J Mater Sci: Mater Med (2009) 20:2455–2463
DOI 10.1007/s10856-009-3825-y

In vitro behavior of layer-by-layer deposited molecular oligoelectrolyte films on Ti–6Al–4V surfaces

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Received: 3 February 2009 / Accepted: 14 July 2009 / Published online: 26 July 2009

Abstract Layer-by-layer self-assembled films of molecular oligoelectrolytes were used to modify Ti–6Al–4V surfaces in order to test their ability as potential drug delivery system. With regard to medical application the in vitro behavior of the modified material was investigated. The Ti–6Al–4V (6% aluminium, 4% vanadium) material was treated in a layer-by-layer (LbL) process with 2, 4, 6 and 8 layers of molecular oligoelectrolytes **1** and **2** and thereby doped with a fluorescent reporter molecule **2**. Human osteoblasts were cultured for a period up to 5 days on the modified material. Ti–6Al–4V surfaces without modification were used as control. In order to investigate the in vitro behavior of the coating as well as the influence of components of the coating on osteoblastic cells, respectively, cell proliferation, differentiation and attachment of hFOB cells were observed by means of cell number, osteoblastic gene expression and fluorescence microscopy. Degradation behavior of the OEM (oligoelectrolyte multilayer film) was examined using optical spectroscopy. Measurement data imply that the layer-by-layer coating was successfully assembled on the Ti surface and endures steam sterilization. The fluorescence signal in cell culture medium increased strictly linear with increasing pre-assembled number of layers on the surface. Proliferation rates of the cells in experimental groups did not differ significantly from each other ($P \geq 0.783$). Differentiation pattern was not significantly changed by the coating. The fluorescent reporter component of the film was absorbed by osteoblastic cells and was detected by fluorescence microscopy.

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1 Introduction

Materials' surface properties, such as surface chemistry, charge and topography, influence the biological performance of medical implants [1]. Common techniques to enhance the biocompatibility of orthopedic and dental titanium-based materials are sandblasting or etching implant surfaces [2], coating with hydroxyapatite [3] or calciumphosphate [4]. For the local application of pharmaceuticals on implants [5] chemical and physical coating methods are of major interest. Primary strategy hereby is to immobilize bioactive molecules such as selective synthetic peptide sequences of extracellular matrix proteins on material surface [6, 7].

The layer-by-layer (LbL) self-assembly [8] is based on Coulomb interaction of charged polyelectrolytes. A negatively charged substrate can be modified by immersing it in a solution of positively charged molecules that bind electrostatically to the surface. As opposed charged molecules attract each other another monolayer is established by dipping the substrate in a solution of a negatively charged electrolyte. Under these iterative conditions a nano-scale

film of opposed charged layers can be built up. Nearly any molecule bearing multiple ionic charges can be assembled on any shape and size of substrate by this technique, making it very advantageous for biomedical applications including new concepts of drug delivery systems [7, 9]. The inherent limitation of the current LbL-method is the use of polymers as building blocks. Polymers are polydisperse and usually do not adopt a defined and controllable shape and orientation within the deposited layers [10]. This leads to a limitation of precise fine tuning of film properties and the manageability of polymeric thin films is therefore very demanding.

Recently, the LbL technique was successfully extended to molecular oligoelectrolytes [11]. These perfect monodisperse molecules do have a concrete number of charges and a defined structure which allows a precise control over the assembly and properties of the thin film. Multilayer films of molecular oligoelectrolytes were built in a consistent monomolecular matter, their degradation behavior is well investigated [11].

A future aim is to immobilize bioactive molecules between the different layers of an LbL nano-architecture such as growth factors. These growth factors can be released in a physiological sequence in order to accelerate and enhance osseointegration of the implants. However, to date there is only little knowledge on the biocompatibility of layer-by-layer coated implants of molecular oligoelectrolytes. Therefore, the aim of the present study was to study the stability and degradability of layer-by-layer coated OEMs on titanium (Ti–6Al–4V) surfaces. In addition its effect on osteoblastic behavior was investigated.

2 Materials and methods

2.1 Titanium scaffolds

Ti–6Al–4V surfaces manufactured by Selective Electron Beam Melting (SEBM) are suitable scaffolds for the attachment, proliferation and differentiation of human fetal osteoblasts [12]. For this reason, Ti–6Al–4V discs (5 mm thick, 11 mm diameter) were prepared by SEBM of a commercially available Ti–6Al–4V powder (particle size 45–100 μm) using EBMS12 system (Arcam AB, Mölndal, Sweden) as described by Heinl et al. [13]. To create a smooth surface, the discs were polished with SiC paper up to 2400 grit in the final step. All samples were sonicated for 30 min in EtOH (70%) and distilled water.

