BIOMIMETIC IMPLANT COATINGS FOR LOCAL DELIVERY OF GROWTH FACTORS AND ANTIBIOTICS

Steffi Grohmann, Manuela Menne, Holger Rothe and Klaus Liefeith

Department of Biomaterials, Institute for Bioprocessing and Analytical Measurement Techniques (iba) e.V.
Rosenhof, 37308 Heilbad Heilgenstadt, Germany
Emails: steffi.grohmann@iba-heiligenstadt.de, web page: http://www.iba-heiligenstadt.de
holger.rothe@iba-heiligenstadt.de
klaus.liefeith@iba-heiligenstadt.de

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ABSTRACT

Two of the most challenging issues with bone implants are the (i) stable osseointegration of the material into the surrounding bone tissue and (ii) prevention of a possible implant-associated infection. Biomimetic coatings are a promising technique to equip bioinert metal and ceramic surfaces with biological cues and thus to both support the osseointegration of the implant and to fight bacterial infections.

This study is focused on thin film coatings containing native extracellular matrix molecules [1-2] (like sulfated glycosaminoglycans) prepared via the "layer-by-layer" technology developed by Decher, et al. [3] (polyelectrolyte multilayer, PEM) and their subsequent loading with biologically active molecules. The bioactive molecules comprise a growth factor and a broad spectrum antibiotic. We report on the effect that the chemical composition of the PEM and the pH of the coating solution impose on the loading capacity of the PEM coatings.

1 INTRODUCTION

Polyelectrolyte multilayer coatings with high analogy to the native extracellular matrix have recently been described by our group [1, 2, 4, 5] and others. Especially, PEM coatings composed of sulfated glycosaminoglycans (like chondroitin sulfate and heparin) are of special interest since they reveal a significant binding capacity for growth factor like bone morphogenetic proteins (BMP). The BMP loaded multilayers induce osteogenic differentiation of osteoblast precursor cells *in vitro*.

In addition to an improved osteointegration, implant-associated infections are still a challenging issue for particular applications. Thus, there is a need for implants that will additionally display an anti-biotic effect and consequently reduce the risk for infections. Simple absorptive/ adsorptive loading of sGAG based PEM coatings was not successful, yet. However, for a different kind of PEM, i.e. coatings composed of a polyanionic polypeptide (poly-L-glutamic acid, PGA) instead of the sGAG, Jiang et al. reported an effective approach to increase the loading capacity [6]. This increase was obtained by simply shifting the pH of the polyelectrolyte solutions to an acidic pH during the film assembly. At this pH not all of the available polyanionic groups were dissociated and thus engaged in ionic bondings to the polycation. When loading the PEM with the antibiotic, the pH was shifted to neutral values. Consequently, acidic groups became deprotonated and bound increased amounts of the slightly positively charged gentamicin. The aim of this study was to transfer this concept from PEM

films with the anionic polypeptide to PEM films with a growth factor binding sGAG, in order to create coatings that will both induce osseointegration and reduce the risk for implant-associated infections.

2 EXPERIMENTAL

2.1 Multilayer Preparation

Chemicals and reagents were purchased from Sigma Aldrich (Taufkirchen, Germany) and used without further purification, unless stated otherwise. PEM films were assembled on QCM-D sensors and borosilicate glass discs (diameter 15 mm, B33 glass, Schott, Jena, Germany). The substrates were cleaned with detergents and deionized water prior to film assembly. Glass discs were etched with concentrated nitric acid immediately before the coating procedure. PEM films were assembled from poly-L-lysine (PLL, 30–70 kDa), poly-L-glutamic acid (PGA, 50–100 kDa) Heparin (HEP, ~12 kDa) and chondroitin sulfate (CS, ~63 kDa, from shark cartilage). The polyelectrolytes were dissolved in either HEPES buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 137 mM NaCl, pH 7.4) or sodium acetate buffer (20 mM, 137 mM NaCl, pH 4.0) at a concentration of 1 mg/ml. Loading of the multilayers was performed with a gentamicin solution (10 mg/ml in phosphate buffered saline, pH 7.4) and/or a BMP-2 solution (0.1 mg/ml in acetate buffer).

2.2 QCM-D Measurements

Both the PEM assembly and the subsequent loading with growth factor and antibiotic were monitored with quartz crystal microbalance measurements. Two deposition regimes were investigated: (i) PEM build-up at pH 7.4 and (ii) PEM build-up at pH 4.0. Subsequent loading of the multilayer with recombinant human bone morphogenetic protein 2 (*rh*BMP-2) and gentamicin was performed at pH 4.5 and 7.4, respectively. In the double loading experiments the multilayers were first loaded with gentamicin and then with the growth factor or vice versa. By means of rinsing the loaded multilayer with buffer, the release of the antibiotic could be estimated.

