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RESEARCH ARTICLE

# Usage of polymer brushes as substrates of bone cells

**Sabine A. LETSCHE[1], Annina M. STEINBACH[1], Manuela PLUNTKE[2], Othmar MARTI[2], Anita IGNATIUS[3], Dirk VOLKMER (✉)[1]**

1 Institute of Inorganic Chemistry II, Ulm University, Albert-Einstein-Allee 11, D-89081 Ulm, Germany
2 Institute of Experimental Physics, Ulm University, Albert-Einstein-Allee 11, D-89081 Ulm, Germany
3 Institute of Orthopaedic Research and Biomechanics, Ulm University, Helmholtzstrasse 14, D-89081 Ulm, Germany



**Abstract** Implant medical research and tissue engineering both target the design of novel biomaterials for the improvement of human health and clinical applications. In order to develop improved surface coatings for hard tissue (bone) replacement materials and implant devices, we are developing micropatterned coatings consisting of polymer brushes. These are used as organic templates for the mineralization of calcium phosphate in order to improve adhesion of bone cells. First, we give a short account of the current state-of-the-art in this particular field of biomaterial development, while in the second part the preliminary results of cell culture experiments are presented, in which the biocompatibility of polymer brushes are tested on human mesenchymal stem cells.

**Keywords** polymer brush, ATRP, micropatterning, bone cell, cell adhesion

## 1 Introduction

Every year, millions of patients receive medical implants to improve their health situation or to save their lives [1,2]. These implants include a variety of medical devices such as artificial hip joints or dental implants, heart valve prostheses or intraocular lenses, and consist of a multitude of materials such as titanium, stainless steel, polyethylene, or polyurethane [1–3]. In hard tissue implants, those materials meet either the mechanical demands, such as titanium and steel [3], or the physiological requirements, such as ceramics [4]. It is, however, still a major challenge to unite both qualities in one material [3–5]. Thus, many problems arise when it comes to the healing process [2]. For hard tissue implants this means that, instead of integrating into the tissue, the foreign body reaction and attachment of fibroblasts lead to a collagenous encapsulation of the medical device [1,3,6,7]. This reduces the stability and half-life of the implant and results in a failure of the implant and a detriment to the patient [2,3,8,9]. On this account, a major focus of biomaterials research has been the improvement of the implant surface for interactions with the human host tissue. At the same time, research for better implant materials accompanies the development of tissue engineering scaffolds [7]. For both, it is essential to understand the complex processes at the interface of proteins, cells and tissues, and biomaterials in order to obtain biocompatible, bioactive devices with suitable mechanical properties to promote regeneration [5,7]. There are several approaches to improve the healing capabilities of biomaterials by altering the physical, chemical, or biological characteristics of the surface.

The physiological environment of cells consists mostly of pores and fibers on the micro- and nano-scale as seen in the basement membrane and extracellular matrices [10, 11]. Moreover, bone material consists of a meshwork of calcified collagen fibers. A microtextured or nanotextured surface will consequently influence cell adhesion and proliferation. Thus, increasing the roughness of implant titanium surfaces on a micro- or nano-scale enhances cell adhesion, integration of implants into the bone, and bone formation by osteoblasts [12–16]. This effect is attributed to the similarity of the surface structure to the pits, which are produced by osteoclasts, when they dissolve the hydroxyapatite bone substance [12]. Pre-osteoblast attachment on titanium material was enhanced by nanostructuring the substrate by high-pressure torsion (HPT). This was ascribed to the higher hydrophilicity of the HPT-processed material compared with the unprocessed titanium surface [17]. The growth and differentiation of mesenchymal stem cells (MSC) was enhanced on nanofibers of polycaprolactone and silk, respectively [10,18,19]. These nanofibers match the size of hydroxyapatite crystals, thus mimicking the natural habitat of bone cells [10].

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E-mail: dirk.volkmer@uni-ulm.de

However, it is not only the surface roughness or texture that influences cell behavior but also the geometrical topographic patterns, such as lines, triangles, hexagons etc., that are able to alter cell reaction. Alaerts et al. [20] showed that pure topography without chemical discontinuities influences cell growth. The fibroblasts adjusted their main axis along the linear grooves of a poly(methyl methacrylate) film. It was confirmed that the patterning technique did not change the physicochemical properties of both groove and ridge surface. Britland and co-workers exposed fibroblastic cells to grooves in fused silica substrates and observed strong cell alignment along the lines [21]. In the experiments of Yu et al. [22], human pulmonary fibroblasts cultivated on micropatterned polycarbonate substrates aligned to lines as well, but did not show a preferred orientation on a point structure. However, the authors observed an inflammatory response on micropatterned samples [22]. Zahor et al. ran experiments with MSCs on linearly micropatterned silicon substrates and noted considerable alignment in the grooves [23].

