cm−1 after film deposition and \( dp \) the penetration depth of...
in the CPD chamber, samples of approximately 1 cm$^2$ were cut with PBS and the 125I-labeled BSA (125I-BSA)-containing fractions were added to PLAd, PTMS, APTMS and PN2/H2, respectively. After exposure to the plasma, the samples were rinsed three times with PBS. Samples were measured in a well-type gamma counter (Wizard 1480, Wallac) along with a known fraction of the total activity added. The disc-associated activity was expressed as a fraction of the added activity.

2.8. Cell culturing

MC3T3-E1 cells (subclone 14) were expanded in basic medium (BM) consisting of α-MEM (Life Technologies Corporation), 10% fetal bovine serum (FBS, Lonza Group Ltd), 2 mM l-glutamine (Life Technologies Corporation) and 100 U ml$^{-1}$ penicillin and 100 g ml$^{-1}$ streptomycin (Life Technologies Corporation). During expansion phase, medium was refreshed every 2 days and cells were trypsinized upon reaching ~80% confluency to subculture on PLAd discs. Subsequently a cell suspension was prepared in fresh BM and directly pipetted on the PLA discs placed in wells of 6 well plates with the non-treated side facing the bottom of the well. Cells were allowed to attach overnight (O/N) and medium was changed to either BM or osteogenic differentiation medium (OM), comprising BM supplemented with 0.2 mM ascorbic acid (AA, Sigma–Aldrich Co., A8960) and 100 ng ml$^{-1}$ of rh-2 (Hangzhou Biodoor Biotechnology Co.). All cell culture experiments were performed at 37 °C in a 5% CO2 humid atmosphere.

2.9. Methylene blue staining

Cells were rinsed with PBS and then fixed in 10% formalin (Sigma–Aldrich Co.) for 15 min. After rinsing twice with dH2O, 1% w/v methylene blue in 0.1 M borax (Sigma–Aldrich Co.) was added drop by drop until the surface of samples was covered. Samples were incubated for 1 min in staining solution and afterwards rinsed several times with dH2O until all excess staining solution was removed. Samples were analyzed with a stereomicroscope (Nikon SMZ-10A with Sony 3CCD camera).

2.10. Polymerase chain reaction

Samples were rinsed with PBS and transferred to new wells of 6 well plates. Total RNA was isolated using the NucleoSpin®RNA II isolation kit (Macherey-Nagel GmbH & Co.) in accordance with the manufacturer’s protocol. RNA was collected in RNase-free water and the total quantity analyzed by spectrophotometry. cDNA was synthesized from 174 ng total RNA using iScript (Bio-Rad Laboratories Inc.). 1 μl of undiluted cDNA was used for quantitative polymerase chain reaction (PCR) analysis, which was performed on a MyIQ single-color real-time PCR detection system (BioRad). MyIQ data was analyzed using iQ$^{	ext{™}}$ optical system software (Bio-Rad Laboratories Inc.). Ct values were normalized to the GAPDH housekeeping gene and the comparative ΔCt method (Ct control–Ct sample) was used to calculate fold inductions. Primer sequences are given in Table 2.

### Table 1

<table>
<thead>
<tr>
<th>Process gas</th>
<th>Film-forming agent</th>
<th>Power [W]</th>
<th>Exposure time [s]$^b$</th>
<th>Sample$^c$</th>
</tr>
</thead>
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<tr>
<td>He</td>
<td>APTMS$^d$</td>
<td>50</td>
<td>20</td>
<td>$P_{\text{APTMS}}$</td>
</tr>
<tr>
<td>He</td>
<td>TMS$^e$</td>
<td>50</td>
<td>40</td>
<td>$P_{\text{TMS}}$</td>
</tr>
<tr>
<td>N$_2$ + 3.4% H$_2$</td>
<td>n/a$^f$</td>
<td>150</td>
<td>36</td>
<td>$P_{\text{N2/H2}}$</td>
</tr>
</tbody>
</table>

$^a$ Process gas can be the vapor phase of a monomer or a mixture of gases.

$^b$ Exposure time to the electric discharge.

$^c$ Resulting samples were named according to treatment received.

$^d$ 3-Aminopropyl-trimethoxysilane.