2.3 Microbiological experiments

The antibiotic effect was assessed by means of agar diffusion assays and additionally release kinetics were recorded employing specific ELISA tests. For the agar diffusion assays loaded PEM films were mounted onto agar plates seeded with *staphylococcus aureus*. The zone of inhibited bacterial growth around the sample was measured and transformed into a loading capacity. For this transformation a standard curve with known concentrations of coated gentamicin ($\mu g/cm^2$) was included in every experiment. For elution experiments the loaded film surface was incubated with a defined volume of buffer at room temperature over an incubation time of up to 14 days. At predefined time points samples of the elution medium were assayed for their gentamicin concentration (commercial gentamicin ELISA test) and their bioactivity.

2.4 Cell biological experiments

For cell biological experiments, MC3T3-E1 preosteoblasts were seeded onto the coated samples and incubated for up to 7 days. The analyses comprised cell morphology (after staining the cell cytoskeleton and nuclei), cell counting and the quantification of osteoblast specific markers like the activity of the bone specific alkaline phosphatase.

3 RESULTS AND DISCUSSION

3.1 QCM-D investigations on the film construction

The multilayer assembly was investigated by means of QCM-D measurements employing three different polyanions for film construction: PGA (poly-L-glutamic acid), CS (chondroitin sulfate) and

HEP (heparin). Poly-L-Lysine (PLL) served as the polycation in all analysed films. Since the approach was to assemble the PEM coatings at a pH that allowed for acidic groups to be less dissociated the pH of the coating solution was adjusted to 4.0. For the assembly of multilayers containing sGAG, it was not known whether the assembly process would still be successful at this acidic pH. The QCM-D data summarized in Figure 1 reveal that a stepwise increase in film thickness could be obtained even at pH 4.0 over a course of ten deposited double layers (polycation + polyanion). Furthermore, when the PEM films were assembled at pH 4.0 they displayed a lower total mass of adsorbed polyelectrolyte per layer as compared to the identical coatings prepared at pH 7.4.

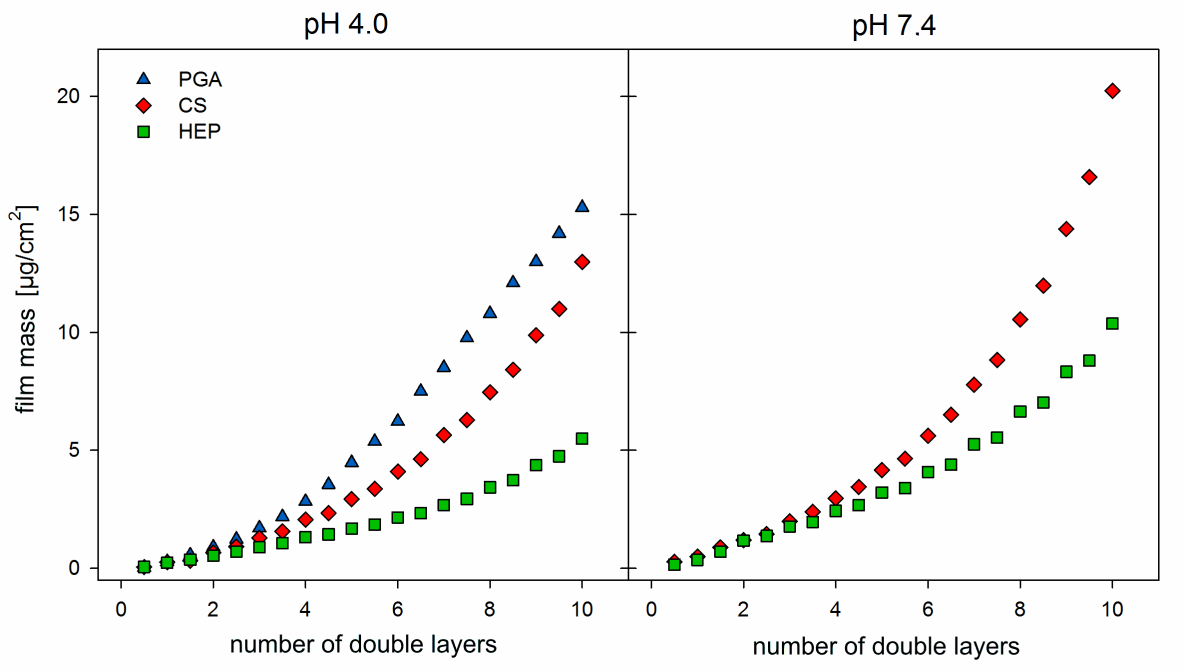

Figure 1: QCM-D results on the film assembly of PEM with the polyanions PGA, CS and HEP at pH values of the coating solution of 4.0 and 7.4.

