Guduru, D., Niepel, M. S., Gonzalez-Garcia, C., Salmeron-Sanchez, M. and Groth, T. (2017) Comparative study of osteogenic activity of multilayers made of synthetic and biogenic polyelectrolytes. *Macromolecular Bioscience*, 17(8), 1700078. (doi:10.1002/mabi.201700078)

Full Paper

Comparative study of osteogenic activity of multilayers made of synthetic and biogenic polyelectrolytes

Deepak Guduru, Marcus S. Niepel, Cristina Gonzalez-Garcia, Manuel Salmeron-Sanchez, Thomas Groth*

_____

D. Guduru
Biomedical Materials Group, Institute of Pharmacy
Martin Luther University Halle-Wittenberg
D-06099 Halle (Saale)

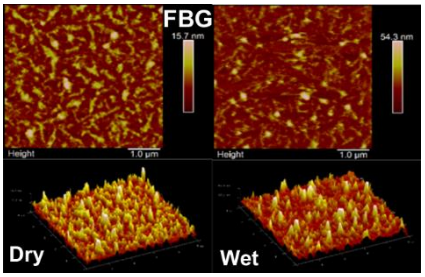
Dr.-Ing. M. S. Niepel, Prof. T. Groth
Biomedical Materials Group, Institute of Pharmacy &
Interdisciplinary Center of Materials Science
Martin Luther University Halle-Wittenberg
D-06099 Halle (Saale)
E-mail: thomas.groth@pharmazie.uni-halle.de

C. Gonzalez-Garcia, Prof. M. Salmeron-Sanchez
Division of Biomedical Engineering, School of Engineering
University of Glasgow
Glasgow G12 8LT, United Kingdom

_____

Polyelectrolyte multilayer (PEM) coatings on biomaterials are applied to tailor adhesion, growth and function of cells on biomedical implants. Here, biogenic and synthetic polyelectrolytes (PEL) are used for layer-by-layer (LbL) assembly to study the osteogenic activity of PEM with human osteosarcoma MG-63 cells in a comparative manner. Formation of PEM is achieved with biogenic PEL fibrinogen (FBG) and poly-L-lysine (PLL) as well as biotinylated chondroitin sulfate (BCS) and avidin (AVI), while poly (allylamine hydrochloride) (PAH) and polystyrene sulfonate (PSS) represent a fully synthetic PEM used as a reference system here. Surface plasmon resonance (SPR) measurements show highest layer mass for FBG/PLL and similar for PSS/PAH and BCS/AVI systems, while water contact angle and zeta potential measurements indicate larger differences for PSS/PAH and FBG/PLL but not for BCS/AVI multilayers. All PEM systems support cell adhesion and growth and promote

osteogenic differentiation as well. However, FBG/PLL layers are superior regarding MG-63 cell adhesion during short-term culture, while the BCS/AVI system increase alkaline phosphatase (ALP) activity in long-term culture. Particularly, a multilayer system based on affinity interaction like BCS/AVI may be useful for controlled presentation of biotinylated growth factors to promote growth and differentiation of cells for biomedical applications.



1. Introduction

Biomaterial properties have to meet certain requirements depending on the type of clinical application (e.g. blood or tissue contact) to achieve the desired performance of a biomedical device. Among parameters that affect the response of body components like proteins and cells to biomaterials are the chemical composition,[1] the surface energy,[2] the surface potential,[3] and topography of surfaces.[4] These properties dictate the adsorption of proteins from surrounding body fluids (e.g. blood or tissue fluid) with potential effects on their bioactivity.[5] Subsequently, adhesion of cells depends on the composition of the protein adsorption layer and the conformation of proteins.[6] Cells interact by gross non-specific physical interaction forces with the surfaces first,[6] followed by receptor-ligand interactions that are based on integrins or other cell adhesion molecules.[7] Hence, physicochemical properties of the material surface that influence protein adsorption affect the subsequent cellular response in terms of adhesion, proliferation, and differentiation.

Biomaterials that meet certain requirements of bulk properties like mechanical strength, etc. can be designed for a specific medical application through surface modification.[5] A large body of methods exists that can be roughly divided into chemical and physical procedures of surface modification.[8] Methods that are based on adsorption and coating represent often simple processes, applicable to different device geometries and type of materials like ceramics, metal alloys, polymers, and combinations thereof.[9] One example is the layer-by-layer (LbL) technique, which is based on the sequential deposition of polyelectrolytes (PEL) on material surfaces to obtain multilayer films.[10] In the past decades, a myriad of diverse synthetic and natural polymers was used to create such multilayer films for different type of applications.[11] Although, LbL technique is based on Coulomb attraction and ion paring in many cases, also hydrogen bonding, van der Waals forces, host-guest interactions and others have been used to form multilayer films.[11, 12] By the selection of PEL, the number of layers and experimental

conditions, such as pH, ionic strength and temperature, multilayer film properties like thickness, elastic modulus, surface energy, zeta potential and topography can be tailored.[13, 14]

Implant materials or tissue engineering scaffolds for replacement, repair and regeneration of bone should possess osteoinductive properties, which involves attachment and growth of osteoblasts or osteogenic progenitor cells and promotion of osteogenic differentiation.[15] Coating of biomaterials with polyelectrolyte multilayers (PEM) by LbL technique was employed previously to support osteogenic differentiation of cells.[16, 17] Beside synthetic PEL, like polystyrene sulfonate (PSS) and poly (allylamine hydrochloride) (PAH) as PEL with good cytocompatibility,[18] particularly molecules of biological origin like proteins and polysaccharides have been used to prepare PEM because of their better biocompatibility and inherent bioactivity.[11, 19] It is interesting to note that mechanical properties of PEM may vary tremendously from high E modulus for synthetic PEL like PSS/PAH of about 500 MPa down to only a few kPa for PEM made of glycosaminoglycans.[20] In this regard, differentiation of osteoblasts and osteogenic cells is generally promoted on stiffer substrata.[21] On the other hand, biospecific cues like matrix proteins and (adsorbed) growth factors like BMP-2 may also promote osteogenic differentiation on soft substrata.[22]

The present work compares three different multilayer systems regarding their osteogenic activity towards MG-63 osteosarcoma cells studying their adhesion, growth, and differentiation. The PSS/PAH multilayer system was used as a cornerstone in this study because of its known osteogenic activity found in previous studies.[13] However, since multilayer systems based on synthetic PEL like PSS/PAH are more difficult to be approved for medical applications, two other systems based on proteins and one glycosaminoglycan were included in this study, as well. The use of avidin (AVI) and biotinylated chondroitin sulfate (BCS) includes an affinity-based interaction, which is to our knowledge the first time that this combination is used for multilayer formation. AVI is widely applied in biomedical studies because of its high

affinity to biotin known as strongest non-covalent interaction ($K_a = 10^{15}$ M^{-1}) between a protein and a ligand. In addition, the bond formation between biotin and AVI is rapid and, once formed, not affected by pH extremes, temperature, organic solvents, and most denaturing agents.[23] Such system would permit potentially the use of other biotinylated ligands to decorate biomaterials surfaces with bioactive cues promoting cell attachment and differentiation. The third system investigated here is a combination of fibrinogen (FBG, Factor I), which represents a major coagulation factor and adhesive cue for cells,[24] used here as polyanion, while poly-L-lysine (PLL) was applied here as polycation. PLL has been widely used for making adhesive coatings, but also as PEL in LbL studies.[25] Beside the investigation of multilayer formation and surface properties, the study focused the impact of each PEM system as surface coatings on osteogenic activity using MG-63 cells, which share many properties with normal bone cells, such as the ability to express alkaline phosphatase and also calcification of tissue and which makes them suitable as a model system.[26]