2.2 Surface modifications

All samples were modified using the electrostatically layer-by-layer technique. As cationic component a permanently nine fold positively charged Newkome-like dendrimer **1** (Fig. 1) was applied [11]. The negatively charged layers were built of a perylene-functionalized Newkome dendrimer second generation **2** [14]. These molecular oligoelectrolytes are perfectly monodispers and guarantee an assembly of hierarchically ordered monolayers [11]. Besides, the perylene derivative serves as reporter electrolyte as it shows high fluorescence efficiencies at low concentrations [14]. On the basis of etched and therefore charged Ti–6Al–4V discs multilayer surfaces with two to eight layers were assembled by iterative dipping circles.

The Ti–6Al–4V samples were etched in a mixture of water, isopropanol and methanol (all HPLC grade) (VWR, Ismaning, Germany) which was saturated with potassium hydroxide KOH. After 30 min of ultrasonication they were washed with water until the washing water showed neutral reaction on pH measurement.

All oligoelectrolytes for the assembly were diluted in a buffer with pH 7, made of 0.1 M NaOH and 0.1 M KH_2PO_4 solution in Millipore water (Fluka, Taufkirchen, Germany). This water was also used for the washing steps. The concentration of the coating solutions is about 10^{-2} mol/l. After diluting in buffer they were all filtrated through a Millipore Millex-GP hydrophilic filter with a pore size of 0.22 μm.

The assembly of one layer requires a period of 30 min followed by a 2 min washing step. The samples were not dried between the different coating steps, but before the measurements they were dried under atmospheric conditions. For the cell experiments all samples were steam sterilized at 121°C for 30 min.

2.3 Cell culture

Human fetal osteoblasts (hFOB 1.19 cells) were cultured in Dulbecco's Modified Eagle Medium F-12 nutrient mixture (DMEM/F12, Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS, Invitrogen GmbH, Karlsruhe, Germany) and 1% penicillin/streptomycin (Invitrogen GmbH, Karlsruhe, Germany). Cells were plated into cell culture flasks and incubated at 34°C and 5% CO_2. Medium was changed every 3 days.

For the cell experiments osteoblasts were seeded on sterile uncoated and coated Ti–6Al–4V discs that were placed into tissue culture polystyrene plastics (24-well tissue culture dish, Nunc, Wiesbaden, Germany).

2.4 Cell proliferation

Osteoblasts were seeded at densities of 2×10^4 cells per disc. After 1, 3 and 5 days of culturing the cell number was determined. The Ti–6Al–4V discs were transferred into unused wells in order to count only cells adhered to the

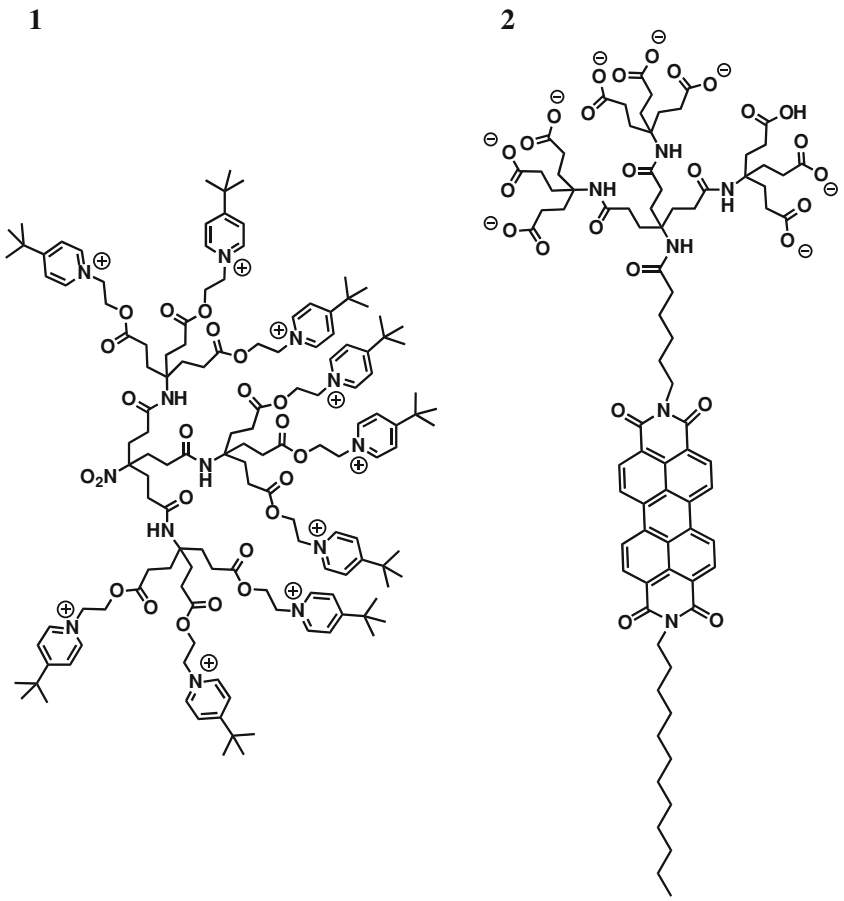

Fig. 1 Molecular structure of cationic Newkome-Dendrimer **1** and anionic fluorescent reporter molecule **2**

