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Multilayer coatings on biomaterials for control of MG-63 osteoblast adhesion and growth

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Abstract Here, the layer-by-layer technique (LbL) was used to modify glass as model biomaterial with multilayers of chitosan and heparin to control the interaction with MG-63 osteoblast-like cells. Different pH values during multilayer formation were applied to control their physicochemical properties. In the absence of adhesive proteins like plasma fibronectin (pFN) both plain layers were rather cytophobic. Hence, the preadsorption of pFN was used to enhance cell adhesion which was strongly dependent on pH. Comparing the adhesion promoting effects of pFN with an engineered repeat of the FN III fragment and collagen I which both lack a heparin binding domain it was found that multilayers could bind pFN specifically because only this protein was capable of promoting cell adhesion. Multilayer surfaces that inhibited MG-63 adhesion did also cause a decreased cell growth in the presence of serum, while an enhanced adhesion of cells was connected to an improved cell growth.

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1 Introduction

There is a broad variety of materials used in the field of tissue replacement and engineering (TE) from ceramics to metals and polymers. The majority of biomedical applications of biomaterials requires both a mechanical support as well as a biocompatible surface e.g. for bone replacement [1]. While many biomaterials approved for medical applications fulfil the first requirement, their biocompatibility may not always be sufficient. Therefore, various surface modification techniques have been developed to control the interaction of implant materials with the surrounding tissue [2, 3]. Particularly important is the enhancement of cell adhesion on the surface of bone implants to promote growth and differentiation of osteoblasts for subsequent integration into the tissue with minimal foreign body reaction [4]. Other biomaterial applications may need a distinct regulation of cell adhesion. This can be favourable to promote growth and differentiation of epithelial cells like hepatocytes [5] or to support growth of epithelial cells and hinder that of connective tissue cells in co-culture systems and clinical settings [6].

Besides the large range of physical and chemical modification techniques to enhance the biocompatibility of implants, alternative approaches focus on biomimetic surface coatings that contain specific ligands for cellular adhesion and growth receptors [7]. A large number of adhesive proteins or biologically active peptides have been immobilized on material surfaces to improve their tissue compatibility [8–10]. However, it can be stated that the binding of bioactive polysaccharides to improve the tissue compatibility of biomaterials has been used less frequently. Indeed, the anticoagulant effects of heparin have been explored by its immobilisation on biomaterial surfaces to

prevent blood clotting since decades [11, 12]. Yet, beside its anticoagulant effects which are mainly based on specific interaction of heparin with antithrombin III [12, 13], heparin has a multitude of other binding partners such as a variety of adhesive proteins e.g. fibronectin and growth factors e.g. fibroblastic growth factors [14, 15]. Therefore, the immobilisation of heparin on materials surfaces may promote the selective binding of bioactive proteins from the surrounding liquids (e.g. serum) which makes this glycosaminoglycan specifically interesting. This is also demonstrated by its increasing use for preparation and modification of implant materials and tissue engineering scaffolds [16, 17].

Many of the surface modification techniques, which are currently in use, are based on covalent binding of bioactive molecules. This may generate certain problems in the modification of biomaterials particularly for hard tissue replacement, because the majority of used materials like metals or ceramics do not provide any bindings sites for organic chemical reactions. Therefore, a first modification step is necessary to generate chemically active species for example by silanization of glass materials, chemical vapour deposition on metals etc. as a preconditioning step for the subsequent reaction with proteins, peptides or other ligands [18]. Within this context, it is also noteworthy that covalent binding of bioactive species like proteins can reduce their activity or even denature them [19]. Therefore, as an alternative approach for binding molecules their physical adsorption has been applied [18] which may lead to stable immobilisation of factors maintaining their biological activity [5].

More than a decade ago, the adsorption of macromolecules with ionogenic groups (polyelectrolytes) to surfaces was introduced as layer-by-layer technique (LbL) by Decher [20]. It requires a charged material surface, which is then alternately covered by polyelectrolytes of opposite charge due to mainly electrostatic interactions. The overcompensation of charge which occurs during the adsorption of each polyelectrolyte leads to a net charge of opposite sign after each step and enables the subsequent adsorption of the corresponding counter polyion. Generally, this process can be repeated many times and leads to the formation of polyelectrolyte multilayers (PEMs) which have been shown to be thermally and mechanically stable [7, 21, 22]. The adsorption process can be controlled by several parameters such as the pH value, ionic strength, temperature etc. due to the dependence of (weak) polyelectrolyte conformation and charge on environmental conditions. Hence, the resulting PEMs may exhibit varying features in terms of layer organization, thickness, topography, surface charge and wettability [22]. Thereby, the LbL method is a very versatile and fairly inexpensive way to modify potentially any kind of charged surface with a remarkable number of molecules. All biological macromolecules like DNA, glycosaminoglycans and proteins may be charged depending on pH value and ionic strength. Hence, they represent polyelectrolytes which can be used to form biomimetic multilayers on biomaterial surfaces [7, 21, 23].

It was the aim of this study to obtain a control over adhesion and growth of osteoblasts by surface modification of biomaterials using the LbL method. In this way, modification of implants can be envisaged which shall facilitate a later integration into the tissue. Biogenic molecules such as chitosan, an anti-inflammatory and anti-microbial polysaccharide [24], and heparin, a glycosaminoglycan with high affinity to a variety of adhesive proteins and growth factors [25], were used to build up to 10 layers (5 bilayers) on glass slides. Glass was chosen as a model substrate due to its comparability to bioactive glass and ceramics used in current implantation practice. Since the pH value of the PEL solution can have a great impact on the multilayer morphology and surface properties [22], the effect of pH value was studied particularly in respect to the adsorption of distinct cell adhesive proteins and the corresponding adhesive response of osteoblast-like MG-63 cells.