2. Experimental Section

2.1. Materials

The PEM assembly was performed on three different material substrates, depending on the requirements of the assays. Glass cover slips (Menzel, Germany) as substrates were cleaned with a 0.5 M NaOH in 96% ethanol solution (Roth, Germany) at room temperature for 2 h followed by excessive rinsing with ultrapure water (10 cycles of 5 min) and dried with a nitrogen flow. New gold-coated sensors, used for surface plasmon resonance (SPR) measurements, were purchased from IBIS Technologies B.V. (Enschede, The Netherlands), cleaned with 99.8% ethanol (Roth), and rinsed with ultrapure water. Sensors were immediately incubated in an ethanol (p.a.) solution of 2 mM mercaptoundecanoic acid (MUDA, 95%, Sigma, Germany) at room temperature overnight to generate a negatively charged surface by

the formation of a self-assembled monolayer (SAM) exposing carboxyl groups.[4] Silicon wafers (Silicon materials, Germany) of (10 x 10) mm² surface were used for atomic force microscopy (AFM) studies. These substrates were treated with a solution of ammonia (Roth, 27%), hydrogen peroxide (Roth, 30%), and water (1:1:5, v/v/v) at 70°C for 15 min, subsequently washed with ultrapure water, and dried with a stream of nitrogen. The following PEL were dissolved in 0.15 M NaCl at a concentration of 2 mg mL^{-1} and adjusted to pH 7.4 by dropwise addition of 0.1 M hydrochloric acid: poly (ethylene imine) (PEI, M_w ~ 750 kDa, Sigma, Germany), polystyrene sulfonate (PSS, 30 wt.% in H_2O, M_w ~ 70 kDa, Sigma), poly (allylamine hydrochloride) (PAH, M_w ~ 120 - 200 kDa, Alfa Aesar, Germany), fibrinogen (FBG, M_w ~ 330 kDa, Merck, Germany), poly-L-lysine (PLL, M_w ~ 1 - 5 kDa, Sigma), biotinylated chondroitin sulfate (BCS, M_w ~ 20 kDa, Innovent e.V., Germany), and avidin (AVI, M_w ~ 66 kDa, Merck).[27]

2.2. Polyelectrolyte Multilayer Assembly

PEM were fabricated on cleaned glass, silicon, and gold substrates. PEI was used as a primary base layer to obtain a positive surface charge. Multilayers were obtained by alternated dip coating of the substrates in the respective PEL solutions for 10 min followed by excessive rinsing with 0.15 M NaCl, pH 7.4 (three cycles for 5 min). In total, six or seven layers were formed, abbreviated as $(FBG/PLL)_2FBG$ and $(FBG/PLL)_3$, $(BCS/AVI)_2BCS$ and $(BCS/AVI)_3$ as well as $(PSS/PAH)_2PSS$ and $(PSS/PAH)_3$.

2.3. Multilayer Characterization

2.3.1. Water Contact Angle Measurements

The wettability of dry PEM was characterized by determining the static water contact angle (WCA) using an OCA15+ device from Dataphysics (Filderstadt, Germany). The sessile drop

method was used here to investigate at least three samples per multilayer combination by dispensing a minimum of three droplets of 1 μL ultrapure water as test liquid on each substrate. For each droplet, at least 10 WCA values were recorded using the ellipse-fitting method.

2.3.2. Surface Plasmon Resonance Studies

The multilayer formation was monitored by SPR using IBIS-*i*SPR (IBIS Technologies B.V.). SPR is based on the detection of changes in the refractive index (RI) at the gold/liquid interface of the gold sensor surface caused by the adsorption of molecules. The resulting change in the SPR angle shift (m°) is proportional to the mass (Γ_{SPR}) of adsorbed molecules on the surface, whereby a shift of 122 m° corresponds approximately to 1 ng mm^{-2}.[28] The MUDA-modified gold sensors were placed into the flow chamber and equilibrated with degassed 0.15 M NaCl pH 7.4 to obtain a stable baseline. Thereafter, each PEL was injected with a flow rate of 3 μL s^{-1} for 10 min starting with PEI and followed by the different polyanion/ polycation combinations mentioned above to form PEM with up to seven layers in total. After each PEL adsorption, the flow chamber was rinsed with 0.15 M NaCl solution pH 7.4 for 15 min to remove unbound molecules. All measurements were done in duplicates.

2.3.3. Zeta Potential Measurements

Streaming potential measurements were carried out with a SurPASS Electrokinetic Analyzer (Anton Paar, Graz, Austria) to obtain zeta (ζ) potentials. Glass coverslips fitting to the adjustable gap cell dimensions were used as substrata for multilayer formation. Two identical modified coverslips were fixed on stamps and placed oppositely into the SurPASS flow cell. The gap of the flow cell was adjusted to a distance that a flow rate of 100 to 150 mL min^{-1} to achieve at a maximum pressure of 300 mbar. 1 mM potassium chloride solution was used as electrolyte for the experiment with pH 10.5 using 1 M sodium hydroxide for pH adjustment.

Hydrochloric acid (0.1 M) was used for pH titration to measure zeta potential in dependence on pH[29] using titration steps of 0.03 μL from pH 10.5 to 5.0 and 0.25 μL from pH 5.0 to 2.25.

2.3.4. Atomic Force Microscopy

The surface topography of the different multilayer systems was evaluated using a multimode AFM equipped with a NanoScope IIIa controller from Veeco (Manchester, UK) and Nanoscope 5.30r2 software. Gold-coated hemispherical silicon cantilevers from Veeco with a tip radius of 30 nm and a spring constant of 0.06 N m^{-1} were used at its resonance frequency of 75 kHz. The phase signal was set to zero at a frequency 5-10% lower than the resonance one. The drive amplitude was 200 mV and the amplitude set-point 2.03 V. The ratio between the amplitude set-point and the free amplitude was kept equal at 1.03. PEM were prepared at pH 7.4 on cleaned silicon wafers (100 mm^2) and dried using compressed nitrogen. Samples were scanned in both air and liquid (10 mM NaCl solution) conditions at room temperature with the AFM operating in the tapping mode®, and height, phase, and amplitude magnitudes were recorded simultaneously. The samples were prepared in triplicates. Surface topography was displayed on the height phase and the roughness was analyzed from these images using the Nanoscope Analysis software (version 1.70).

2.4. Cell Response

2.4.1. Cell Culture

Cryopreserved human osteosarcoma MG-63 cells were thawed and grown in Dulbecco's modified Eagle's medium (DMEM, Biochrom AG, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom) and 1% antibiotic-antimycotic solution (AAS, Promocell, Germany) at 37°C in a humidified 5% CO_2/ 95% air atmosphere. Cells of almost confluent cultures were washed once with sterile phosphate buffered saline (PBS) followed by treatment

with 0.25% trypsin/ 0.02% EDTA (Biochrom) at 37°C for 5 min to detach the cells. Trypsin was neutralized with DMEM containing 10% FBS and the cells were resuspended in DMEM containing 10% FBS after centrifugation at 250g for 5 min. Finally, the cells were seeded on plain and PEM-coated glass coverslips at a density of 25,000 cells mL^{-1}.

2.4.2. Cell Adhesion and Spreading

Short-term cell adhesion and spreading studies were conducted on multilayer coatings prepared under sterile conditions on glass coverslips. MG-63 cells were incubated at 37°C in a humidified atmosphere under 5% CO_2 for 4 h. After incubation, the culture medium was removed and the samples were rinsed with sterile PBS once. Thereafter, samples were fixed and stained using 0.5% (w/v) crystal violet (Roth) in methanol (Roth) at room temperature for 30 min. All samples were carefully washed with ultrapure water three times and dried in air. Images were taken in transmission mode with an Axiovert 100 (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a CCD camera (Sony, MC-3254, AVT-Horn, Aalen, Germany). Cell count and morphology, such as cell area and aspect ratio, were evaluated from five images per sample using "BioVoxxel Toolbox for ImageJ and Fiji" image processing software. All experiments were run in triplicate.