$^e$ Tetramethylsilane.

$^f$ Not applicable.

### Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>GAPDH$^a$</td>
<td>5'-TGGCAACTTGAGATTTGCTGC-3'</td>
</tr>
<tr>
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<td>5'-AGATGCTATGAGGCTCCCG-3'</td>
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<tr>
<td>Bone sialoprotein</td>
<td>5'-CTCGAGCAGCGACTGCCGA-3'</td>
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<tr>
<td></td>
<td>5'-TGGCAAGAAGCAACGGCCATCCCC-3'</td>
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<tr>
<td>Osteocalcin</td>
<td>5'-CAGACTTACAGACACATCACAGG-3'</td>
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<td></td>
<td>5'-AGTCAACGAGAGACAGAGACA-3'</td>
</tr>
<tr>
<td>Osterix</td>
<td>5'-ATGGGCTTCTCCTGTTCG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCCTGGCTGCGCCCTCAAG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AGCATTCTCCAGTGCCGGC-3'</td>
</tr>
</tbody>
</table>

$^a$ Glyceraldehyde 3-phosphate dehydrogenase.

$^b$ Runt-related transcription factor.
2.11. Statistical analysis

For each test, three replicates of each condition were used and statistical analysis performed using SPSS Statistics 18.0, with one-way ANOVA and Tukey’s multiple comparison test or Student’s t-test, in the case of Fig. 5, between time points for each individual treatment. In all cases the P-values were considered statistically significant as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

3. Results

3.1. Gas plasma treatment changes the chemical composition of PLA surfaces

PLAd were treated with gas plasma at atmospheric pressure in a dielectric discharge barrier configuration (Table 1). Addition of TMS or APPTMS caused a thin coating (50–150 nm) to be deposited on the surface. In contrast, when treating the samples with N2/H2 (PN2/H2), only the chemical composition of the original surface was modified and no coating was deposited. However, to confirm that all treatments effectively changed the PLAd surface chemical composition, FTIR-ATR was performed (Fig. 1). Surface modification with N2/H2 led to incorporation of N-containing functional groups (e.g. primary and secondary amino groups, amidates, imides), evidenced by the characteristic absorption band of the vibrational band N–H at wave-numbers larger than 3000 cm$^{-1}$ as well as the C–N deformation vibration at ~1650 cm$^{-1}$. Due to residual oxygen in the gas mixture hydroxyl groups were introduced on the surface as well. Primary amino groups were also achieved by film deposition of APPTMS, as shown in Fig. 1 (PAPPTMS). The film is characterized by a Si–O–Si network, as well as by Si–O–C groups, which did not undergo a condensation reaction (absorption band at ~1100 cm$^{-1}$). Hydrogen bonds, characterized by the broad band at wave-numbers larger than 3000 cm$^{-1}$, belong to the Si–O–H and N–H vibrational bands. Deposition of TMS led to a thin film mainly consisting of Si–(CH$_3$)$_x$ (x = 1, 2, 3). Characteristic absorption bands (2900–2800 cm$^{-1}$) C–H, 1290–1850 cm$^{-1}$ Si–(CH$_3$)$_x$Si–C) can be observed in the corresponding FTIR spectrum. The small absorption at ~2100 cm$^{-1}$ shows that Si–H bonds are also formed during film deposition.

3.2. Altered biomaterial properties on the treated surfaces

Hydrophobicity was determined by measuring water contact angles on both non-treated samples (PLAd) and samples immediately after gas plasma treatment. In addition, wettability was assessed after the disinfection procedure using SPSS Statistics 18.0, with one-way ANOVA and Tukey’s multiple comparison test or Student’s t-test, in the case of Fig. 5, between time points for each individual treatment. In all cases the P-values were considered statistically significant as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

Based on AFM analysis, roughness was quantified as root mean squared roughness Rq. Treatment with N2/H2 increased Rq from 1.14 to 12.2 nm, whereas PPTMS and APPTMS had lower Rq than that of PLAd (0.9 and 0.61 nm, whereas before the treatment it had been 1.14 nm, see Fig. 3A).

Next, we used HIM to evaluate surface topology after incubation with FBS, the protein source in cell culture. Fig. 3B shows that FBS roughens the surface of PLAd, relative to unexposed PLAd. Among the different samples, PPTMS exhibited the roughest surface.