2 Materials and methods

2.1 Materials

Glass cover slips (Menzel, Germany) were cleaned using 0.5 M NaOH (Roth, Germany) in 96% ethanol (Roth, Germany) for 2 h at room temperature followed by excessive rinsing with MilliQ water (0.055 μS/cm). New gold coated sensors for SPR measurements were cleaned using ethanol p.a. (99.8%, Merck, Germany) and excessive rinsing with MilliQ water. Sensors were immediately incubated over night in a solution of 2 mM mercaptoundecanoic acid (95%, Sigma, Germany) diluted in ethanol p.a. to obtain a negatively charged surface similar to glass. Rinsing was realized using ethanol followed by MilliQ water.

Polyelectrolyte solutions were prepared as follows: poly (ethylenimine) (MW 750,000 g/mol; 18,197–8, Sigma, Germany), heparin (min. 150 I.U./mg, MW 8,000–25,000 g/mol; Applichem, Germany) and chitosan with a deacetylation degree of 85% (MW 500,000 g/ mol; 85/500/A1, Heppe, Germany) were dissolved in MilliQ water containing 0.14 M NaCl (Roth, Germany) at a concentration of 2 mg/ml under stirring. Chitosan solution contained 0.05 M acetic acid (Roth, Germany) and was solubilised at 50°C for 1 h. The pH values of the solutions were adjusted using HCl (Merck, Germany) and NaOH followed by a filtration step using 0.45 μm and 0.2 μm pore size PES (poly (estersulfon)) filters.

Plasma fibronectin (pFN, Roche, Germany) reconstituted stock solution was diluted on demand to 0.5 μg/ml or 5 μg/ml in phosphate buffed saline (PBS) pH 7.4. Genetically engineered fibronectin type III repeats (Sigma, Germany) and collagen I (Cytonet, Sofia, Bulgaria) were diluted in the same manner with an additionally higher concentration of 20 μg/ml and 100 μg/ml, respectively.

2.2 Preparation of multilayers—LbL

Polyelectrolyte multilayers (PEMs) on glass cover slips were prepared by covering them first with a poly (ethylenimine) (PEI; P) layer followed by the adsorption of heparin (H) and chitosan (C). Subsequently, heparin and chitosan were used alternately up to the 9th and 10th layer abbreviated as $P(HC)_4$ (PEI plus 4 bilayers of heparin and chitosan) and $P(HC)_4H$ (PEI plus 4 bilayers of heparin and chitosan plus heparin), respectively. As a crucial parameter of the adsorption process the pH value of the PEI and heparin solution were adjusted to pH 5.0, 7.0 or 9.0, respectively, and controlled throughout the LbL process. The pH value of the chitosan solution was kept constant at pH 5.0 because becomes insoluble at higher pH values. The, following abbreviations are used depicting the pH value of the corresponding solution: P5H5C5, P7H7C5 and P9H9C5. Each polyelectrolyte coating step was followed by washing the cover slips for 15 min with MilliQ water of the same pH value as the previous polyelectrolyte solution. Coated cover slips were dried under a stream of nitrogen and stored in an exsiccator.

2.3 Physico-chemical characterization of multilayers

Static contact angles using fresh ultra pure water (MilliQ) were measured using the sessile drop method with an OCA15+ device from Dataphysics (Filderstadt, Germany) to obtain information about the wettability of the samples. Three cover slips per pH combination and terminal layer were measured with five droplets of 3 μL each. Angles below 10°C were considered as zero.

Surface plasmon resonance measurements were done with an iSPR from IBIS Technologies (Hengelo, The Netherlands) to monitor the increase in mass with each adsorption step. The shift in the resonance angles from 4 regions of interest per sensor were measured using the SPR software 3.1.21 and later on evaluated with the iSPR analyse data software.

2.4 Cell culture

MG-63 osteoblast-like cells were used to measure cell adhesion on the different multilayer coatings. Cells were cultured in Dulbeccos modified Eagle medium (DMEM, Biochrom AG, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom, Germany) and 1% antibiotic mixture (Sigma, Germany). For cell adhesion experiments MG-63 cells were harvested with Trypsin/EDTA (Sigma, Germany). Cells were subsequently washed with DMEM and finally resuspended in DMEM with or without 10% FBS at a concentration of 25,000 cells/ml.

2.5 Cell adhesion

Modified cover slips were placed into 12-well tissue culture plates. Sterilisation was done using 70% ethanol for 10 min followed by excessive washing with sterile PBS. For some of the experiments multilayer modified slides were pre-coated with different proteins: plasma fibronectin (pFN), fibronectin type III repeat (FN III) and collagen I (coll I) which were dissolved in PBS pH 7.4 in defined concentrations and used separately to cover the PEMs $P(HC)_4$ and $P(HC)_4H$. After rinsing the modified cover slips with PBS the corresponding protein solution was introduced and incubated for 30 min at 37°C in humidified CO_2 incubator followed by twice washing with PBS. Uncoated multilayers and plain glass surfaces were used for comparison (incubation in PBS only).

To investigate cell adhesion and morphology on the different PEMs 2.5×10^4 cells/well were seeded in 1.0 ml serum free medium. After 4 h of incubation the samples were washed with PBS to remove non-adherent cells followed by fixation with 4% (w/v) phosphate buffered formaldehyde solution (Roti-Histofix, Roth, Germany) for 5 min at room temperature. Phase contrast pictures were taken at the end to analyse the overall cell morphology and to quantify cell adhesion and spreading. Morphological parameters such as the cell area and the perimeter were evaluated using automated image analysis software (analySIS 3.00, Soft Imaging System GmbH, Germany). The roundness of the cell was calculated giving a value between 0 and 1.0, where 1.0 represents a perfectly circular cell shape. For statistics, the cell adhesion experiments were done in triplicate and 5 photographs were taken per cover slip. Mean values and standard deviations were calculated accordingly.