scaffolds and not those attached to the plastic dish bottom. Then cells were detached with trypsin/EDTA (Invitrogen GmbH, Karlsruhe, Germany) for 5 min and counted in a cell counter (Casy I, Schärfe System GmbH, Reutlingen, Germany). All cell experiments were repeated 2–3 times with at least three samples in each experimental run.

2.5 Cell differentiation

For the quantitative real-time polymerase chain reaction (real-time PCR) of selected osteogenic differentiation genes hFOB cells were seeded at a density of 1×10^5 cells/disc and cultured in osteoblastic differentiation medium (DMEM/F12 supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 10^{-8} M dexamethasone (Sigma Diagnostics, Inc, St. Louis, USA), 50 μM L-ascorbic acid-2-phosphate (Sigma Diagnostics, Inc, St. Louis, USA) and 10 mM β-glycerol phosphate (Sigma Diagnostics, Inc, St. Louis, USA)) at 37°C under 5% CO_2. After 3 days of incubation, differentiation medium was removed and RNA was isolated using a commercially available kit (RNeasy Micro Kit, Qiagen, Hilden, Germany) according to the manufacturer's protocols. The integrity and purity of the total RNA was verified spectrophotometrically. The relative grade of differentiation of hFOB cells cultured on the scaffolds was defined by quantifying the relative expression of alkaline phosphatase (ALP), osteocalcin (OC), collagen type I and bone sialo protein, four selected osteogenic differentiation genes, by real-time PCR. The genes were normalized against 18s rRNA, a housekeeping gene with RNA levels that are constitutively transcribed. Gene specific primer sequences (Table 1) were designed using Primer Express 3.0 software (Applied Biosystems, Darmstadt, Germany). The primers were synthesized by Invitrogen GmbH, Karlsruhe, Germany. Isolated RNA was reverse-transcribed with the High Capacity cDNA Archive Kit (Applied Biosystems,

Table 1 Primer sequences used for quantitative real-time PCR

Gene	Sequence
Alkaline phosphatase	F: 5′-CCGTGGCAACTCTATCTTTGG-3′
	R: 5′-CAGGCCCATTGCCATACAG-3′
Osteocalcin	F: 5′-GGAGGTGTGTGAGCTCAATCC-3′
	R: 5′-CCGTAGAAGCGCCGATAGG-3′
Bone sialo protein	F: 5′-AAGCAATCACCAAAATGAAGACT-3′
	R: 5′-TGGAAATCGTTTTAAATGAGGATA-3′
Collagen type I	F: 5′-TGACCTCAAGATGTGCCACT-3′
	R: 5′-ACCAGACATGCCTCTTGTCC-3′

Darmstadt, Germany) in the thermo cycler PTC-200 (Bio-Rad Laboratories GmbH, Munich, Germany) according to the manufacturer's protocols. Each reverse-transcription reaction contained 2 µg RNA. The cDNA was amplified with the Power SYBR green PCR Master Mix in the 7300 real-time PCR System (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocols. Each real-time PCR reaction contained 40 ng of cDNA, 0.3 µmol of gene-specific primers and 12.5 µl of the SYBR green PCR master mix. The cycling conditions used for the real-time PCR were as follows: initial denaturation/enzyme activation for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min. The production of a single product was confirmed by melting curve analysis.

2.6 Microscopic analyses

Surface topography was examined using scanning electron microscopy (SEM, Philips XL 30, Philips, Eindhoven, Netherlands) operating with an accelerating voltage of 25 kV and measured by confocal three-dimensional white light microscopy (µSurf, NanoFocus AG, Oberhausen, Germany) to calculate the three-dimensional roughness parameter S_a (average height deviation) of the prepared specimens. Three samples from each group were measured and five individual measurements per sample were made over a 0.160×0.160 mm^2 area.

After 5 days of culturing, the absorbance of the fluorescent reporter molecule by the osteoblasts was assessed by microscopic examination. The medium was removed and the cells were washed once with PBS, fixed with 4% formalin for 10 min and dehydrated in a graded alcohol series at room temperature. All samples were microscopically examined (Axio Imager A1, Carl Zeiss, Jena, Germany).

2.7 Fluorescence emission in cell culture medium

All cell culture samples were filtrated through a Millipore Millex-GP hydrophilic filter with a pore size of 0.22 µm. The fluorescence emission measurements were recorded using a Shimadzu Spectrofluorometer RF-5301 PC. The excitation wavelength was 500 nm.