3.2 Investigations on the loading capacity with the antibiotic

After the successful film assembly at pH 4.0 all PEM were loaded with the antibiotic gentamicin at pH 7.4. Due to this pH shift the acidic groups of the polyanions become deprotonated and thus were expected to adsorb more of the slightly positively charge antibiotic. The loading of the multilayer with gentamicin (GS) was assessed by means of QCM-D measurements. The respective results are summarized in Figure 2. While the mass of PGA based multilayers increased by more than 100% , the mass of the sGAG based multilayers only increased by ~5% upon loading with gentamicin. This suggests that the binding mechanism is of the antibiotic to the polyanion is not solely based on electrostatic interactions since the zetapotential and the isoelectric points of PGA- and CS-based multilayers are very similar.

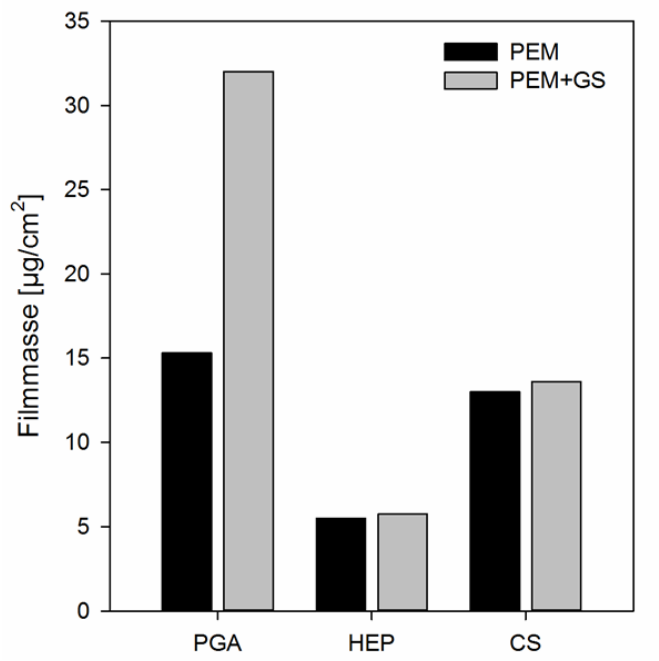

Figure 2: Results of QCM-D measurements on the total film mass before and after loading with the antibiotic gentamicin (films assembled at pH 4.0).

Based on the results of the agar diffusion test based experiments on the loading density of gentamicin in PEM films, no biological activity was determined for any of the CS based PEM films, implicating that either no antibiotic was bound or bound antibiotic was released immediately during the rinsing steps. Consequently, CS based coatings were excluded from the loading experiments with gentamicin.

The second prominent result is that the pH of the coating solution significantly affected the loading density for PGA and HEP based multilayers (Figure 3). A twofold up to more than a tenfold increase in loading capacity was observed for films assembled at pH 4.0 as compared to pH 7.4. This finding indicates that the mechanism proposed by Jiang et al. is successfully transferable to HEP based multilayers. Furthermore, a clear increase of the loading capacity with the number of double layers was observed (10 to 30 double layers).

However, similar to the results obtained from the QCM-D measurements PGA based multilayers display an up to 15-fold increased binding capacity for the antibiotic as compared to the HEP based multilayers of interest. For HEP based multilayers more than 10 double layers are necessary, in order to achieve a substantial antibiotic effect. Consequently all further comparative experiments were performed on PGA and HEP based multilayers composed of 20 and 30 double layers.