2.6 Cell proliferation

Glass slides with PEMs were placed into 12-well tissue culture plates. Sterilisation was done using 70% ethanol for 10 min which was followed by excessive washing with sterile PBS. Then, 1 ml of serum-containing cell suspension (2.5×10^4 cells/well in DMEM with 10% FBS) was added and incubated for 1, 3 and 6 days at 37°C in a humidified 5% CO_2 incubator. A modified LDH (lactate dehydrogenase) cytotoxicity assay (number II, BioVision, USA) was applied to quantify the number of cells. Briefly,

if the culture period was finished, samples were transferred to new 12-well-plates. Then, 500 µl of 0.5% (v/v) Triton X-100 were added to each well for lysis of all viable cells. The plates were incubated for 30 min at room temperature under slow rotation. Following, 10 µl of the supernatant was transferred to a transparent 96-well-plate. To start the reaction 100 µl of the LDH reaction mix were added to each well. The plates were wrapped up in aluminium foil and agitated for 30 min at 160 rpm at room temperature. To terminate the reaction 10 µl stop solution from the LDH assay kit were added to each well. The absorbance of the solution was measured at 492 nm using a plate reader (FLUOstar, Optima, Germany). For statistics, the cell proliferation experiments were performed in triplicate. Mean values and standard deviations were calculated.

2.7 Statistics

Results are shown as mean values ± standard deviation. For statistical evaluation the student's *t*-test has been applied. The number of samples has been indicated in the figure captions. The significance level is $\alpha = 0.05$ (indicated by asterisks).

3 Results

3.1 Physico-chemical investigations

The surface wettability of layers was investigated by static water contact angle (CA) measurements. Figure 1 shows the CAs of clean glass and all subsequent layers up to the 10th (H terminated) layer $P(HC)_4H$. Dry clean glass, which was stored in an exsiccator over night showed a CA value of about 40° (hatched column). In general, contact angles increased upon poly (ethylenimine) adsorption (layer number 1). Heparin terminated multilayers (even numbers) were more hydrophilic than chitosan terminated ones (all other odd numbers) which was especially valid for pH combinations P5H5C5 and P7H7C5. An oscillation of the values (layer after layer) between H and C terminated layers was detected which indicates a change of the terminating molecule. It was also observed that the CA was dependent on the pH value of the polyelectrolyte solutions. The CAs varied in absolute height and difference between H and C terminated PEMs from P5H5C5 (white) to P7H7C5 (patterned) and P9H9C5 (black). In tendency, the CA value difference from one to the next layer was highest for P5H5C5, i.e. chitosan terminated layers show the highest CAs and heparin terminated layers show the lowest values among the pH setups. The difference between C and H terminated layers was smaller for P7H7C5, especially for the lower number of layers. Absolute values for H terminated layers were higher than for P5H5C5 and smaller for C terminated layers compared to P5H5C5. The pH combination P9H9C5 resulted in the lowest difference between C and H and occasionally led to heparin terminated layers with higher CAs than the corresponding chitosan terminated layers. Focussing on the 9th ($P(HC)_4$) and 10th ($P(HC)_4H$) layer it can be seen that for the pH combinations P5H5C5 and P7H7C5 the contact angles for chitosan terminated PEMs are significantly higher compared to heparin terminated multilayers. For P9H9C5 on the other hand, the CA values for chitosan terminated layers ($P(HC)_4$) are lower than for $P(HC)_4H$. Over all, a layer formation up to ten layers can be demonstrated.

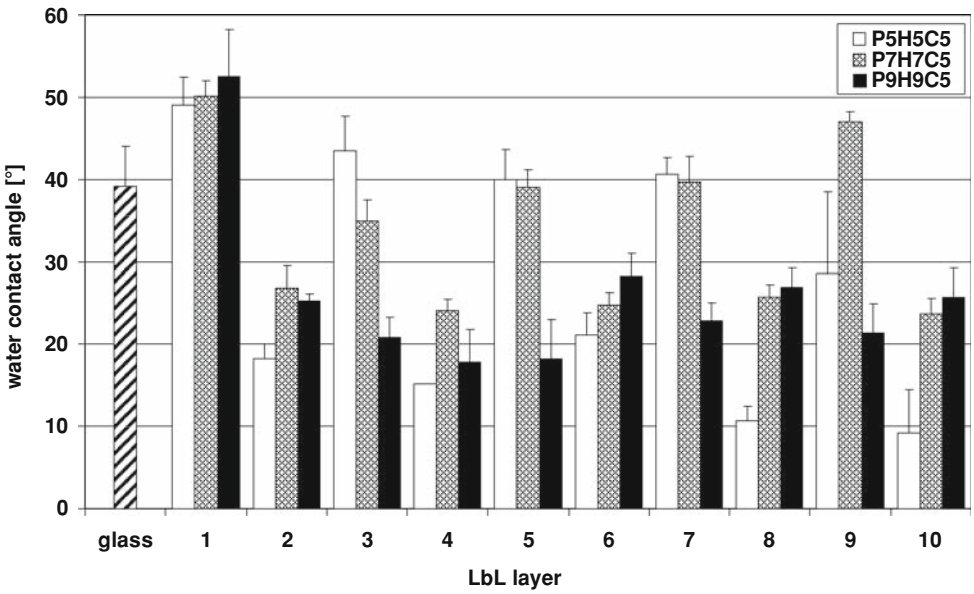

Fig. 1 Static water contact angles of plain glass (hatched bar) and PEMs up to 10 layers (1..10; 1 = P, all other odd numbers = C, even numbers = H); wettability is plotted as a function of pH during layer formation: P5H5C5 (white), P7H7C5 (dotted), P9H9C5 (black)