3.3. BSA adsorption

After 3.5 h of incubation at 37 °C, excess 125I-BSA was washed off the surface by rinsing the samples three times in PBS and the adhered protein was quantified (Fig. 4). Adhesion of 125I-BSA was highest on PAPPTMS (67%) and lowest on PN2/H2 (21%). Adsorption of PLAd and PPTMS was 40% and 36%, respectively, but the difference was not statistically significant. Overall, the results demonstrated that differential protein binding occurs through surface treatment.

3.4. MC3T3 adhesion and proliferation

We hypothesized that cellular behavior would be affected by the physicochemical properties of PLAd, PN2/H2, PPTMS, and APPTMS. To investigate this, we chose the pre-osteoblast mouse cell line MC3T3-E1 as a model cell because its transcriptional response to osteogenic signals is well documented. PLAd, PPTMS, PAPPTMS and PN2/H2 were incubated in BM overnight and the next day $50 \times 10^3$ MC3T3-E1 cells were seeded per sample and cultured for 24 and 48 h. At both time points we qualitatively analyzed the cell distribution on the surface by methylene blue staining. We chose early time points to describe the early molecular response of the cells to the surfaces, rather than to describe the downstream consequence on osteogenesis at a later time point.

After 24 h, MC3T3-E1 cells were homogeneously distributed on PPN2/H2 and PAPPTMS discs, whereas on PLAd and PPTMS they were not (Fig. 5A, top row). The characteristic of PPTMS was cell alignment in certain areas of the surface. Methylene blue staining indicated that the cell numbers decreased after 48 h in the case of PPTMS, and this was confirmed by DNA assay (Fig. 5B). In contrast, cell density seemed higher after 48 h, in PAPPTMS, consistent with DNA assay, although in this case the difference was not statistically significant. Cell numbers did not seem to be significantly affected in the case of PLAd and PN2/H2 within the time frame studied.

3.5. Osteogenesis of MC3T3-E1 cells

Two and four days after seeding MC3T3-E1 cells on PLAd, PN2/H2, PPTMS and APPTMS, gene expression of Runx-2, osterix, osteocalcin and bone sialoprotein was analyzed by PCR. Overall, we observed that, as expected, cells treated with OM exhibited higher expression of osteogenic markers than cells treated with BM (Fig. 6). For all tested genes, cells cultured in BM on PAPPTMS exhibited lower expression than cells cultured on control samples (PLAd), but not when cultured in OM, suggesting that rhBMP-2 and AA can rescue a possible negative effect of PAPPTMS on osteogenesis of MC3T3 cells.

Cells cultured on PN2/H2 showed levels of expression for all genes at both time points very similar to those of cells cultured on PLAd. In the case of PPTMS, expression of osterix and Runx-2 genes was enhanced in OM when compared to the other treatments on day 2. Most notable, however, were the levels of BSP and OC gene expression, for both BM and OM. Expression of BSP and OC genes were increased approximately 10-fold in BM and 30-fold in OM, compared to PLAd and PN2/H2 when cultured in OM, suggesting that rhBMP-2 and AA can rescue a possible negative effect of PAPPTMS on osteogenesis of MC3T3 cells.

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approximately 20-fold higher in BM relative to cells cultured in PLAd in BM.