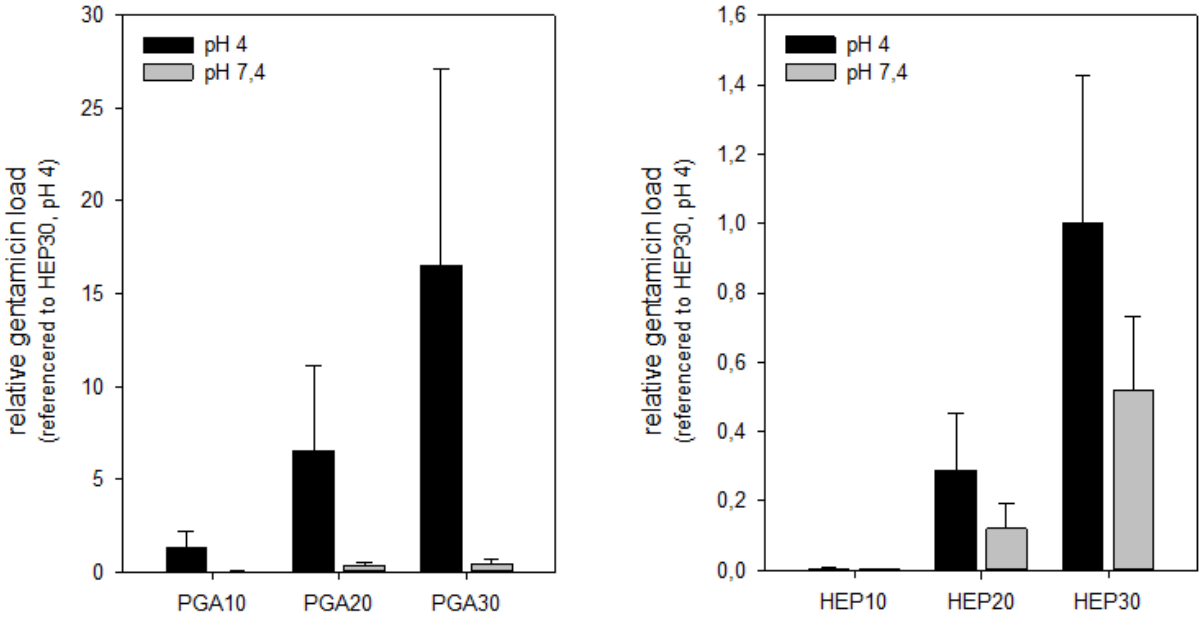

Figure 3: Relative gentamicin load on PEM films assembled from different polyanions (PGA vs. HEP), with increasing numbers of double layers (10, 20 and 30) and different pH values of the coating solution (pH 4.0 vs. pH 7.4). Results were obtained by means of agar diffusion assays.

Elution studies on the aforementioned loaded multilayers over an incubation period of up to 14 days revealed that significant amounts of gentamicin were released from PGA based multilayers with 20 and 30 double layers. Complete inhibition of *staphylococcus aureus* growth was achieved with these samples over the entire time period. For HEP30 coatings a complete inhibition was obtained until day 4 and reduced proliferation until at least day 7. HEP20 coatings were only able to completely inhibit proliferation for 24 hours. After 14 days no residual antibacterial effect was detectable for any of the HEP based coatings.

3.3 Coatings with dual bioactivity

In addition to loading the films with a single target molecule we also performed loading experiments with two biologically active components. Although PGA based multilayers reveal superior binding capacity for the antibiotic they reveal no specific binding affinity for the growth factor. On the other hand the growth factor possesses a binding domain with high affinity for heparin. Since HEP based multilayers further displayed a fair binding capacity for gentamicin they were chosen as the basis for the development of coatings with dual bioactivity.

Due to the fact that the two bioactive molecules were not soluble in the same buffer (pH) sequential loading with gentamicin at pH 7.4 and the growth factor at pH 4.5 had to be performed and evaluated. The antibiotic effect was most pronounced for coatings solely loaded with gentamicin (Figure 4, left panel). However, if additionally loaded with the growth factor, the best results were obtained when gentamicin was applied as the last layer. This is probably due to the fast release of the antibiotic. For the growth factor activity a similar trend was observed (Figure 4, right panel). The most effective osteoblast stimulation was obtained for the coatings that were exclusively loaded with the growth factor. When the antibiotic was co-loaded onto the PEM films (especially when the antibiotic was deposited prior to the growth factor), the osteoblast response decreased to a certain extent, probably related to binding sites that are blocked by the adsorbed gentamicin. Yet, a significant bioactivity remained available for the dually loaded ECM-analogous PEM films that make these coatings

interesting for applications in the fields of implantology that face an increased risk for implant-associated infections.