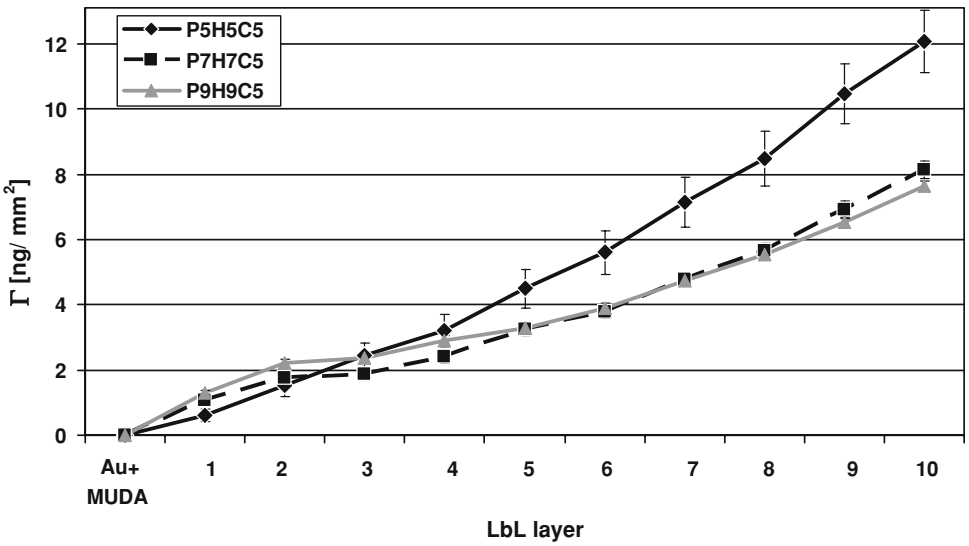

Fig. 2 Surface plasmon resonance measurements of the layer-by-layer assembly of PEMs up to 10 layers (1..10; 1 = P, all other odd numbers = C, even numbers = H) showing the layer mass increase per area Γ as a function of solution pH value combination: P5H5C5 (spades, black full line), P7H7C5 (squares, black dotted line) and P9H9C5 (triangles, grey line)

SPR measurements were conducted to evaluate the mass increase by adsorption of polyelectrolytes on the sensor surface during multilayer formation which can be seen in Fig. 2. The adsorption of poly (ethylenimine) reveals a strong dependence on the pH of the solution. The higher the pH is the more mass was adsorbed to the surface. When focussing on the subsequent adsorption it could be demonstrated that the highest multilayer mass after 10 layers was obtained for the P5H5C5 combination while the gain in mass was significantly reduced for P7H7C5 followed by P9H9C5.

3.2 Biological investigations

First, multilayers with chitosan (middle three groups of columns) and heparin (right three groups of columns) as terminating layer with and without pre-coating of pFN were investigated with respect to MG-63 cell adhesion. The results shown in Fig. 3 reveal that all plain multilayers (white columns) either terminated with C or H were fairly cytophobic since only a small number of cells adhered. There was also no significant effect of the pH value during multilayer formation on cell adhesion except for the chitosan terminated PEMs of P7H7C5 (pH 7) with a slightly elevated number of cells. The pre-adsorption of only 0.5 μg/ml pFN caused a considerable increase in cell adhesion on all terminal layers (patterned columns). Chitosan terminated PEMs from setups P5H5C5 (pH 5) and P7H7C5 provide higher values than heparin terminated multilayers from the same pH combination. Generally, it was observed that the pH combination P9H9C5 (pH 9) was more adhesive for MG-63 cells than P7H7C5 followed by P5H5C5. Figure 3 also shows that the adhesion of MG-63 cells after pre-adsorption of 5 μg/ml pFN (black columns)

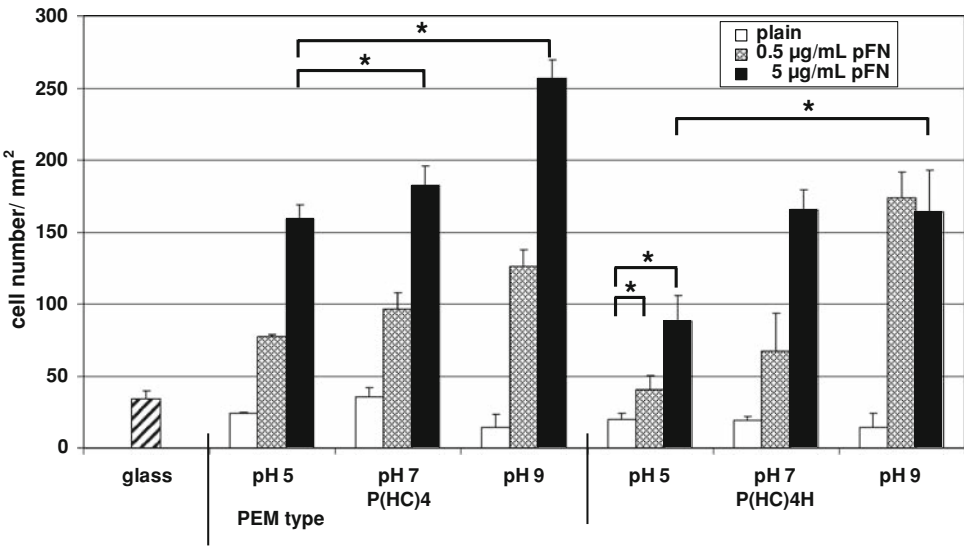

Fig. 3 Adhesion of MG-63 cells after 4 h incubation on plain glass (hatched bar), plain PEMs (white) and PEMs coated with 0.5 μg/ml pFN (dotted) or 5 μg/ml pFN (black), respectively, and produced under various pH value conditions (P5H5C5 = pH 5/ P7H7C5 = pH 7/ P9H9C5 = pH 9); middle group of columns: $P(HC)_4$ and right group of columns: $P(HC)_4H$ ($n = 15$)