2.4.3. Proliferation of MG-63 Osteosarcoma Cells

Proliferation studies were performed in serum-containing medium. The quantity of cells was determined after 24 h and 72 h with a QBlue® cell viability assay kit (BioChain, USA), which quantifies the amount of metabolically active cells. Before measuring, the old medium was carefully aspirated and the cells were washed with sterile PBS once. Then, 300 μL of pre-warmed colorless DMEM supplemented with QBlue® reagent at a ratio of 10:1 were added to each well, and the samples were again incubated at 37°C for another 2 h. Thereafter, 100 μL of supernatant from each well were transferred to a black 96-well-plate (Greiner, Germany) and

fluorescence intensities were measured with an excitation wavelength of 544 nm and at an emission wavelength of 590 nm using a plate reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany).

2.4.4. Osteogenic Activity of MG-63 Cells

The osteogenic activity of MG-63 cells on multilayers was quantified by measurements of alkaline phosphatase (ALP) using the p-nitrophenol (pNP) assay. MG-63 cells were cultured on terminal layers for one, five, seven, and eleven days in osteogenic medium containing 10 mM β – glycerophosphate (Alfa Aesar, Germany), 50 ng mL^{-1} BMP-2 (PeproTech GmbH, Germany), 50 µg mL^{-1} ascorbic acid (Sigma, Germany), 10 nM dexamethasone (Sigma, Germany), 1% AAS. The adherent cells were washed with sterile PBS pH 7.4 and lysed with 0.2% Triton X-100 (Sigma) in 10 mM Tris-HCl (Applichem, Germany) pH 7.2 at 4°C for 20 min. The resulting supernatant was collected and centrifuged at 13,000g at 4°C for 7 min. Then, 100 μL of 1 mg mL^{-1} p-nitrophenyl phosphate (pNPP, Sigma, Germany) prepared in 50 mM glycine (Roth) in 1 mM $MgCl_2$ (Roth) pH 10.5 were added to 50 μL of cell lysis supernatant per well in a 96-well-plate and incubated at 37°C in the dark for 2 h to allow the reaction from p-NPP into p-NP. The reaction was stopped by addition of 1 M sodium hydroxide and the absorbance was read at 405 nm in a plate reader (FLUOstar OPTIMA).

The total protein content was quantified to normalize the ALP activity. A commercial bicinchoninic acid (BCA) test (Pierce®, ThermoFisher Scientific, Germany) was used to measure the protein concentration. Thereby, 200 μL BCA working solution were added to 25 μL cell lysate per well in a 96-well-plate and the mixture was incubated at 37°C for 30 min. Thereafter, the absorption was measured at 550 nm with the microplate reader and the ALP concentrations were normalized against the BCA concentrations of the same samples to determine the cellular ALP activity.

2.5. Statistics

All data are represented as mean values ± standard deviations (SD). Statistical analysis was performed using Origin 8 Pro software (OriginLab, USA) with one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test. The number of samples has been indicated in the respective figure and table captions. Statistical significance was considered for $p \leq 0.05$ and indicated by asterisks in the figures.

3. Results and Discussion

3.1. Characterization Multilayer Formation and Surface Properties

The multilayer formation process was monitored by surface plasmon resonance (SPR). The resulting shift of the SPR resonance angle (m°) is proportional to the mass (Γ_{SPR}) of adsorbed molecules on the surface.[28, 30] As expected, an increase in layer mass was observed for all PEM systems as the number of assembled layers increased, displayed in **Figure 1**. The highest layer growth was found for the FBG/PLL system with a total of 2.413 ng cm^{-2} after the adsorption of the final PLL layer (7^{th} layer), which reflects the higher molecular weight of FBG compared to the other PEL applied here. The other two multilayer systems expressed a linear growth behavior with a slightly higher layer mass for the synthetic PSS/PAH system. The lowest layer mass of the BCS/AVI system is probably attributed to the relatively small molecular weights of both binding partners (BCS: ~20 kDa, AVI: ~68 kDa) compared to FBG and both synthetic PEL PSS and PAH (see materials section for more details).

Static WCA measurements are applied frequently to characterize the change of terminal layer composition during assembly of multilayers and were used here to study wetting properties of multilayers, because of their relevance for cell adhesion. In general, differences in contact angles between polyanions and polycations during multilayer formation can be used as a first indicator whether well separated or more fuzzy multilayer systems exist.[31] **Figure 2** shows the

WCA values of clean glass, PEI as first layer, polyanions (even numbers), and polycations (odd numbers). The formation of FBG/PLL multilayers was characterized by the largest WCA of this study with small variations between FBG and PLL. WCA for PLL layers were similar to those found in other studies,[32] while WCA measured for FBG films on glass have been found to be about 90°, previously.[33] This explains why WCA of FBG layers were higher than that of PLL, but indicates a dominance of PLL in this multilayer system due to the lower WCA of FBG layers compared to the previous study. Hence, it can be assumed that the FBG/PLL multilayers represent a more fuzzy system due to possible interpenetration of the smaller PLL within the larger FBG molecules as found also in other multilayer studies with PLL.[25] By contrast, PSS/PAH multilayers had WCA values located between the two other systems with a large difference in terminal anion and cation layer and were similar to those found elsewhere.[18] This strongly supports the idea that PSS/PAH formed a more well-separated layers system,[18] if compared to the FBG/PLL and BCS/AVI system. The BCS/AVI system was found to be the most hydrophilic with contact angles lower than 30°. Moreover, WCA were decreasing to about 20° with no alternation between AVI and BCS, which indicates rather an intermingling of both molecules than the formation of well-separated layers.[31]

Zeta potentials of PEM with terminal polycation and polyanion layers prepared at pH 7.4 on glass slides were determined in dependence on the pH value of the titration solution (1 mM KCl). Surface charges play a central role during multilayer formation by LbL method, because a charge reversal is considered as a prerequisite for the adsorption of next PEL layer.[10] When comparing the three terminal polyanion layers at pH 7.4 (**Figure 3**A), PSS, BCS and FBG all exhibited a negative zeta potential as also illustrated in **Table 1**. Only at pH values below pH 5.0, positive values were obtained. On the other hand, zeta potentials of the terminal polycation layers of PAH and PLL shown in Figure 3B, were positive, which undermines the claim that multilayers from PSS/PAH and FBG/PLL were formed predominantly by ion pairing. By contrast, both AVI and BCS exhibited both a negative potential if the pH was higher

than 5.0. This indicates that Coulomb attraction and ion pairing are not the prevailing mechanism of multilayer formation here. Hence, as supposed initially, the affinity of biotin to avidin seems to make a considerable contribution to the multilayer formation process here. Furthermore, the point of zero charge (PZC) was about 4.5 pH units lower for AVI in comparison to PAH and PLL (Table 1), which supports the idea that higher quantities of BCS contribute to the multilayer mass, which is also well in line with the low and almost constant WCA of this system with progression of multilayer formation. A further observation during the zeta potential measurements was that particularly the potentials of the terminal polyanions layer became positive at low pH values (see Figure 3A). This finding is due to the fact that the surface potential of multilayers represents not only the charge density of the outermost layer, but do represent the surface potential of a permeable swollen surface layer, which includes also the charge of the polycation, which becomes dominant at low pH values.[34]