4. Discussion

Here we showed that gas plasma technology at atmospheric pressure could modify PLA to successfully enhance osteogenic differentiation of pre-osteoblast cells. PLA discs were subjected to an electric discharge at atmospheric pressure in the presence of He containing tetramethylsilane (PTMS), 3-aminopropyl-trimethoxysilane (PAPTMS) or N₂/H₂ (PN₂/H₂). The surface of these samples was modified, as confirmed by FTIR-ATR (Fig. 1), according to the treatment: in PTMS, methylene (–CH₂–) and methyl (–CH₃) groups were mainly present; in PN₂/H₂ amine groups (–NH– and –NH₂) and on PAPTMS silanol groups (Si–OH–) were detected, in addition to amine groups. The hydrophobicity of the discs differed and could be summarized from more hydrophobic to least as PTMS > PLAd > PAPTMS > PN₂/H₂, immediately after the treatment (Fig. 2). Disinfection with ethanol altered hydrophobicity to PTMS > PLAd > PN₂/H₂ > PAPTMS, but the original order was recovered after incubation in BM (Fig. 2). Although used here as the disinfection agent, ethanol had an impact on the surface properties, as did O/N incubation in BM, possibly due to protein adsorption. Hence it is important to realize that the properties of these biomaterials do not remain unaffected once the treatment is finished; rather, subsequent steps can alter them before cells come into contact with the surface. In a clinical scenario, where it is more likely that γ-irradiation will be used as sterilization agent in preference to ethanol, the properties of these discs could be altered in different ways to that described here. Therefore, we also assessed the wettability behavior after γ-irradiation and observed that γ-irradiation affected the wetting behavior in a similar fashion to ethanol by increasing the wettability of PAPTMS and PLAd and decreasing that of PN₂/H₂. However, PTMS became slightly more hydrophilic with γ-irradiation, whereas with ethanol it became more hydrophobic. Nevertheless PTMS remained, after both ethanol and γ-irradiation treatment, the most hydrophobic sample.

All treatments had an effect on surface roughness as measured by AFM (Fig. 3A). The results showed the following trend from
highest \( R_q \) to lowest: \( \text{PN2/H2} > \text{PLAd} > \text{PTMS} > \text{PAPTMS} \). This suggests that film deposition smoothens the surface (\( R_q \) values close to \( \text{PLAd} \)), whereas treatment with \( \text{N2/H2} \) not only changes chemical groups at the surface but also roughens it. However, protein adsorption can again induce topographical changes, just as it did with the wettability of some samples. For instance, although AFM showed that \( \text{PAPTMS} \) and \( \text{PTMS} \) surfaces after treatment, incubation in FBS (\(+FBS\)) changed this and Fig. 3B suggests that \( \text{PTMS} \) exhibited a rougher surface.

In respect to cellular adhesion, after 24 h, representative images of cellular patterning suggest that there were fewer MC3T3-E1 cells adhering to \( \text{PTMS} \) than to the other samples. This treatment also had a negative effect on proliferation, as confirmed by DNA quantification (Fig. 5B). It was also observed that MC3T3-E1 cells were heterogeneously distributed on \( \text{PTMS} \), forming characteristic cell lines (Fig. 5A). Most likely this was not due to mechanical injury of the surface: if that had been the case, injuries in discs treated differently would be expected as well. It might be that with this particular treatment the distribution of chemical groups leads to uneven protein adsorption and consequently cell adhesion. Nonetheless, Philips et al. also showed that adhesion of cells to –CH3 surfaces was poor and could only be comparable to other surfaces when coated with fibronectin [16]. In addition, Curran et al. showed that cells cultured on –CH3 surfaces appeared in clusters and were not homogeneously spread on the surface, similar to what we observed. By contrast, cells cultured on –NH2 and –SH surfaces showed well-spread morphology and by day 7 were well distributed on the surface [43]. We also observed homogeneous cell distribution in surfaces containing amine groups (\( \text{PN2/H2} \) and \( \text{PAPTMS} \)). Within the time points studied, none of the surfaces seemed to have had a significant positive effect on cell proliferation, although methylene blue staining of cells cultured on \( \text{PAPTMS} \) suggested an increase from 24 to 48 h. Although not statistically significant, observations were consistent with DNA quantification (Fig. 5B). A note should be made though that some cells could have detached during washing steps, which was not evaluated, that could have led to an underrepresentation of cell amounts in Fig. 5.