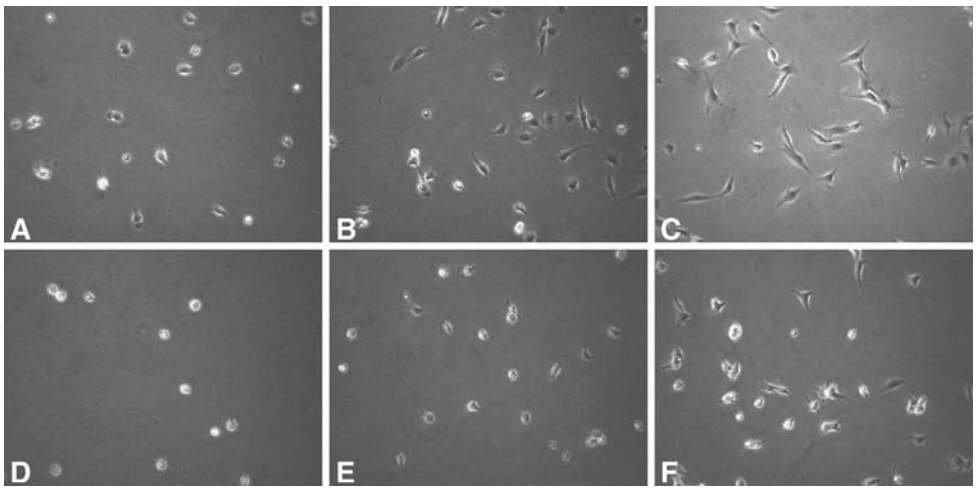

Fig. 4 MG-63 cell photographs on PEMs coated with 0.5 μg/ml pFN; chitosan terminated $P(HC)_4$ (**a**–**c**) and heparin terminated $P(HC)_4H$ (**d**–**f**) on P5H5C5 (**a**, **d**), P7H7C5 (**b**, **e**) and P9H9C5 (**c**, **f**), magnification 200×

was more than twice as high as for the 0.5 μg/ml pFN samples in most cases. In agreement with the lower pFN concentration most cells were detected on P9H9C5 followed by P7H7C5 and P5H5C5. A lower adhesion of MG-63 cells was observed on all heparin terminated multilayers which expressed the same dependence on the pH value as found for the chitosan terminated PEMs in tendency.

Besides cell numbers the morphology of cells can be considered as a complementary measure of cell adhesion. The better the cell-biomaterial interaction the more spread and polarized the cell shape becomes. In Fig. 4a–f MG-63 cells show a different behaviour on the different PEM setups after pre-adsorption of 0.5 μg/ml pFN. As a result the shape of the cells is in general more round on heparin terminated PEMs (d–f) and cells spread more on chitosan terminated PEMs (a–c). That means $P(HC)_4$ promotes the cell adhesion whereas $P(HC)_4H$ leads to a lower attachment and spreading. There are also differences between the pH setups during multilayer build-up. MG-63 on P5H5C5 (a and d) show the lowest degree of spreading which advances with increasing pH values. Best spreading can be seen on P9H9C5 (c and f). Considering the advantages of chitosan over heparin terminated PEMs the sample P9H9C5 with $P(HC)_4$ (Fig. 4c) demonstrates the most developed cell spreading with very large cells.

In a second approach different proteins were adsorbed onto glass and PEMs, such as plasma fibronectin (pFN), a FN III protein fragment which represents a multimer of the RGD domain but lacks the heparin and cell binding domain of pFN and collagen I (coll I). Since results reveal similar trends for all pH combinations, here only results for the pH combination P7H7C5 are shown. All values in Fig. 5 are depicted as normalized against plain glass (corresponds to the value 1). The pre-adsorption of 5 μg/ml of each protein solution on glass (patterned columns) caused a moderate increase in cell number compared to plain glass. It is important to note that the FN III fragment had the same effect on cell adhesion as pFN. By contrast if the different proteins were pre-adsorbed to PEMs only pFN had a promoting effect on cell adhesion. For comparison see the cell

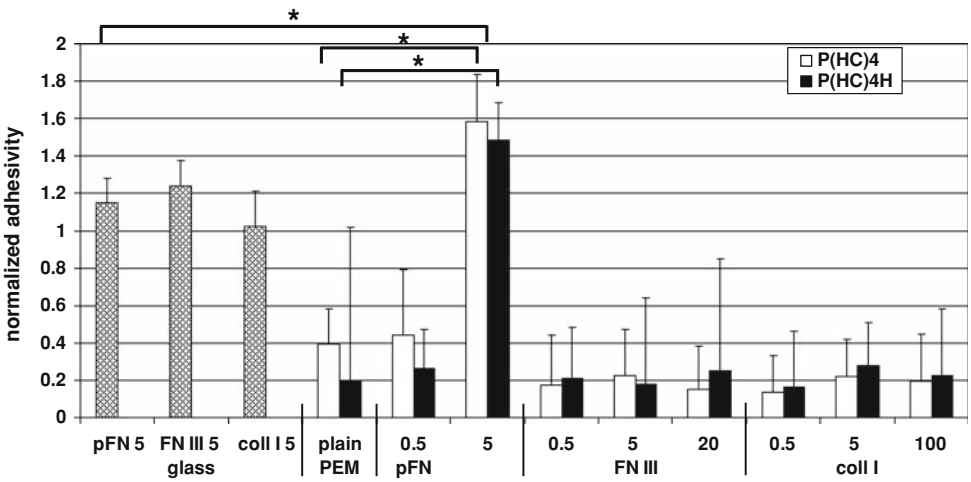

Fig. 5 Adhesion of MG-63 cells after 4 h incubation on different surfaces normalized against plain glass as a control. Plain glass and with protein solutions pFN, FN III, coll I (patterned); PEMs (chitosan terminated—white, heparin terminated—black): plain, with pFN, FN III and coll I, respectively, protein solutions have varying concentrations as indicated in (μg/ml) ($n = 10$)