2.8 Statistics

All the values are given as means and standard deviation. For the analysis of all data the software program SPSS (Version 16.0 for Windows) was used. After the assessment of significant differences by one-way analysis of variance (ANOVA), differences among groups were established with the post-hoc Tukey Test. Values of $P < 0.05$ were considered to be statistically significant.

3 Results

3.1 Surface characterization

The macroscopic view of the Ti–6Al–4V discs changed after the layer-by-layer treatment. The silvery Ti–6Al–4V surface of untreated discs changed to multicolored, indicating the successful coating procedure (Fig. 2). On the microscopic level, the surface features were also modified (Fig. 3). The untreated surface had parallel grooves, which are typical of the grinding process with silicon carbide papers and oriented along the polishing direction. These parallel grooves produced by polishing disappeared on the etched surface and an irregular topography emerged. Surface measurements of the discs showed an increased surface roughness on etched and coated surfaces. The S_a value increased from 0.07–0.10 µm.

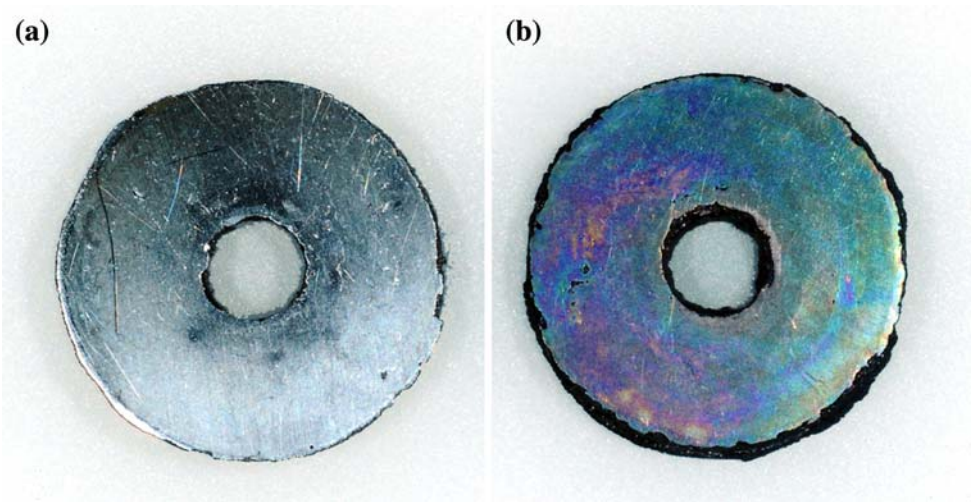

Fig. 2 **a** Untreated and **b** layer-by-layer coated Ti–Al6–V4 scaffold

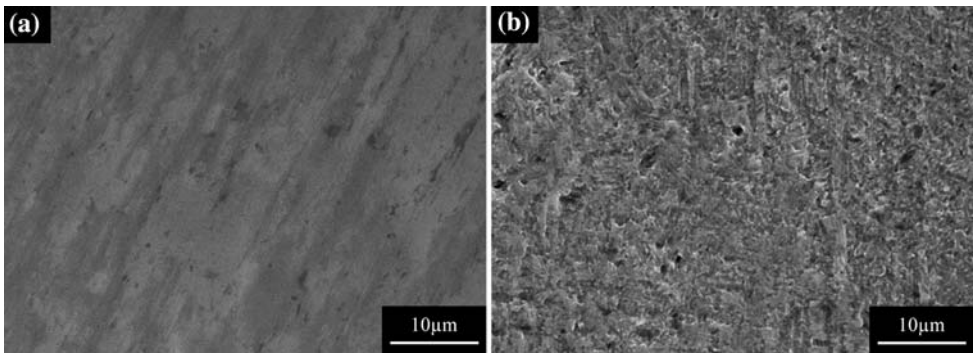

Fig. 3 SEM view of **a** untreated and **b** etched Ti–6Al–4V scaffold

3.2 Cell proliferation

In the main, the number of cells attached to the surfaces increased constantly during the study (Fig. 4). During the first day of culturing the cell number decreased slightly on all surfaces compared to the initial cell density (2×10^4 cells per well). The highest cell number was detected on the 2-layer surfaces, where it reached 1.396×10^4 cells per well. The lowest cell number was observed on 6-layer surfaces with 0.638×10^4 cells per well. On day 3 the cell number was similar on all surfaces with the highest value on 8-layer surfaces (4.94×10^4 cells per well) and the lowest value on 2-layer surfaces (3.924×10^4 cells per well). Between day 3 and day 5 only little increase of viable cells could be seen on 4-, 6- and 8-layer surfaces, while non-coated titanium and 2-layer surfaces demonstrated an intense increase of cell number during this time. After 5 days of culturing the cell number on untreated scaffolds reached 7.167×10^4 cells per well which was the highest at that point in time. On 6-layer surfaces the cell number was the lowest and reached only 4.994×10^4 cells per well. In no case the differences between cell numbers reached significance.