Besides the topographical pattern, chemical structure influences cell growth. Cell adhesion always depends on the composition and the conformation of the underlying layer of adsorbed proteins. The proteins' conformation in turn depends on the physicochemical characteristics of the surface [24–26]. Keselowsky and co-workers investigated the effect of functional groups such as methyl, hydroxyl, carboxylate, and amine groups on fibronectin adsorption and consequently its impact on cell adhesion [27]. They found that hydroxyl groups outperformed the other functionalities. Healy and co-workers [28] undertook studies with linearly patterned methyl and amino-functionalized substrates. Bone cells preferably spread on the amino-functionalized surface and mineralized it subsequently. Even small chemical changes lead to significant changes in the integration of polymer pins into the bone tissue in the works of James et al. [6].

Extracellular matrix proteins that are involved in the cell attachment process, such as fibronectin, are often used themselves as surface modification to promote cell adhesion selectively [29]. Tugulu and co-workers used protein-functionalized polymer brushes to alter surface properties [30]. A similar approach is the use of specific adhesion sequences like the well-known RGD peptide. This was also used by Tugulu et al. to model cell attachment [31].

In the work of Brock and co-workers, cells sensed the geometry of such RGD peptides' adhesive fibronectin islands and arranged their cytoskeleton towards the edges of triangles, squares, hexagons, and the like [29].

Zapata and co-workers [32] found an optimum of the size of chemical structure, when they fabricated substrates of demixed poly(ε-caprolacton)/poly(D,L-lactide) (PCL/PDLA) blends by phase separation. The MC3T3-E1 osteoblast-like cells adhered preferably on the PDLA islands, bridging the PCL parts. At places where the distance was too large, this bridging caused stress on the cytoskeleton resulting in reduced proliferation. The earliest and highest maximum in cell growth was observed at a structure of 21–33 μm.

Whether the chemical or the topographical cues rank higher in the cells' reception hierarchy is unclear. Charest and co-workers [33] prepared substrates with grooves and ridges with the same width. In the right angle to this pattern a line pattern of chemical adhesives was deposited. On these substrates osteoblast-like MC3T3-E1 cells aligned along the mechanical topography (grooves). The contrary was demonstrated in the experiments of Britland et al. [21]. Their work showed that cells on similar substrates orient themselves towards the chemical lines more than towards the topographical grooves. Both results are illustrated in Fig. 1.

This contradiction is alleviated by the fact that Charest and co-workers applied their adhesive lines only on the ridges and used osteoblast-like cells. Britland and co-workers, however, produced substrates with continuing lines for fibroblast cell adhesion on the ridges and the grooves. It seems likely that chemistry and topography act synergistically, as both when superimposed cause even greater alignment than each individual pattern does alone [21].

By the great majority of studies on the interaction of cells with biomaterials it is shown that the adhesion, proliferation, and activity of cells are influenced by a variety of material properties. Roughness or micro- and nano-structural features promote cell adhesion as long as the natural environment of the specific cell type is imitated, for example the pores, fibers, and crystals of bone tissue. Apparently, cells can sense topography alone without chemical discontinuities.

Besides the influence of surface topography, chemical and biochemical cues are major parameters for cell attachment and function, as is generally well known. For the chemical guidance, even the geometry of these cues is a factor. Furthermore, there is evidence that cells are influenced by both topography and (bio)chemistry synergistically.

From the previous summary we can thus deduce that cells interact with their environment in a complex manner, which is due to the intricate design of biological tissues.

Another aim of biomaterials scientists is to make the implant "invisible" for the immune system by mimicking the natural material. For the hard tissue implants, this material would be hydroxyapatite on a collagenous organic matrix [5,34].

As James and co-workers observed, the increase of carboxylate groups on implanted polymer pins improved the integration into the surrounding tissue by chelating $Ca^{2+}$ ions [6]. These $Ca^{2+}$ ions are the foundation for the build-up of hydroxyapatite and connect the biomaterial to the bone.