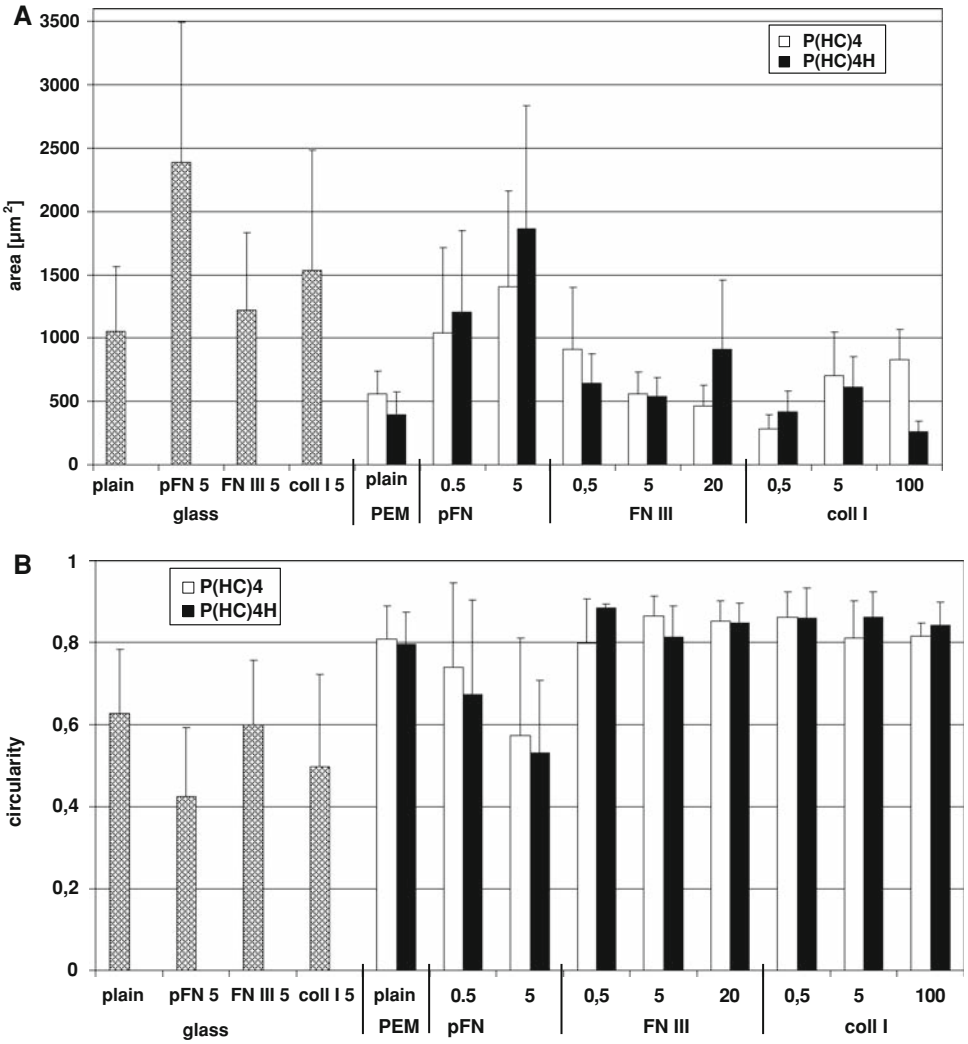

Fig. 6 MG-63 cell morphology evaluation after 4 h incubation on different surfaces: area (**a**) and circularity (**b**) of plain glass and with protein solutions pFN, FN III, coll I (patterned); PEMs (chitosan terminated—white, heparin terminated—black): plain, with pFN, FN III and coll I, respectively, protein solutions have varying concentrations as indicated in (µg/ml)

adhesion at 5 µg/ml pFN, 20 µg/ mL FN III and 100 µg/ml coll I. The same effect at a different magnitude was also observed on PEMs formed at pH setups P5H5C5 or P9H9C5 (data not shown).

Quantitative data of cell area and circularity depicted in Fig. 6a and b, respectively, support cell adhesion numbers from Fig. 5. For samples with a high cell number also an advanced spreading was observed. As a consequence, the area of the cells increase (Fig. 6a) and the shape becomes more irregular which corresponds to low values of circularity (Fig. 6b). As has been stated by adhesion numbers MG-63 on both PEM types ($P(HC)_4$-black and $P(HC)_4H$-white columns) reveal the best spreading on 5 µg/ml pFN coated samples. Lower concentrations of pFN result in lower spreading and consequently rounder cells. On the other hand, the FN III domain and collagen I cannot promote cell spreading. Numbers of cell area are rather low and comparable to plain PEMs since cells remain round. Good spreading on glass covered with various proteins could be observed (patterned columns).

Cell proliferation tests on plain PEMs were conducted without protein pre-adsorption but in FBS containing medium. As can be seen in Fig. 7, the values demonstrate the ability of MG-63 cells to initially adhere and subsequently divide over the course of 6 days. As expected cell numbers increase from 1 d (white columns) to 3 d (patterned columns) to 6 d (black columns). Values are below or very similar to the glass control (left group of columns). In most cases C terminated PEMs show a comparable cell growth to H terminated ones for the same pH setup. Furthermore, cell proliferation on PEMs is best on the pH combination P9H9C5 (pH 9) on both terminating layers with a small advantage of C terminated PEMs. These cell proliferation numbers are followed by the pH combinations P7H7C5 (pH 7) and P5H5C5 (pH 5). For pH setup P7H7C5 it is noteworthy that C terminated multilayers are much more proliferation supporting than H terminated ones.