Atomic force microscopy (AFM) gathered information on the surface topography of all terminal layers under dry and wet conditions, shown in **Figure 4.** AFM investigations demonstrated that dry and wet conditions affected the multilayer surface structure regarding average roughness (**Table 2**). All surfaces had a granular surface morphology with roughness increasing from dry to wet conditions except for the terminal PAH layer. The morphology demonstrated an island-like structure for all multilayers, except for the terminal FBG layer, where a rather fibrillar structure was observed. Figure 4 A&B show that the nominal height of the PAH layer was increased over the preceding PSS layer probably due to the higher molecular weight of PAH. Dry PSS-terminated layers in wet state had an almost doubled roughness value (1.62 nm to 2.70 nm for dry and wet PEM, respectively). In contrast, the succeeding dry PAH layer possessed a higher roughness at dry compared to wet conditions (5.17 nm dry to 3.86 nm wet). The topography of these multilayers was very similar to findings in other studies.[35] Large aggregates were observed in the FBG/PLL system, probably due to the high molecular weight of FBG and the tendency of fibrinogen to polymerize (Figure 4C, D). Further, the FBG layer

had a large increase in surface roughness from dry (2.15 nm) to the wet state (4.49 nm). A similar result was found for the succeeding PLL layer whose roughness almost tripled upon swelling (1.51 nm dry to 4.26 nm wet). The aggregates found in the BCS/AVI system where much smaller probably due to the lower molecular weight of both PEL (Figure 4E, F). However, also for the BCS layer together an increase in surface roughness from dry (2.48 nm) to wet state (5.52 nm) was observed. It is known that the surface roughness of multilayers can increase with film thickness, but depends also on the type and size of molecules, the growth regime and complexation conditions.[36] Due to the different nature of molecules used in this study, a comparative interpretation of surface topography is difficult. Indeed, it was obvious that all surfaces possess a certain roughness also under wet conditions, which may have also some effect on the interaction with cells.

The results of physical studies of multilayer formation and surface properties indicate that the PSS/PAH reference system possessed an intermediate wetting behavior with a clear change of surface potential from negative of PSS to a positive of PAH terminal layers at pH 7.4. Its surface roughness was similar to previous studies.[18, 35] The FBG/PLL multilayer system was characterized by an intermediate wetting behavior that promotes cell adhesion in general,[6] with surface potentials alternating between both terminal layers. The large roughness indicates that FBG may start to polymerize to fibrin. The positive potential of terminal PLL layer at pH 7.4 and the fact that many cells possess integrins that recognize the RGD sequence in FBG may also promote adhesion of cells on both terminal layers.[1, 3, 7] Finally, the most hydrophilic BCS/AVI system has lowest WCA and negative surface potentials at pH 7.4, which normally are not supportive for cell adhesion. However, the presence of BCS in this system may allow for the binding of adhesive proteins with heparin-binding domains like fibronectin (FN) and vitronectin (VN) from serum, which may promote cell adhesion as demonstrated for other functionalized chondroitin sulfates in previous studies.[37, 38]

3.2. Studies on osteogenic activity of the different multilayer systems

Cell adhesion studies were carried out by seeding MG-63 cells on both terminal layers in serum-containing medium, which contains adhesive proteins like FN and VN, that can be recognized by integrin adhesion receptors of cells.[39] In addition, cells like MG-63 osteoblasts secrete proteins like collagen rapidly, which can adsorb on the material surface.[40] The highest number of MG-63 cells was found on terminal FBG and PLL layers after 4 h of culture with slightly more cells on the PLL layer (**Figure 5**). By contrast, the number of cells adhering on the synthetic PSS/PAH system was significantly lower, especially on the terminal PSS layer, as observed in other studies with cells, too.[18] The difference in cell count between polyanion and polycation terminal layers was largest in this system, while it was lowest in the BCS/AVI system. In the latter one, the difference in cell adhesion on both terminal layers was not significant. The quantification of cell area and circularity led to similar trends as observed for the number of adhering cells. The spreading was largest in cells plated on the FBG/PLL system, but also on the BCS/AVI system (**Figure 6**A) with no significant differences between both terminal layers. By contrast, cell area was smallest, while circularity was highest of cells seeded on the terminal anionic PSS layer; cells were larger and more elongated on the PAH terminal layers (**Figure 6**A&B). The increased cell count on the FBG/PLL system was reasonable because FBG is blood plasma protein[41] that promotes cell adhesion by integrin-related pathways,[42] while PLL is a commonly used coating agent to increase cell adhesion.[43] The large difference in cell adhesion between PSS and PAH can be explained by their difference in surface charge and wettability. Thereby, the highly negative surface potential and low WCA of PSS obviously suppressed MG-63 cell adhesion as found for such type of surfaces in other studies.[3, 6, 28] Since charge and wettability of BCS and AVI terminal layers were almost identical, it was not surprising that cell adhesion and spreading did not differ. Surface roughness

seemed to play a minor role in cell adhesion in this study, since the roughness values were not significantly different under wet conditions between the different multilayer systems.

To see whether initial effect of multilayers on cell adhesion would also prevail during longer culture of cells, proliferation studies were performed with MG-63 cells for one and three days. Quantification of viable cells was performed with QBlue assay, which is equivalent to the quantity of metabolic active cells (**Figure 7**). No significant differences were found in the amount of metabolic active cells after one day of culture (Figure 7, left panel), which is also in accordance to the similar extent of cell spreading on most of the terminal layers. The quantity of metabolic active cells increased after three days of culture on all surfaces with some variations among the different layers. Significant differences were only found with lesser quantity of cells on AVI terminal layers in comparison to PLL and PSS. Interestingly, more viable cells were found on PSS than on PAH after 7d. This observation might be related to the presence of aromatic rings in PSS, that promote the adsorption of proteins as found in other studies with an effect on longer culture of cells.[35] In the FBG/PLL system, a higher metabolic activity was observed on the PLL layer, which points to the cell-adhesive and growth-promoting properties of PLL.[43] In contrast, the growth rate was the lowest on AVI in the BSC/AVI system. Here, the high negative zeta potential together with the hydrophilic nature of both terminal layers may be responsible for the reduced growth of cells.

Studies on the expression of alkaline phosphatase (ALP) by MG-63 cells were performed to investigate the effect of the multilayer composition on osteogenic differentiation of cells. MG-63 osteoblast-like cells were selected as a useful model like in previous studies.[44, 45] ALP is highly expressed in early osteogenesis in vitro and is also considered as a sign of bone formation and mineralization for all types of pluripotent stem cells including osteoblast-like cells.[46] Hence, a significant increase in ALP activity during differentiation medium conditions suggests a cell-commitment to the osteoblastic lineage. MG-63 cells were cultured in osteogenic medium

containing BMP-2 and reduced amounts of serum (1% FBS) over a period of eleven days as done in a previous study.[46] The ALP activity per total protein was quantified after one, five, seven, and eleven days and the results are displayed in **Figure 8**. In general, ALP activity increased on all surface with culture time. In particular, the highest increase was found from day five to day seven. Here, ALP activity almost tripled on each surface. The highest difference between polyanion and polycation layers was found for the PSS/PAH system, indicating that the polycation is supporting osteogenic differentiation, which is in accordance with other studies that cationic surface modifications promote osteogenic differentiation of cells.[47] However, such observation was not made for the FBG/PLL system, were relatively low values of ALP activity were observed similar to that of cells cultured on terminal PSS layers. By contrast, the terminal BCS/AVI multilayer system, which was inferior regarding cell adhesion and partly proliferation, expressed the highest averaged ALP values, indicating that this most biogenic system is more suitable than the others to promote osteogenic differentiation of cells. One of the possible reasons for this observation could be the interaction of chondroitin sulfate with BMP-2 as found in other studies with sulfated polysaccharides of one of the multilayer systems presented here.[48]