Consequently, many experiments were run to find

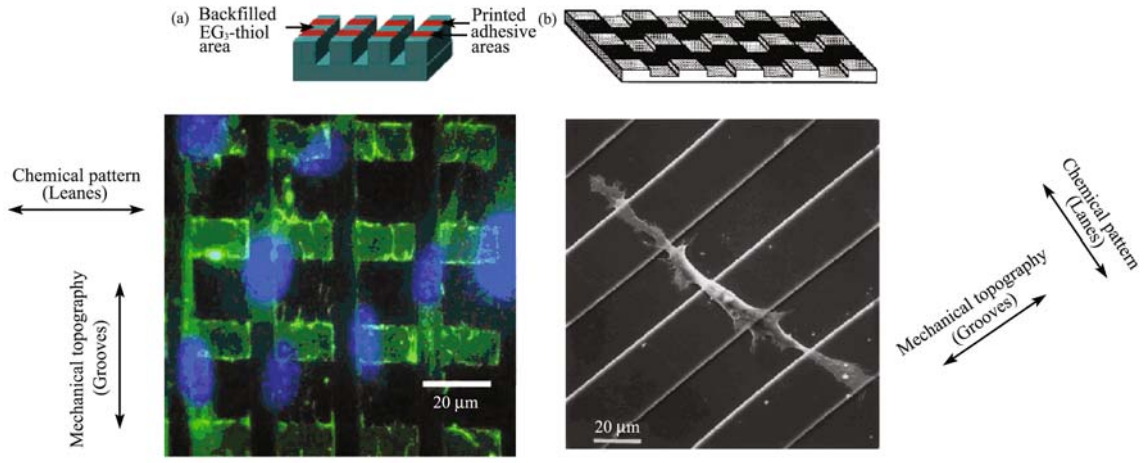


**Fig. 1** Topography or chemical structure? Schematic illustration of the substrates, which Charest et al. **(a)** and Britland et al. **(b)** used and images of the cells on the chemical and topographic patterns; **(a)** is a fluorescence micrograph of Charest et al., 2006: The alignment of the osteoblast-like cells along the topographical structure is visible thanks to the DAPI blue nuclei, the chemical structure is arranged at the right angle; **(b)** shows the results of Britland et al., 1996 as a scanning electron microscope image: here the cell is oriented along the chemical cues, which run across the linear topography at the right angle; images modified after Britland et al., 1996 and Charest et al.,

methods to coat titanium devices with hydroxyapatite [3, 35–40]. It was shown that hydroxyapatite-coated implants displayed a higher percentage of direct bone-implant contact than titanium implants without coatings do [13, 35–37]. Crystalline calcium phosphate improved differentiation of rat bone marrow cells and calcification of the substrate material over titanium controls [41].

The sputtering methods, such as right angle magnetron sputtering, improved the hydroxyapatite coatings over the ones deposited by plasma spraying, but there is still potential for improvement [39]. Mao and co-workers [42] adsorbed ethylenediaminetetraacetate (EDTA) on titanium substrates to generate nucleation sites for a biomimetic calcium phosphate coating.

For those coatings, one of the crucial points for a good biomaterial is their long-term stability. Here, many hydroxyapatite coatings fail to meet the requirements [3, 43].

During the mineralization of the collagen matrix in the bone under physiological conditions, carboxyl and carbonyl groups of the collagen polymer serve as nucleation sites [44]. Figure 2 shows the orientation of the hydroxyapatite crystals (dark) along the collagen fibrils (light grey).

The control over the crystallization, in particular, is in the spotlight of materials scientists. Costa and Maquis [4] confirmed in a biomimetic approach that calcium phosphate crystal growth was influenced by a Langmuir-Blodgett film with calcium carboxylate head groups. The Cui group [34] observed that a monolayer of stearic acid profoundly changes calcium phosphate crystallization. The desired hydroxyapatite was built only in the presence of the carboxylate head groups.

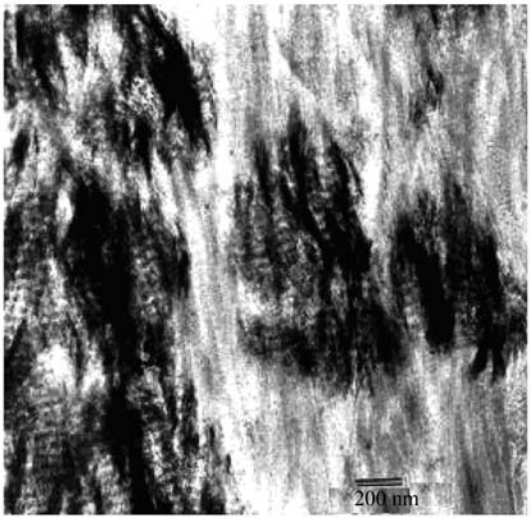


**Fig. 2** Transmission electron micrograph showing the orientation of the hydroxyapatite crystals relative to collagen fibrils (Reprinted with permission from Ref. [45], © 2008 American Chemical Society)

Taubert and co-workers illustrated that a well-defined (yet non-crystalline) interface, even a rather flexible matrix like an amphiphilic poly(acrylic acid)-block-poly(n-butylacrylate) block copolymer film, is able to serve as a template for nucleation by controlling calcium phosphate mineralization. Moreover, the block copolymer film at the air/water interface also acts as a tool for the 2D arrangement of the resulting particles in a near-crystalline order [46]. The Wentrup-Byrne group has demonstrated that the amount of phosphate groups in the matrix polymers [poly(monoacryloxyethyl phosphate) and

poly(2-(methacryloyloxy)-ethyl phosphate)] had great impact on the calcium phosphate crystallization. Besides the degree of cross-linking of the polymeric matrix, the accessibility of the phosphates themselves plays an important role in both the amount and type of formed mineral [47].