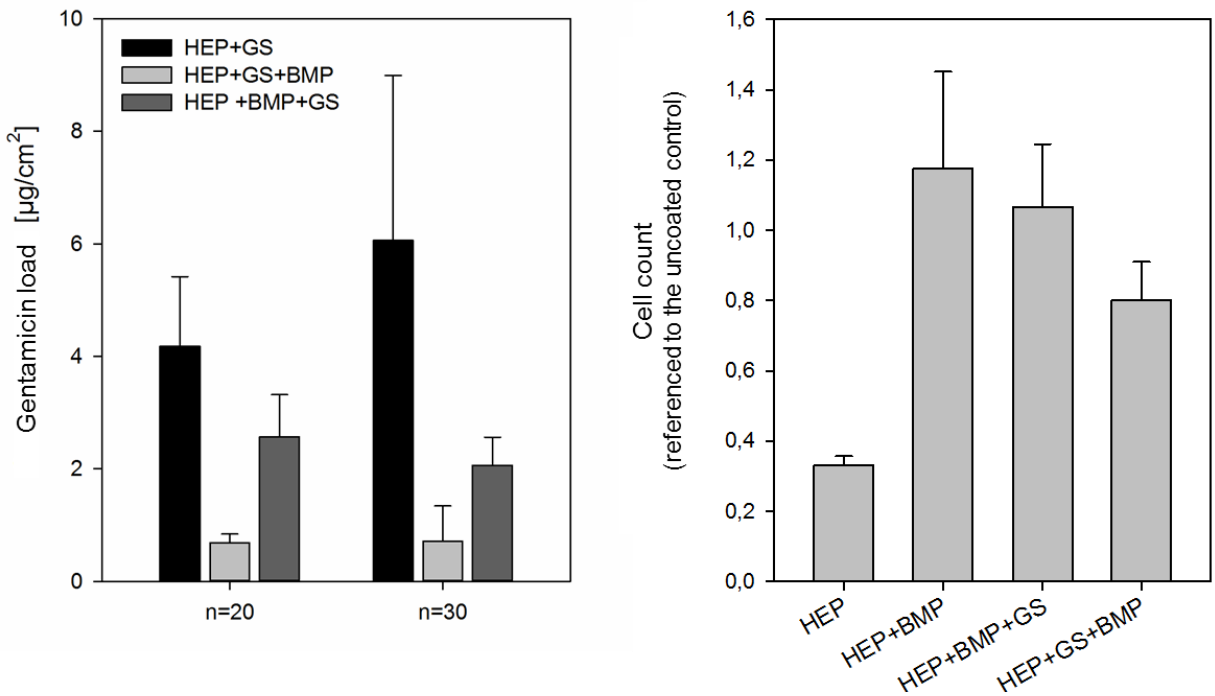

Figure 4: Analyses on the two bioactive effects with respect to the order of loading the coatings. Left) antibiotic activity of loaded HEP20 and HEP30 coatings. Right: cell count on loaded HEP20 coatings.

4 CONCLUSION

Implant coatings with dual bioactivity can successfully be prepared applying the layer-by-layer technology. The basic LbL approach was improved by incorporating a pH shift between the assembly process and the loading process. Our data reveal that the dual delivery of both a growth factor and an antibiotic is possible if loaded in the correct sequence. The best biologically relevant activities can be obtained by first depositing the growth factor and subsequently the antibiotic. The resulting coatings display a toxic effect against bacteria and a stimulating effect on the differentiation of preosteoblastic cells. This promising potential of these multilayers (with both with single and dual bioactivity) for implants in bone contact will further be investigated by means of *in vivo* animal experiments.

ACKNOWLEDGEMENTS

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REFERENCES

[1] Grohmann S, Rothe H, Frant M, Liefeith K. *Colloidal force spectroscopy and cell biological investigations on biomimetic polyelectrolyte multilayer coatings composed of chondroitin sulfate and heparin.* Biomacromolecules 2011; **12**:1987-97.

[2] Grohmann S, Rothe H, Liefeith K. *Investigations on the secondary structure of polypeptide chains in polyelectrolyte multilayers and their effect on the adhesion and spreading of osteoblasts.* Biointerphases 2012; **7**:1-13.

[3] Decher G. *Fuzzy Nanoassemblies: Toward Layered Polymeric Multicomposites.* Science 1997; **277**:1232-7.

[4] Grohmann S, Weiss T, Liefeith K. *Biointerfaces between Biomaterials and Biosystems in Tissue Engineering: Potential Contributions of Two-Photon Polymerisation and Polyelectrolyte Multilayer Coatings.* in: Biomaterials for Stem Cell Therapy: State of the Art and Visions for the Future De Bartolo, L and Bader, A 2013;CRC Press, Boca Raton, London, New York:110-52.

[5] Grohmann S, Rothe H, Eisenhuth S, Hoffmann C, Liefeith K. *Biomimetic assembly of polyelectrolyte multilayers containing phosvitin monitored with reflectometric interference spectroscopy.* Biointerphases 2011; **6**:54-62.

[6] Jiang B, Li B. *Tunable drug loading and release from polypeptide multilayer nanofilms.* International Journal of Nanomedicine. 2009; **4**:37-53.