4. Conclusions

Different polyelectrolyte multilayer systems of synthetic like PSS/PAH and biological origin such as FBG/PLL and BCS/AVI were used here to study their activity towards osteogenic differentiation of cells. Although the synthetic PSS/PAH system is not suitable for a clinical application it was used here as a kind of reference system because of its known cytocompatibility.[18] The other two systems were included due to the known osteogenic activity of fibrinogen [37, 49] and the inherent bioactivity of chondroitin sulfate towards proteins with heparin-binding domains.[48] Physical studies showed that the PSS/PAH multilayer system was well separated with intermediate wetting properties, opposing sign of charge of terminal PSS

and PAH layers, which was also the case for BCS/FBG multilayer system. However, in both biogenic multilayer systems the polyelectrolytes seemed to be more intermingled, which was related to non-significant differences in cell adhesion, growth, and differentiation between both terminal layers. Most importantly was the finding that multilayers made of BCS and AVI had a superior osteogenic activity compared to FBG/PLL, which was comparable to that of PAH. This finding opens new avenues for the application of such affinity-based multilayer systems since avidin-containing terminal layers may be used for localized presentation of biotinylated adhesive-proteins and growth factors reducing their dosage but increasing their activity to avoid undesirable side-effects as suggested recently.[50]

Appendix/Nomenclature/Abbreviations

Acknowledgements:

This work was supported by Marie Curie Industry-Academia Partnerships and Pathways (FP7-PEOPLE-2012-IAPP) and IOF-Marie Curie fellowship program (Protdel 331655) as well as the German Research Society (DFG) through Grant GR 1290/10-1. We thank Innovent e.V. Jena for generously providing us the BCS.

Received: February 28, 2017; Revised: Month XX, XXXX; Published online:

DOI: 10.1002/mabi.

Keywords: poly (allylamine hydrochloride), poly-L-lysine, avidin, MG-63 cells, differentiation

References

[1] N. Faucheux, R. Schweiss, K. Lutzow, C. Werner, T. Groth, *Biomaterials* **2004**, *25*, 2721.

[2] G. Altankov, F. Grinnell, T. Groth, *J Biomed Mater Res* **1996**, *30*, 385.

[3] G. Altankov, K. Richau, T. Groth, *Materialwissenschaft Und Werkstofftechnik* **2003**, *34*, 1120.

[4] M. S. Niepel, B. Fuhrmann, H. S. Leipner, T. Groth, *Langmuir* **2013**, *29*, 13278.

[5] D. G. Castner, B. D. Ratner, *Surface Science* **2002**, *500*, 28.

[6] P. Parhi, A. Golas, E. A. Vogler, *Journal of Adhesion Science and Technology* **2010**, *24*, 853.

[7] R. O. Hynes, *Cell* **2002**, *110*, 673.

[8] A. S. Hoffman, *Macromolecular Symposia* **1996**, *101*, 443.

[9] P. Roach, D. Eglin, K. Rohde, C. C. Perry, *J Mater Sci: Mater Med* **2007**, *18*, 1263.

[10] G. Decher, *Science* **1997**, *277*, 1232.

[11] R. R. Costa, J. F. Mano, *Chem Soc Rev* **2014**, *43*, 3453.

[12] J. F. Quinn, A. P. R. Johnston, G. K. Such, A. N. Zelikin, F. Caruso, *Chemical Society Reviews* **2007**, *36*, 707.

[13] V. Gribova, R. Auzely-Velty, C. Picart, *Chemistry of Materials* **2012**, *24*, 854.

[14] T. Boudou, T. Crouzier, K. Ren, G. Blin, C. Picart, *Adv Mater* **2010**, *22*, 441.

[15] X. Yu, X. Tang, S. V. Gohil, C. T. Laurencin, *Adv Healthc Mater* **2015**, *4*, 1268.

[16] Z.-M. Liu, Q. Gu, Z.-K. Xu, T. Groth, *Macromolecular Bioscience* **2010**, *10*, 1043.

[17] M. Zhao, L. Li, C. Zhou, F. Heyroth, B. Fuhrmann, K. Maeder, T. Groth, *Biomacromolecules* **2014**.

[18] L. Mhamdi, C. Picart, C. Lagneau, A. Othmane, B. Grosgogeat, N. Jaffrezic-Renault, L. Ponsonnet, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems* **2006**, *26*, 273.

[19] T. Serizawa, M. Yamaguchi, T. Matsuyama, M. Akashi, *Biomacromolecules* **2000**, *1*, 306.

[20] C. Picart, *Current Medicinal Chemistry* **2008**, *15*, 685.

[21] A. J. Engler, S. Sen, H. L. Sweeney, D. E. Discher, *Cell* **2006**, *126*, 677.

[22] E. Migliorini, A. Valat, C. Picart, E. A. Cavalcanti-Adam, *Cytokine Growth Factor Rev* **2016**, *27*, 43.

[23] C. Dundas, D. Demonte, S. Park, *Appl Microbiol Biotechnol* **2013**, *97*, 9343.

[24] M. W. Mosesson, *Journal of Thrombosis and Haemostasis* **2005**, *3*, 1894.

[25] T. Crouzier, C. Picart, *Biomacromolecules* **2009**, *10*, 433.

[26] B. Vagaska, L. Bacakova, E. Filova, K. Balik, *Physiol Res* **2010**, *59*, 309.

[27] C. Gonzalez-Garcia, S. R. Sousa, D. Moratal, P. Rico, M. Salmeron-Sanchez, *Colloids and Surfaces B-Biointerfaces* **2010**, *77*, 181.

[28] N. Aggarwal, N. Altgarde, S. Svedhem, G. Michanetzis, Y. Missirlis, T. Groth, *Macromol Biosci* **2013**, *13*, 1327.

[29] M. S. Niepel, D. Peschel, X. Sisquella, J. A. Planell, T. Groth, *Biomaterials* **2009**, *30*, 4939.

[30] R. B. Schasfoort, A. J. Tudos, "*Handbook of surface plasmon resonance"*, Royal Society of Chemistry, 2008.

[31] D. Yoo, S. S. Shiratori, M. F. Rubner, *Macromolecules* **1998**, *31*, 4309.

[32] J. J. Medina Benavente, H. Mogami, T. Sakurai, K. Sawada, *PLoS One* **2014**, *9*, e90189.

[33] R. E. Baier, R. C. Dutton, *J Biomed Mater Res* **1969**, *3*, 191.

[34] J. F. L. Duval, D. Küttner, M. Nitschke, C. Werner, R. Zimmermann, *Journal of Colloid and Interface Science* **2011**, *362*, 439.

[35] A. Quinn, E. Tjipto, A. M. Yu, T. R. Gengenbach, F. Caruso, *Langmuir* **2007**, *23*, 4944.

[36] C. Picart, P. Lavalle, P. Hubert, F. J. G. Cuisinier, G. Decher, P. Schaaf, J. C. Voegel, *Langmuir* **2001**, *17*, 7414.

[37] A. Köwitsch, M. S. Niepel, G. P. A. Michanetzis, Y. F. Missirlis, T. Groth, *Macromolecular Bioscience* **2016**, *16*, 381.

[38] N. Altgarde, E. Nileback, L. de Battice, I. Pashkuleva, R. L. Reis, J. Becher, S. Moller, M. Schnabelrauch, S. Svedhem, *Acta Biomater* **2013**, *9*, 8158.

[39] J. G. Steele, G. Johnson, P. A. Underwood, *J Biomed Mater Res* **1992**, *26*, 861.

[40] B. Fournier, P. A. Price, *J Cell Biol* **1991**, *114*, 577.

[41] Y. I. Veklich, O. V. Gorkun, L. V. Medved, W. Nieuwenhuizen, J. W. Weisel, *J Biol Chem* **1993**, *268*, 13577.

[42] D. Gugutkov, C. Gonzalez-Garcia, G. Altankov, M. Salmeron-Sanchez, *Journal of Bioactive and Compatible Polymers* **2011**, *26*, 375.

[43] B. S. Jacobson, D. Branton, *Science* **1977**, *195*, 302.

[44] K. Kirchhof, K. Hristova, N. Krasteva, G. Altankov, T. Groth, *Journal of Materials Science-Materials in Medicine* **2009**, *20*, 897.