3.3 Cell differentiation

Figure 5 shows the quantitative real-time PCR analysis of the selected gene expression pattern. This analysis revealed that, for the most part, the investigated osteoblastic markers were overexpressed in the cells cultured on coated surfaces compared to the cells on uncoated surface (ALP: 302.6% on 2-layer-surface, 154.1% on 4-layer-surface, 288.2% on 6-layer-surface and 196.6% on 8-layer-surface; OC: 134.7% on 2-layer-surface, 467.8% on 4-layer-surface and 132% on 6-layer-surface; Coll I: 207.5% on 2-layer-surface, 136% on 4-layer-surface, 150.7% on 6-layer-surface and 115.1% on 8-layer-surface; BSP: 129.3% on 2-layer-surface, 142.2% on 4-layer-surface and 121.5% on 8-layer-surface). Only the osteocalcin expression on the 8-layer-surface (83%) and the bone sialo protein expression on the 6-layer-surface (89.5%) are slightly underexpressed. In no case over- or underexpression were significant.

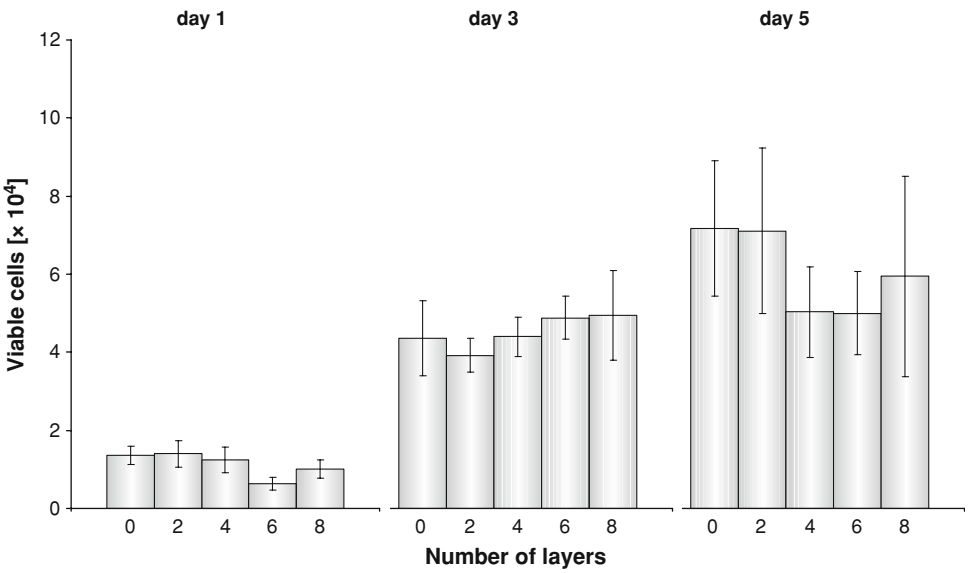

Fig. 4 Proliferation test of hFOB cells cultured on titanium discs with different numbers of fluorescence layers. Each reading represents the mean and error bars represent a standard deviation for $n = 6$. * $P \leq 0.05$ relative to uncoated surface (0)