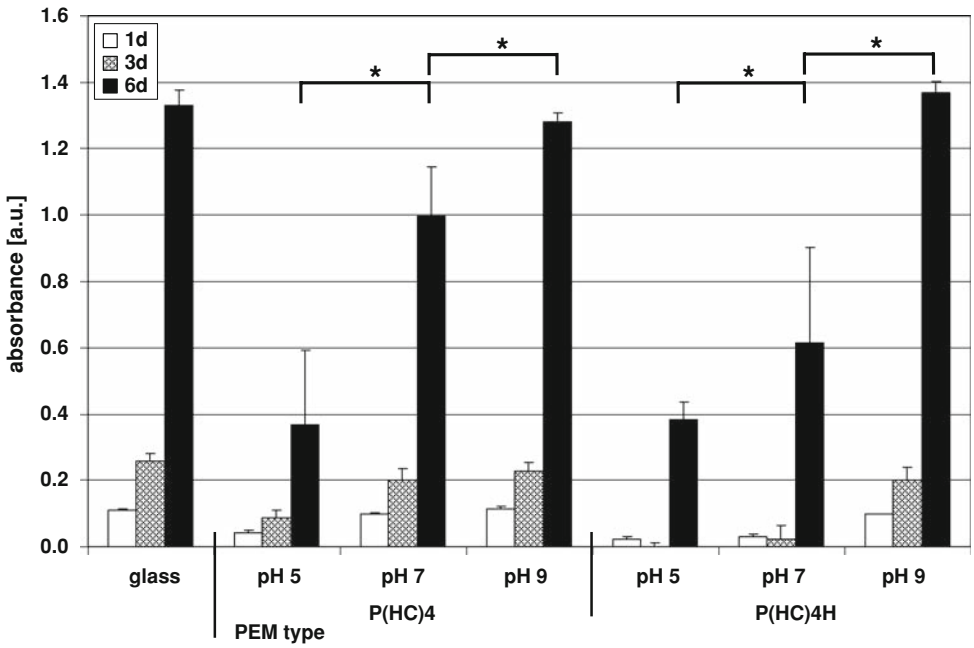

Fig. 7 Cell proliferation of MG-63 after 1 day (white), 3 days (patterned) and 6 days (black) incubation on glass (left three columns) and PEMs of varying pH setup during layer formation (P5H5C5 = pH 5/ P7H7C5 = pH 7/ P9H9C5 = pH 9); $P(HC)_4$ (middle group of columns) and $P(HC)_4H$ (right group of columns) ($n = 6$)

4 Discussion

It was shown in this article that the coating of glass as model biomaterial with biogenic polyelectrolyte multilayers can be used to control the adhesion of MG-63 cells. It is particularly important to note that a simple parameter—the pH value of the polyelectrolyte solution—can be utilized as a tool to control MG-63 osteoblasts adhesion and their subsequent growth. While plain PEMs are per se only weakly adhesive they acquire adhesivity by binding minute amounts of proteins like fibronectin which are present in the serum or secreted by cells. The binding of proteins is driven by specific interactions between the glycosaminoglycan heparin and the respective binding domains of fibronectin and potentially also other proteins possessing similar specificities.

In general, the characterization of biomaterials surface properties is crucial to understand cell behaviour. It is one of the characteristics of the LbL method that the surface chemistry changes from one to the next adsorption step [7, 21, 22]. Both poly (ethylenimine) and chitosan are weak polycations bearing amine groups. They render the surface less hydrophilic demonstrated by higher contact angles which are close to that found for amine-terminated self-assembled monolayers on glass [26]. On the other hand, heparin as a polyanion contains sulphate and carboxylic functionalities leading to very hydrophilic surfaces. This results in an oscillation of the contact angles between polycation and polyanion with progressing layer deposition. It has been found for weak polyelectrolytes that the pH value of the polyelectrolyte solutions controls the charge and conformation of polyelectrolytes [22, 27–29]. Hence, pH changes have an impact on the properties of multilayers composed of weak polyelectrolytes like poly (ethylenimine) and chitosan. A first example for this interrelationship is the effect of pH variations on the poly (ethylenimine) adsorption. The measurements illustrate that the CA increases after its adsorption with increasing pH value. SPR measurements as well show that poly (ethylenimine) adsorption is greatest at pH 9, although it is least charged at this pH value (pKa of about 8.5). However, zeta potential measurements demonstrate that glass acquires an increasing negative charge at higher pH values (not shown here). Hence, it is the surface charge which drives the adsorption process. Chitosan as a weak polycation (pKa of around 6.5) is influenced by pH changes as well. However, since chitosan is insoluble at higher pH values its adsorption was carried out at pH 5. Nevertheless, the charge and conformation of the adsorbed chitosan can be altered by pH changes of surrounding liquids as well. Heparin being a strong polyelectrolyte, however, is well dissociated for all adjusted pH values (pKa of around 3). Therefore, pH changes in this work had no direct influence on its charge and conformation. Hence, for the setup P5H5C5 when chitosan is highly charged, it can adsorb well onto the previous layer of heparin which leads to the characteristic overcompensation of charge and high masses can be adsorbed. This in turn, allows for a strong adsorption of heparin on the positively charged multilayer surface composed of chitosan during the next adsorption step [30]. Both the highest mass adsorption measured by SPR and largest differences in CA prove the anticipated relationship. By contrast, in case of the pH setup P9H9C5 the chitosan previously adsorbed to the PEM is least charged and can