[45] S. W. Tsai, H. M. Liou, C. J. Lin, K. L. Kuo, Y. S. Hung, R. C. Weng, F. Y. Hsu, *PLoS One* **2012**, *7*, e31200.

[46] D. Peschel, K. Zhang, S. Fischer, T. Groth, *Acta Biomater* **2012**, *8*, 183.

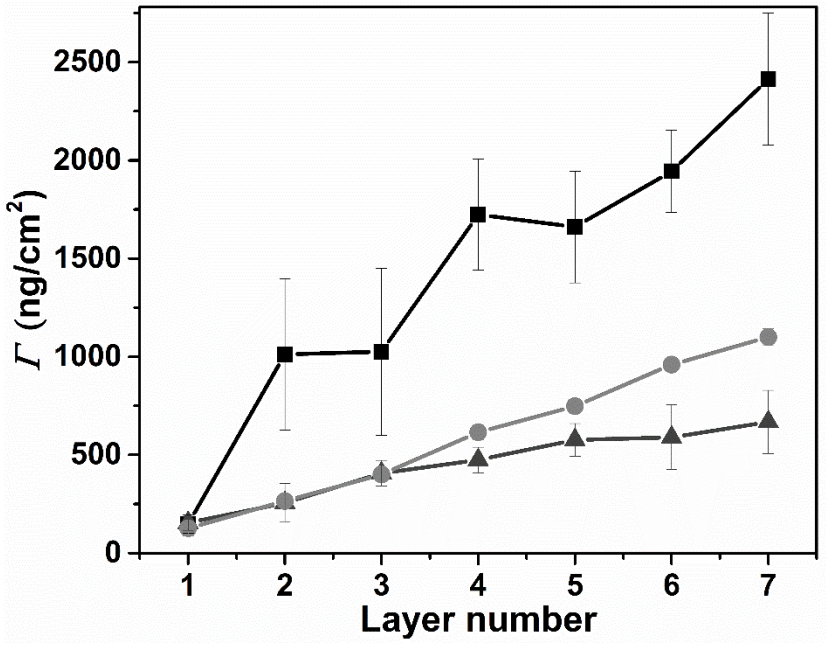
[47] H. J. Griesser, "Woodhead Publishing Series in Biomaterials A2", in *Thin Film Coatings for Biomaterials and Biomedical Applications*, Woodhead Publishing, 2016, p. xi.

[48] M. Buttner, S. Moller, M. Keller, D. Huster, J. Schiller, M. Schnabelrauch, P. Dieter, U. Hempel, *J Cell Physiol* **2013**, *228*, 330.

[49] F. Kidwai, J. Edwards, L. Zou, D. S. Kaufman, *Stem Cells* **2016**, *34*, 2079.

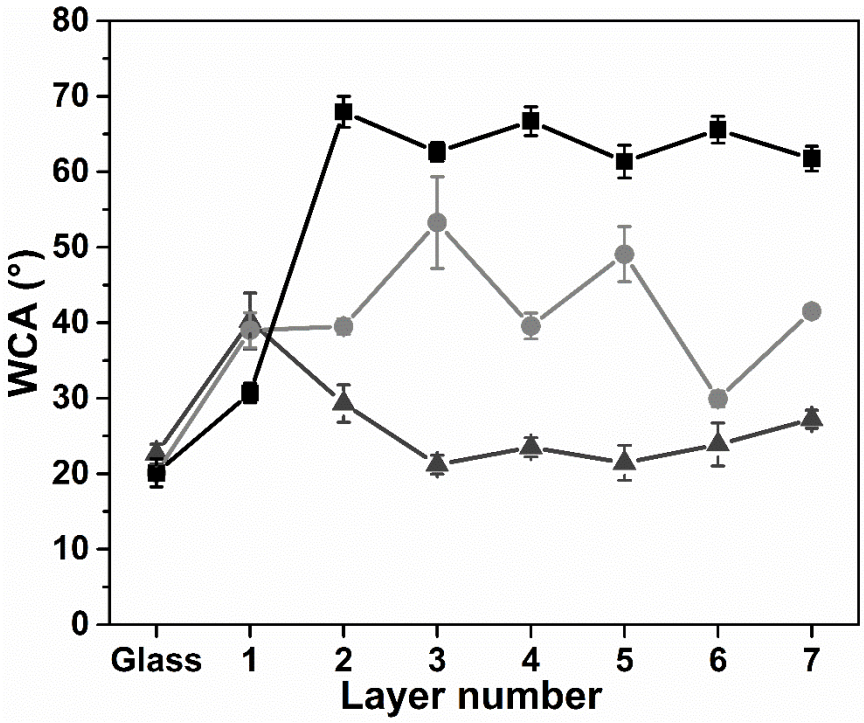
[50] V. Llopis-Hernandez, M. Cantini, C. Gonzalez-Garcia, Z. A. Cheng, J. Yang, P. M. Tsimbouri, A. J. Garcia, M. J. Dalby, M. Salmeron-Sanchez, *Sci Adv* **2016**, *2*, e1600188.

Figure 1.



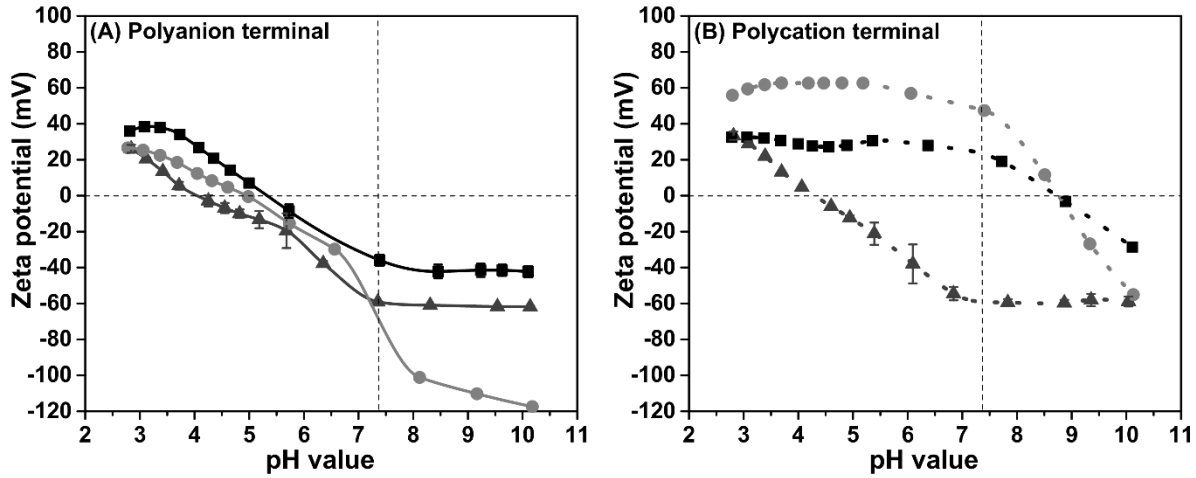
PEL adsorption onto MUDA-modified gold sensors obtained with SPR. The total layer mass (Γ) is shown for the following PEM systems: polystyrene sulfonate/ poly (allylamine hydrochloride) (PSS/PAH) (●), fibrinogen/ poly-L-lysine (FBG/PLL) (■) and biotinylated chondroitin sulfate/ avidin (BCS/AVI) (▲). [Layer 1 = poly (ethylene imine) (PEI), odd numbers = polycation, even numbers = polyanion]

Figure 2.