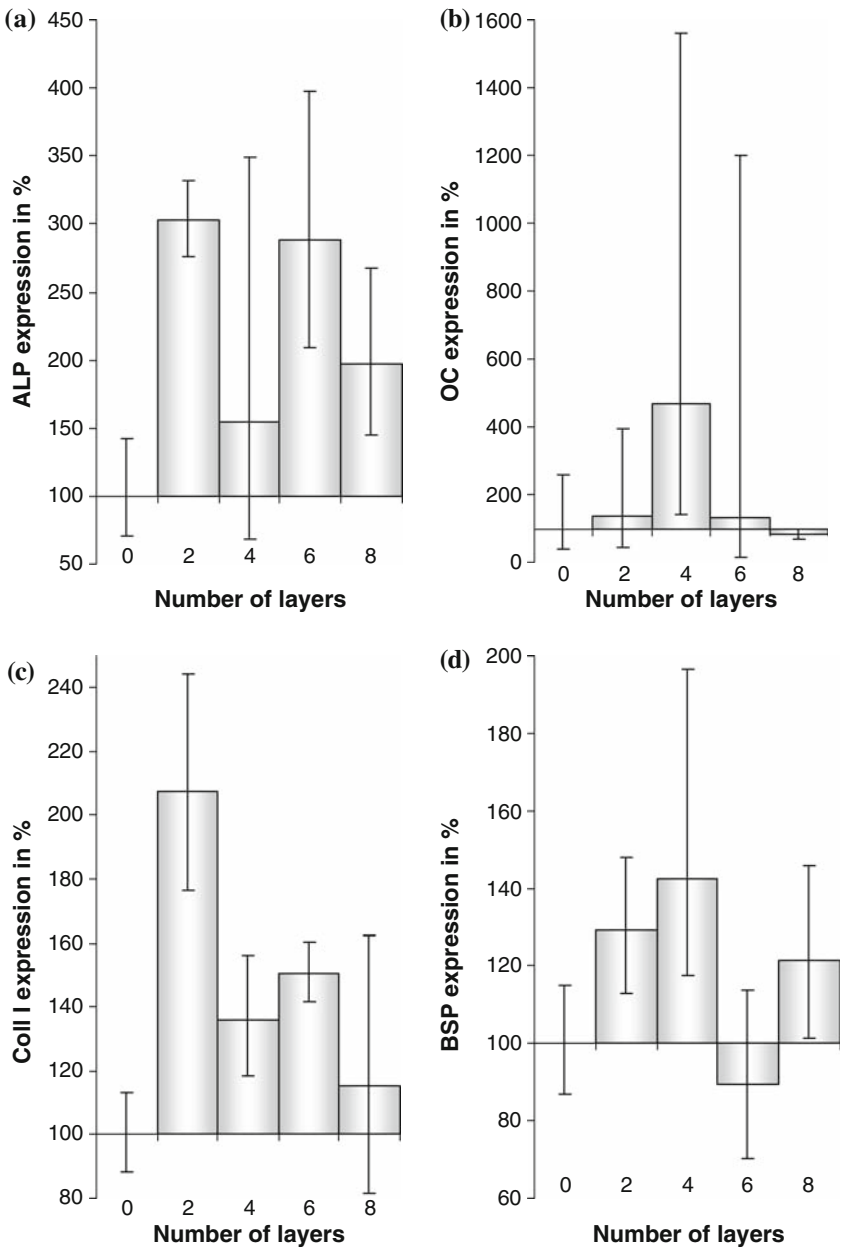

Fig. 5 Gene expression of **a** Alkaline Phosphatase (ALP), **b** Osteocalcin (OC), **c** Collagen Type I (Coll I) and **d** Bonesialoprotein (BSP) on coated surfaces compared to uncoated surfaces and expressed as percent. Each reading represents the mean and error bars represent a standard deviation for $n = 6$. * $P \leq 0.05$ relative to uncoated surface (0)

3.4 Release of the fluorescence molecule

The fluorescence emission of the cell culture medium after 1 day of culturing was investigated. According to known literature all oligoelectrolyte layers should be decomposed at that point [11]. The typical fluorescence spectrum of compound **2** could be detected in every cell medium. Figure 6a shows a summary of the fluorescence emission spectra at an excitation wavelength of 500 nm from the 2-layer surface solution to the 8-layer surface solution (from bottom up) with an obvious ascent of intensity of emission signal. To back up this observance the number of layers was correlated to the maximum of emission at 545 nm (Fig. 6b). The emission intensity increases strictly linear with increasing number of layers.

3.5 Cell attachment and morphology

Figure 7 shows osteoblastic cells attached to coated surfaces after 5 days of culturing. Cells are well spread on the surfaces and establish their typical filopodia. The fluorescent reporter molecule was incorporated into the cells (Fig. 7a). The nuclei appear more intensely colored (*arrows*). On the uncoated surfaces no fluorescence signal can be seen (Fig. 7b).

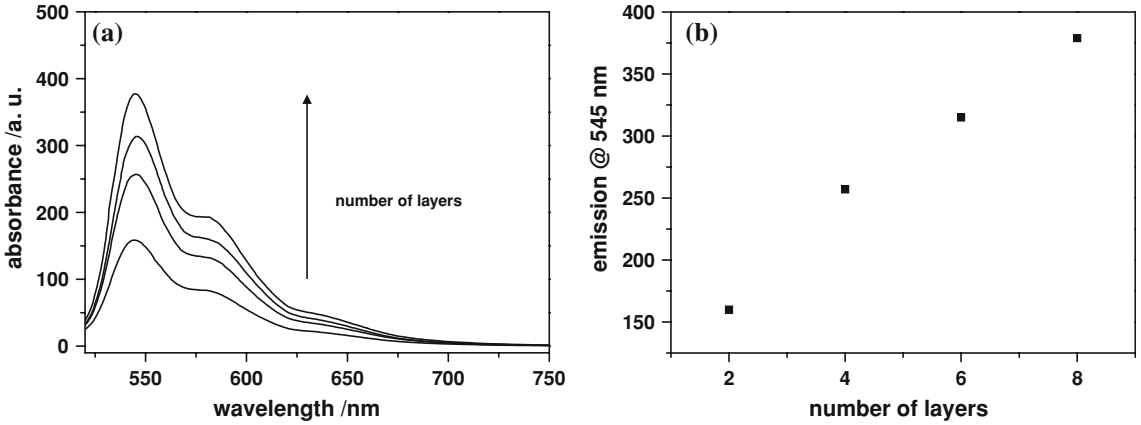

Fig. 6 **a** Fluorescence spectra of **2** in cell culture medium after 1 day from Ti-samples with 2, 4, 6 and 8 altering layers of molecular oligoelectrolytes **1** and **2**. **b** Correlation of emission @ 545 nm versus number of layers