Expression of osteogenic markers on MC3T3 cells varied according to the sample in which they were cultured (Fig. 6), implying that the specific gas plasma treatments changed the key physico-chemical properties of PLA that play a role in cell differentiation. Overall, gene expression in less pro-osteogenic surfaces seemed to be time dependent, since expression of most markers is increased by day 4 compared to day 2. Thus osteogenic differentiation could be enhanced at later time points in the case of, for instance, \( \text{PAPTMS} \). Such delay could prove beneficial as it would enable a combination of high cell numbers and osteogenic differentiation to occur in the same sample.
The combination of PAPTMS and BM had a negative effect on expression of all markers, rescued by AA and rhBMP-2. Cells cultured on PN2/H2 exhibited similar levels of expression for all genes and time points as that of cells cultured on PLAd, suggesting that treatment with N2/H2 did not change surface key parameters that might regulate cell differentiation. Treatment with TMS had a positive effect on osteogenesis of MC3T3 cells, since expression of BSP and OC genes was induced in cells cultured on PTMS already in BM. Although these large fold inductions were not observed in the case of osterix and Runx-2, PTMS slightly enhanced expression of these genes at day 2 in OM. It is also interesting to note that treatment with TMS induced aggregation of cells on certain areas of the surface rather than a homogeneous distribution. A positive effect of cell aggregation on osteogenesis has been reported previously in hMSCs [44,45].

If only the chemical groups that are characteristic of these surfaces are considered (and ignoring other surface characteristics), one could point out that –CH2 and –CH3 groups had a positive effect on osteogenesis of MC3T3-E1 cells. However, others have shown that osteogenesis of MC3T3 cells was favored by –NH2 and –OH groups [22], consistent with the effects observed on osteogenesis of hMSCs (also favored by –NH2 groups) [15,16]. In our work, –NH2 and –OH were mainly found on PN2/H2 (–NH2) and PAPTMS (–NH2) and these samples had a neutral or even negative effect on osteogenesis of MC3T3-E1 cells. Such contradictory results can be explained by the fact that the substrates used by others, silica or gold, were different than the substrate we used, PLAd. Furthermore, other chemical groups besides the ones indicated were deposited by gas plasma, and therefore a straightforward comparison is not possible. In addition, Curran et al. showed that introducing the same chemical group using different silane-introducing chemistries can already lead to differences in the expression of some markers within the same cell type [46].

A clear link between the physicochemical properties of PLA-modified surfaces and the cellular responses of MC3T3-E1 cells could not be established. However, cellular adhesion was favored by surfaces with similar chemistry but different roughness (PN2/H2 and PAPTMS), and, furthermore, osteogenic differentiation was enhanced by PTMS which had a roughness, though not a chemistry, comparable to that of PAPTMS. These results suggest that the chemical composition of the PLA surface is more important in determining cellular adhesion and osteogenic differentiation of MC3T3-E1 cells than roughness. Interestingly, the relevance of chemistry to roughness effects was also highlighted by the attachment, proliferation and viability of hUVECs when cultured on gas plasma treated PLA [31]. In Fig. 4 we also showed that amounts of protein adsorbed are dependent on the surface properties. Besides the quantity, the quality of adhered proteins might also change, or even the domains of the same protein presented to the cells might vary according to the surface characteristics. This is relevant in the case of cell-adhesion proteins such as fibronectin, fibrinogen, vitronectin, laminin and collagen [20,21,47,48], which could have a determining effect in regulating cell adhesion, spreading and consequently fate.

Future work will include the response analysis of more clinically relevant cell types, such as hMSCs, to these surfaces and in 3-D scaffolds, since the treatment can be easily applied to 3-D structures. The ease of gas plasma treatment makes it an appealing technique for quickly transforming biomaterials properties and the results shown here make it a promising tool for the development of an effective generation of bone graft substitutes.
5. Conclusions

Gas plasma at atmospheric pressure was used to modify the physicochemical properties of PLA discs. The chemical composition, hydrophobicity/hydrophilicity and topography of the surface changed according to the treatment used. MC3T3-E1 cell adhesion and proliferation was markedly influenced by the surface in which they were cultured. Treatment with TMS resulted in adhesion of few cells, aggregated heterogeneously across the surface, and had a negative effect on proliferation. By contrast, APTMS treatment allowed cells to spread homogeneously across the surface and enhanced proliferation. Interestingly, TMS enhanced expression of OC and BSP genes, whereas APTMS treatment had a negative effect on cell differentiation. Cells cultured on PLA without any treatment showed similar responses in terms of differentiation as cells cultured on surfaces treated with N₂/H₂.

Modifications in PLA by gas plasma treatment at atmospheric pressure helped to successfully guide osteogenesis in vitro. These results provide a solid basis for further investigation of gas plasma modified 3-D scaffolds as potential bone graft substitutes.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1, 3 and 5, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.04.021.