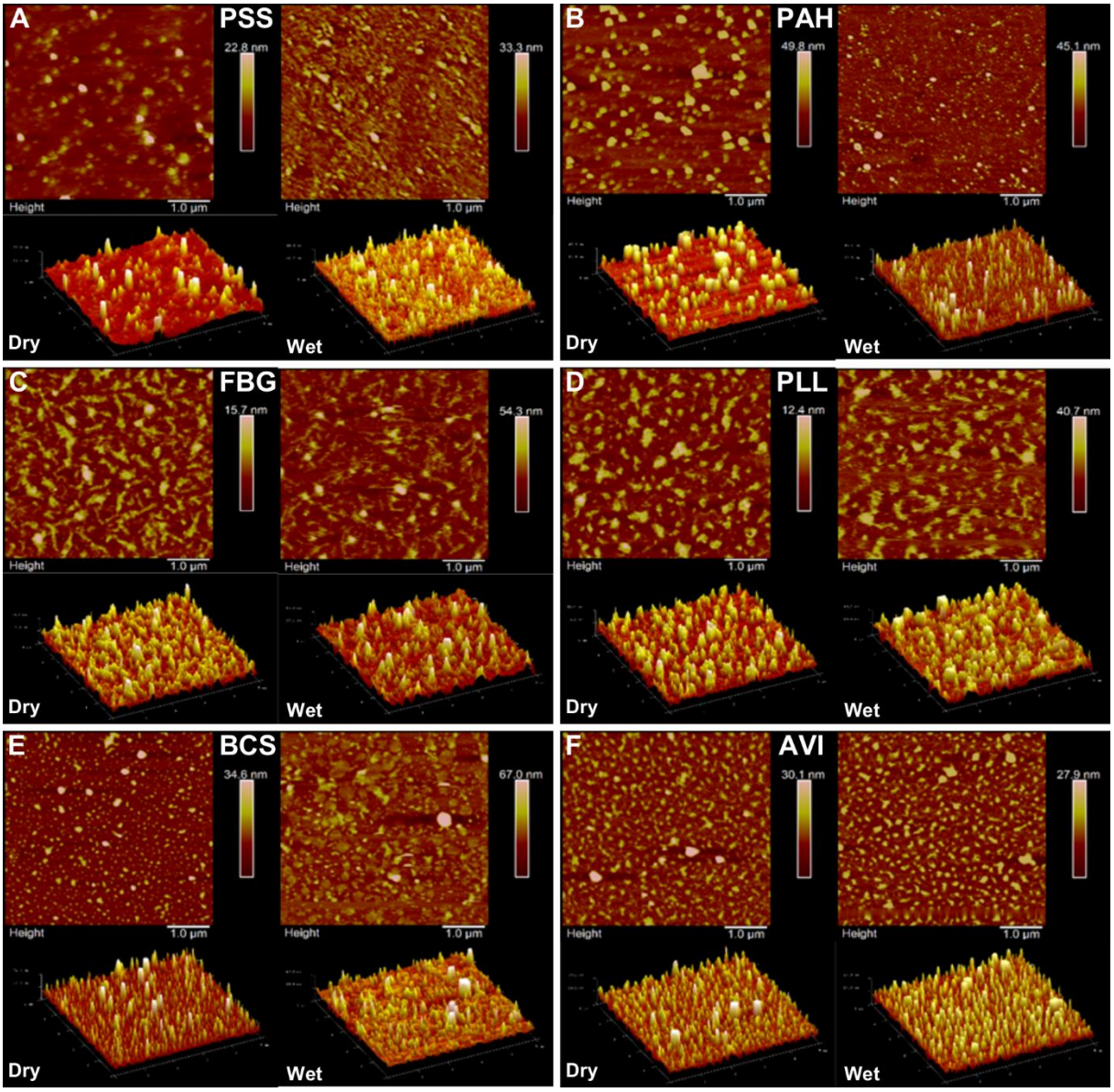
Static WCA measurement during multilayer formation up to seven layers. Results are means ± SD, n = 10. The initial layer is PEI (i.e. 1), while odd numbers refer to cations and even numbers to anions; polystyrene sulfonate/ poly (allylamine hydrochloride) (PSS/PAH) (-●-), fibrinogen/ poly-L-lysine (FBG/PLL) (-■-) and biotinylated chondroitin sulfate/ avidin (BCS/AVI) (-▲-) multilayer systems.

Figure 3.



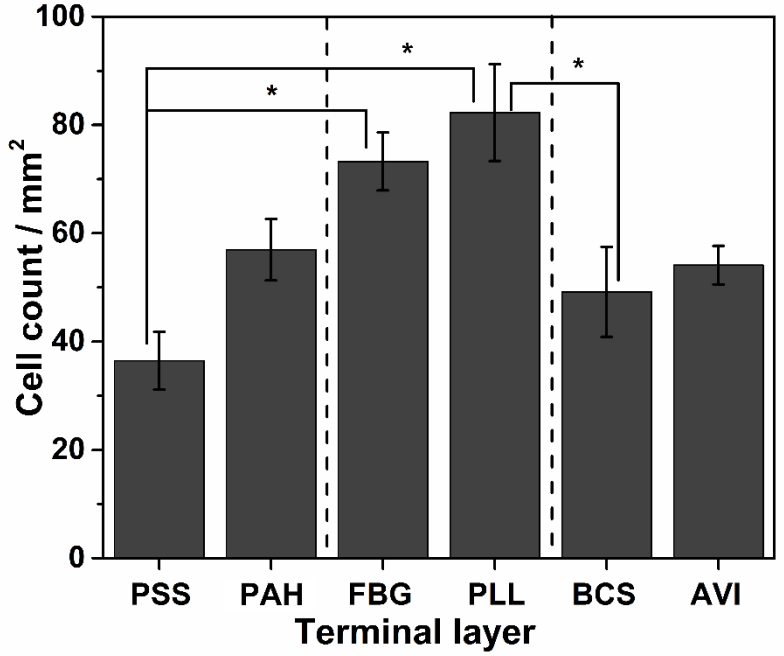
Zeta potential measurements are plotted as function of pH value. Results are means ± SD, n = 2. (A) Outermost polyanion layers, solid lines: Black (–■–) fibrinogen (FBG), dark gray (–▲–) biotinylated chondroitin sulfate (BCS), and light gray (–●–) polystyrene sulfonate (PSS). (B) Outermost polycation layers, dotted lines: Black (…■…) poly-L-lysine (PLL), dark gray (…▲…) avidin (AVI), and light gray (…●…) poly (allylamine hydrochloride) (PAH). [Polyanions = outermost sixth layer, Polycations = outermost seventh layer]

Figure 4.



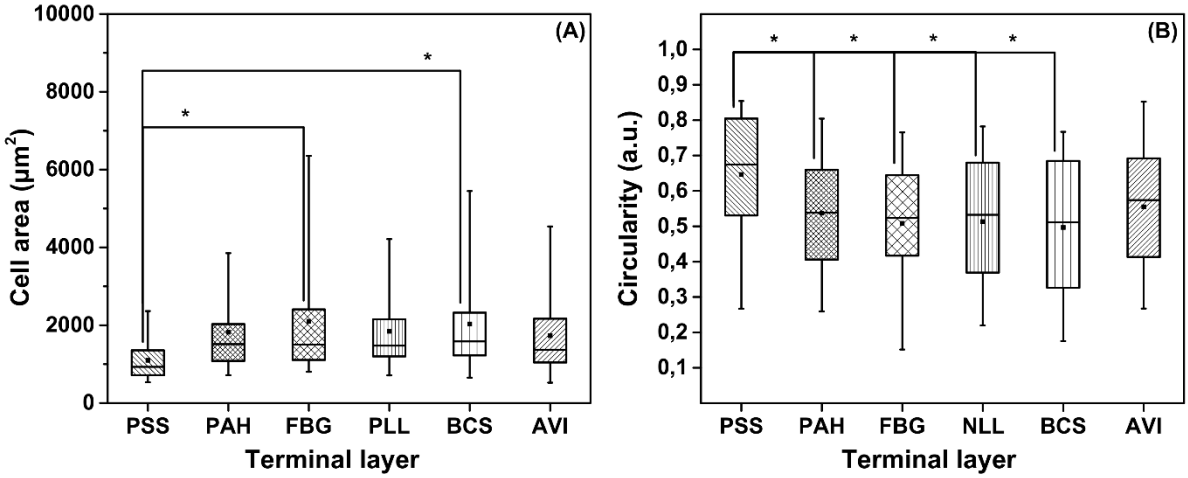
AFM images of LbL systems at both dry and wet (10 mM NaCl) conditions. The height magnitute image for terminal polyanions (left) and terminal polycations (right) is shown together with a 3D reconstruction of the surface [polystyrene sulfonate (PSS), poly (allylamine hydrochloride) (PAH), fibrinogen (FBG), poly-L-lysine (PLL), biotinylated chondroitin sulfate (BCS), avidin (AVI); polyanions = outermost sixth layer, polycations = outermost seventh layer].