therefore bind less heparin although it is highly dissociated. This leads to lower mass adsorption shown by SPR data and least differences in water contact angles. The setup P7H7C5 is the intermediate case to some extent. Chitosan might be partly charged depending on its exact composition and deacetylation degree and heparin is well dissociated. As a consequence, the layer growth can take place but not as much mass as in the low pH case can be adsorbed. As an effect of the corresponding charge conditions and resulting conformation of the polyelectrolytes after adsorption PEMs formed at low pH are expected to be thicker and single layers might be arranged more separately while those formed at higher pH values may be thinner and both polyelectrolytes should be rather intermingled [22]. A further consequence of the different layer thickness may be also different visco-elastic properties of multilayers. In a study concerning this matter it was shown that more visco-elastic multilayers inhibit cell adhesion and spreading while those being more rigid promote these processes [31]. This finding is supported by an earlier publication regarding the impact of substrate mechanics on cell adhesion [32]. Overall, the different physico-chemical properties of multilayers detected in this study gave reason for the distinct variation in cell adhesion and will be investigated more detailed and published elsewhere.

The physico-chemical properties of biomaterials surfaces control protein adsorption and subsequent adhesion and proliferation of cells [33]. In particular the biomaterial wettability which can be characterized by water contact angle measurements has been found to be important [18]. Being a controversial subject it is a common understanding that surfaces with moderate wettability greatly support the cellular interaction while highly wettable materials like hydrogels do not support the adsorption of proteins and adhesion of cells [26, 34, 36]. Also, the surface charge of biomaterials has an effect on protein binding, cell adhesion and proliferation. It has been found that biomaterials with a strong negative surface potential may reduce the adsorption of specific proteins like FN and limit the adhesion of cells while surfaces with basic functionalities like amino groups that lead to a less negative or positive surface potential may attract proteins and cells [37, 38]. In this context one can discuss some findings of this article such as the low adhesion of MG-63 cells on plain heparin terminated PEMs which possess a high wettability and have a negative surface potential under physiological conditions (data not shown here). Since all cells have a net negative charge [39] plain heparin terminated PEMs function cell repellent. Chitosan, on the other hand, possesses amine groups as basic functionalities and therefore demonstrates rather cell attractive effects.

Osteoblasts possess a variety of integrins that enable them to bind to a number of extracellular matrix proteins such as fibronectin, osteonectin, different collagens, etc. [1]. Therefore, the low cell attraction of plain PEMs could be compensated by the adsorption of plasma proteins such as fibronectin. Indeed, on all of the PEM configurations plasma fibronectin enhanced cell adhesion. Chitosan terminated layers promoted cell adhesion to a higher extend which can be due to its positive charge (i.e. basic amino groups) compared to the negative heparin (i.e. acidic sulphate and carboxyl groups). Within this context it was shown recently that more pFN binds to amino than to carboxyl groups [40]. The fact that FN is slightly negatively charged under physiological conditions (pKa of around 5.6–6.1) supports results from the present study [38]. Besides the pure mass of adsorbed protein, however, its conformation and type of binding play a role [41]. Additionally to the general improvement of the cell attraction by pFN, there are differences among the pH setups during LbL that can be explained with the properties of multilayers. For a low pH value setup (P5H5C5) hydrogel-like layer properties go along with lower cell adhesion whereas higher pH adjustments (P9H9C5) lead to multilayers that are more compact with possible mixing of polyelectrolytes of adjacent layers and an inhomogeneous surface. In the latter case, the presence of both heparin and chitosan within one layer may be responsible for the elevated adhesion of MG-63 cells. Analogous explanations can be given for the cell proliferation which is dependent on the previous processes of protein adsorption and cell adhesion [35]. Both cell adhesion and proliferation show the same trends. Especially the multilayer organization and resulting mechanical differences and the degree of heterogeneity between the PEMs seem to have a big impact on the cell growth showing clear advantages for the high pH combination P9H9C5.

The present study also shows that PEMs can be used to trigger the specific interaction of molecules such as heparin and plasma FN [14, 25]. In contrast to pFN, FN III and collagen I could not improve cell adhesion at comparable or even higher protein concentrations on multilayers. In fact, all of them have the ability to interact with MG-63 cells due to the presence of cell binding motifs such as RGD [15]. But only pFN has a binding domain for heparin whereas FN III and coll I do not. This indicates that the high cell binding capacity of pFN must be due to its biospecific interaction with the heparin from within the PEMs. The similarity of the chitosan and heparin terminated multilayers after pFN pre-adsorption in terms of cell adhesion can be used as an additional sign for a certain intermingling of chitosan and heparin within one layer which was mentioned above. Similar findings on mixed or instable multilayers have been reported in the literature [22, 42]. Hence, it is possible that heparin can bind pFN even if it is not the terminating layer molecule. In

opposition to that, the specific heparin-pFN binding is absent on plain glass and all proteins stimulate cell adhesion to a very similar degree. Cell morphology studies can further support this assumption. Due to the superior binding conditions cell-substrate interactions are significantly improved on PEMs with a pFN pre-adsorption compared to FN III revealed by much better cell spreading and stronger polarization of cells.

This study shows that the LbL method can be employed to modify biomaterial surfaces in order to control cell behaviour. The adhesion of MG-63 osteoblast cells was altered exploiting the specific interaction of glycosaminoglycans and proteins. This cooperation is part of natural processes in tissues like bone [43] and can be mimicked as demonstrated in this article. A further interesting application of these multilayers on bone implants is the incorporation of growth factors like BMP-2 due to its high affinity to heparin. Thus, multilayers from heparin and chitosan could be used as reservoir for controlled release to further promote healing of implants and tissue engineering scaffolds. Moreover, the tuning of the adhesivity of substrates may pave the way for other biomedical applications, when growth and differentiation of selected cell types is desired while others like fibroblasts shall be suppressed [5, 6, 34, 44].

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