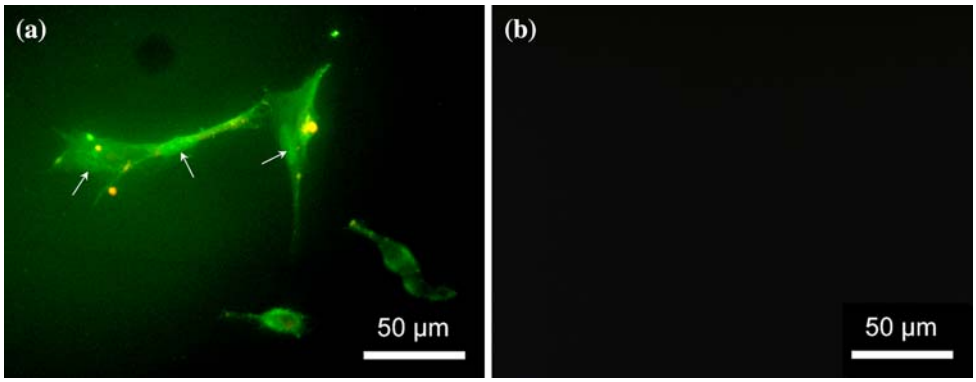

Fig. 7 Fluorescence micrographs of hFOB cells cultured for 5 days on **a** titanium surfaces coated with fluorescence loaded layer-by-layer assemblies and **b** uncoated titanium surface. Nuclei appear more intensely colored (*arrows*). (Color figure online)

4 Discussion

The objective of this paper was to examine whether a layer-by-layer assembly can be deposited on Ti–6Al–4V surfaces, as titanium and its alloys are routinely used in orthopedic surgeries because of their excellent biocompatibility and good mechanical properties [15–17]. Additionally the in vitro behavior of the coating and its effect on osteoblastic behavior was investigated. To create assemblies on the SEBM processed Ti–6Al–4V surfaces the samples were immersed in a solution of potassium hydroxide KOH. Alkaline treatment of titanium surfaces often leads to a modified surface topography [18]. Despite SEBM processed Ti–6Al–4V surfaces showed slightly increased surface roughness, the enlarged S_a value of etched surfaces about 0.10 µm still can be considered as smooth. Thus, the layer-by-layer assembly is an adequate method to modify the chemical properties of an implant surface without influencing the surface topography in a noteworthy manner. This is very important as an adjusted surface roughness itself already increases attachment and proliferation of cells [17, 19, 20].

The incorporation of bioactive substances like hormones and proteins into layer-by-layer coatings is a very interesting field of research. The controlled release of such agents in a physiological environment is of particular interest for medical applications [21, 22]. Recently, Rosenlehner et al. found that molecular oligoelectrolyte coatings are released from a silica glass substrates in a controlled matter [11]. The present study shows that these results are assignable to titanium surfaces. The layers decompose in physiological environment and thereby release components of the film. Through fluorescence measurements of the cell culture medium of coated surfaces the applicability of molecular oligoelectrolytes for the modification of titanium surfaces could be proofed. The fluorescent signal in the cell culture medium derived from oligolectrolyte **2** and shows, that the layer-by-layer coating was dispensed to the cell medium. Samples with a higher number of dye layers showed increased fluorescence emission in the corresponding cell medium. The coherence between number of layers and fluorescence emission is strictly linear. We therefore conclude that the assembled film is still intact after steam sterilization at 121°C and the