Figure 5.



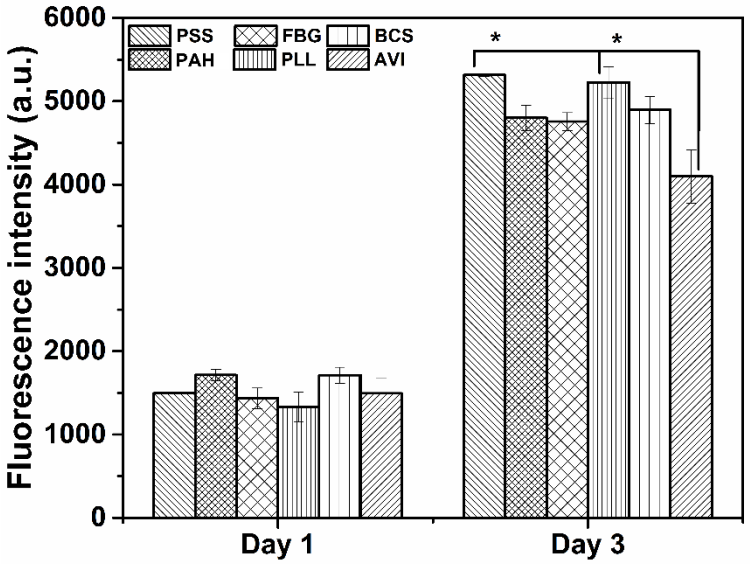
Adhesion of MG-63 cells to PEM after culture in DMEM with 10% FBS for 4 h. Results are means ± SD, n = 5. Asterisks indicate statistical significance at $p \leq 0.05$ [polystyrene sulfonate (PSS), poly (allylamine hydrochloride) (PAH), fibrinogen (FBG), poly-L-lysine (PLL), biotinylated chondroitin sulfate (BCS), avidin (AVI); polyanions = outermost sixth layer, polycations = outermost seventh layer].

Figure 6.



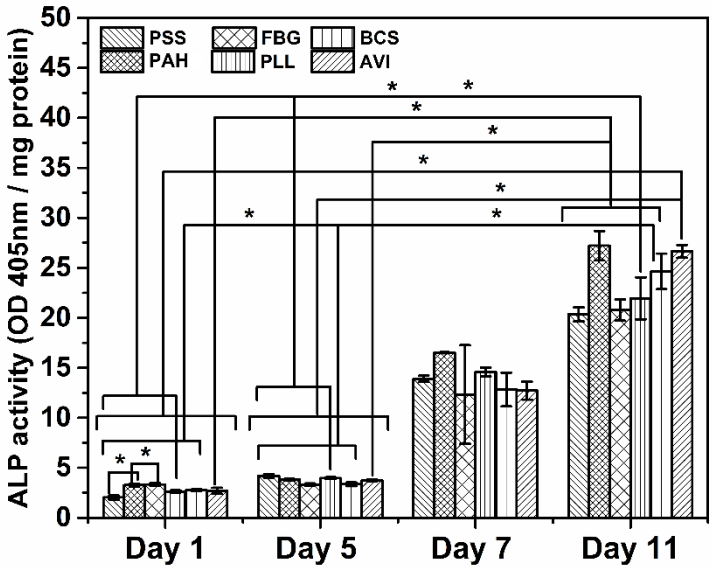
Spreading (A) and circularity (B) of MG-63 cells after culture in DMEM with 10% FBS for 4 h. The box indicates the 25th and 75th percentile, the median (dash), and mean value (black square), respectively, whereas the 95-5% confidence interval is represented by the whiskers. Asterisks indicate statistical significance at $p \leq 0.05$ [polystyrene sulfonate (PSS), poly (allylamine hydrochloride) (PAH), fibrinogen (FBG), poly-L-lysine (PLL), biotinylated chondroitin sulfate (BCS), avidin (AVI)].

Figure 7.



Proliferation of MG-63 cells determined with the QBlue® assay for indication of metabolically active cells after one and three days of culture. Results are means ± SD of three independent experiments. Asterisks indicate statistical significance at $p \leq 0.05$ [polystyrene sulfonate (PSS), poly (allylamine hydrochloride) (PAH), fibrinogen (FBG), poly-L-lysine (PLL), biotinylated chondroitin sulfate (BCS), avidin (AVI)].

Figure 8.



ALP activity per mg protein on different terminal layers. Asterisks indicate statistical significance at $p \leq 0.05$ [polystyrene sulfonate (PSS), poly (allylamine hydrochloride) (PAH), fibrinogen (FBG), poly-L-lysine (PLL), biotinylated chondroitin sulfate (BCS), avidin (AVI)].

Table 1. Zeta potential (ζ) values at pH 7.4 and pH values at point of zero charge (PZC) for terminal anionic and cationic layers.

Terminal layer	ζ at pH 7.4 [mV]	PZC at pH
PSS 6th	-68 ± 1.11	4.9 ± 1.72
PAH 7th	47 ± 0.37	8.8 ± 0.41
FBG 6th	-36 ± 3.07	5.4 ± 0.05
PLL 7th	23 ± 0.41	8.8 ± 0.82
BCS 6th	-60 ± 0.60	4.0 ± 0.45
AVI 7th	-55 ± 1.83	4.3 ± 0.43

Polystyrene sulfonate (PSS), poly (allylamine hydrochloride) (PAH), fibrinogen (FBG), poly-L-lysine (PLL), biotinylated chondroitin sulfate (BCS), avidin (AVI); 6th = sixth layer, 7th = seventh layer

Table 2. Roughness average (Ra) [nm] for individual terminal layers obtained from AFM height images under dry and wet conditions.

Terminal layer	Dry [nm]	Wet [nm]
PSS 6th	1.62 ± 0.13	2.70 ± 0.14
PAH 7th	5.17 ± 0.63	3.86 ± 0.81
FBG 6th	2.15 ± 0.24	4.49 ± 1.04
PLL 7th	1.51 ± 0.04	4.26 ± 0.04
BCS 6th	2.48 ± 0.29	5.52 ± 0.20
AVI 7th	2.62 ± 0.16	4.55 ± 1.73

Polystyrene sulfonate (PSS), poly (allylamine hydrochloride) (PAH), fibrinogen (FBG), poly-L-lysine (PLL), biotinylated chondroitin sulfate (BCS), avidin (AVI); 6th = sixth layer, 7th = seventh layer

A comparative study of synthetic and biogenic polyelectrolyte multilayer systems on osteogenic activity showed that multilayers composed of fibrinogen and poly-L-lysine have superior effect on cell adhesion and growth, while multilayers made of biotinylated chondroitin sulfate and avidin promoted osteogenic differentiation of cells, which was more effective than multilayers made of polystyrene sulfonate and poly (allylamine).

Deepak Guduru, Marcus S. Niepel, Cristina Gonzalez-Garcia, Manuel Salmeron-Sanchez, Thomas Groth*

Osteogenic Activity of Polyelectrolyte Multilayers