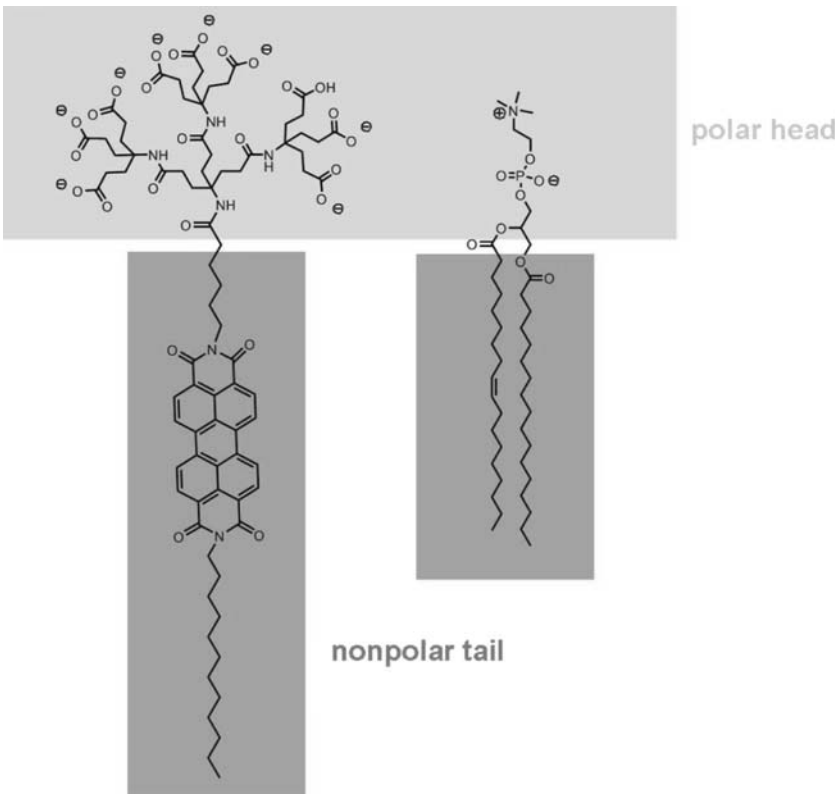

Fig. 8 Analog amphiphilic structures of fluorescent reporter **2** (*left*) and membrane building phospholipids (lecithin) (*right*)

arrangement of layers has not been damaged. Next to the cell medium the applied fluorescence dye was found to be integrated in the hFOB cells, shown by fluorescence microscopy. The LbL coated titanium samples turned out to be an efficient transporting and delivery system for film components into cells. Incorporated bioactive agents could be transported in an easy and effective way and an initial new drug delivery system could be established. As the molecular structure of the used reporter dye corresponds to that of phospholipids that are a major component of all biological membranes (Fig. 8), we assume that the dye was incorporated into the cell membrane of the hFOB cells.

To date, literature provides only little information on cell response to layer-by-layer nano-assemblies. The results of the present study demonstrate for the first time that the proliferation of human fetal osteoblasts is not negatively affected by the coating with molecular oligoelectrolytes. Differentiated function of hFOB cells was assessed by monitoring the expression of both early (alkaline phosphatase and collagen type I) and late (osteocalcin and bone sialo protein) osteoblastic genes. A significant difference on samples modified with the oligoelectrolyte coating was not observed when compared to that of uncoated surface. No negative influence of the coating and its components on the differentiation of the cells has been observed. However, the expression of almost all investigated genes and groups was even higher than that of the control. These results suggest that the differentiation of osteoblasts as well does not depend markedly on the layer-by-layer assembly. Therefore we assume that the LbL treatment itself, as well as the compounds used to modify the surface, is not cytotoxic for hFOB cells on Ti–6Al–4V surfaces. The excellent properties of the titanium samples as implant material were preserved and not negatively influenced by the treatment with molecular oligoelectrolytes.

The present study of layer-by-layer coating on the Ti–6Al–4V surface shows that a transport of molecules or active agents via implant modification can be accomplished by LbL assembly and accordingly e.g. bone regeneration can be influenced directly. The enhancement of the concept of layer-by-layer assembly with oligoelectrolytes to titanium surfaces offers now great opportunities for the future development of this promising technique in terms of drug delivery and implant modification.

5 Conclusions

The present study demonstrates that layer-by-layer assembly of molecular oligoelectrolytes represents a simple but effective way to deposit adjustable thin films on SEBM manufactured Ti–6Al–4V surfaces. This technique allows incorporation of a multitude of molecules such as bioactive drugs or proteins in the film and their efficient

transport into cells. The response of e.g. bone cells can thus be positively affected and the performance of biomaterials can be enhanced. This work demonstrates the creation of hierarchically ordered layer-by-layer assemblies of oligolectrolytes on Ti–6Al–4V surfaces and their controlled release. The enrichment of the coating can be extended to numerous therapeutic agents and therefore offers great opportunities in terms of drug delivery and implant improvement. The LbL technique will attain an increasing application in the biomedical field, particularly in the processing of implant materials.

Acknowledgements The investigations were supported by the DFG GZ: NE 339/5-1 and Hi 468/16-1. Cells were kindly provided by the Department for Functional Materials in Medicine and Dentistry, University of Würzburg, Würzburg, Germany. The authors gratefully acknowledge the excellent technical assistance of Ms. Andrea Krautheim-Zenk and the likeable support of Mr. Peter Heinl in scanning electron microscopy.

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