To elucidate the mechanisms of calcium phosphate bio- and biomimetic mineralization, it should help to think out of the box and have a closer look at another common biomineral. Calcium carbonate, along with calcium phosphate, belongs to one of the most ubiquitous minerals in nature. This is reflected in the vast number of publications that are concerned with the biomimetic formation of calcium carbonate in the presence of organic templates and/or additives, as reviewed in detail by Xu et al. [48].

Acidic macromolecules are involved in the production of calcified biomaterials such as bone, teeth, or nacre. Intensive studies on the mineralization process of calcium phosphates, oxalates, and carbonates were accomplished. In these processes macromolecules with carboxylate functional groups play a major role [49]. It has been pointed out by Arias et al. that sulfated biopolymers, especially polysaccharides and proteoglycans, have an important role in the biological mineralization process, one putative function being to promote calcium carbonate nucleation [50,51].

There are a lot of proteins in organisms that contribute to controlled crystal nucleation and growth. These normally are highly negatively charged due to carboxylate, sulfate, or phosphate groups and are therefore predestined to bind calcium ions [51].

In the bone tissue, beside the main component collagen, other bone-specific proteins exist, which are believed to regulate, inhibit, or promote calcium phosphate crystallization [52,53]. The typical non-collagenous bone proteins are osteopontin, bone sialoprotein, and the dentin matrix protein 1 [53]. Their common feature is that they possess acidic groups, mostly carboxylates, and exhibit hydroxyapatite binding ability [53].

In marine algae (coccolithophorides) it has been demonstrated that polyanionic polysaccharides accompany calcite crystal formation from nucleation to growth and also regulate crystal morphology by enhancing precipitation of calcium carbonate ions on the acute crystal face [54, 55].

When calcium carbonate precipitation is assayed in solutions of glycosaminoglycans (GAG), a clear effect of the sulfate groups has been observed. When hyaluronan, a nonsulfated but carboxylated GAG, was added, unmodified calcite crystals were observed as compared with calcite crystallization in the absence of the polymer [51]. The specific pattern of sulfation of the GAGs seems to be crucial as these groups may act as recognition sites for the nucleation and growth of the mineral on the GAG matrix [51].

It has also been shown that functionalized self-assembled monolayers (SAM) with sulfate groups are more active than other negatively charged groups in inducing calcium carbonate nucleation, and the sulfate groups induce a face-selective nucleation [56,57]. Calcium carbonate crystallization on self-assembled monolayers of 1-ω-funtionalized alkylthiols deposited on precious metal substrates depends on the spacing, ordering, and orientation of the terminal group. These studies suggest that a promising approach towards oriented crystal growth should be the use of synthetic polymers functionalized with specific groups in precise locations relative to the backbone in order to correlate the influence of these groups with their ability to affect the nucleation, growth, and morphology of inorganic crystals.

However, recent studies suggest that the many biogenic highly ordered mineral structures may form via an amorphous precursor such as amorphous calcium carbonate (ACC) or amorphous calcium phosphate. Politi and co-workers [58] investigated the regeneration of the adult sea urchin spine. The skeletal calcium carbonate hard part forms in this organism through an amorphous precursor phase. ACC as a transient precusor could be observed also *in vitro* to produce microstructured single-calcite crystals [59,60] or aragonite tablets [61].

Although the biologically inspired compounds mimicking the architectural concept of nacre were described before, the approach followed by the Volkmer group led to highly oriented laminated crystal architectures without a structurally preorganized organic matrix [60]. Their thin polycrystalline calcite films originate from an amorphous phase serving as a template for epitaxial overgrowth of calcite platelets.

Volkmer and co-workers also showed that a metastable ACC film is formed using polymethacrylic acid (PMAA) brushes as a template [62]. The process is schematically shown in Fig. 3. After thermal treatment, this amorphous film transforms into a thin polycrystalline calcite film.

The Volkmer group mineralized the polymer film, as published [60]. The textured polymer brushes were exposed to a continuous flow of a supersaturated calcium carbonate solution by combining a 10 mmol/L calcium chloride and a 10 mmol/L sodium carbonate solution in a mixing chamber. After a defined time, the substrates were removed from the perfusion cell, washed, dried in air, and analyzed with diverse microscopic techniques. According to optical micrographs taken with crossed polarizers, the samples showed no birefringence which is indicative of a predominantly ACC film. After a thermal treatment at 250°C for 2 h, a thin polycrystalline calcite film could be observed. The crystalline character of the mineralized patterns could be proved by polarization microscopy, whereas ATR-FTIR measurements were employed to distinguish between the different $CaCO_3$ modifications.

As our main interest is focused on the improvement of implant surfaces, we recently turned our attention to

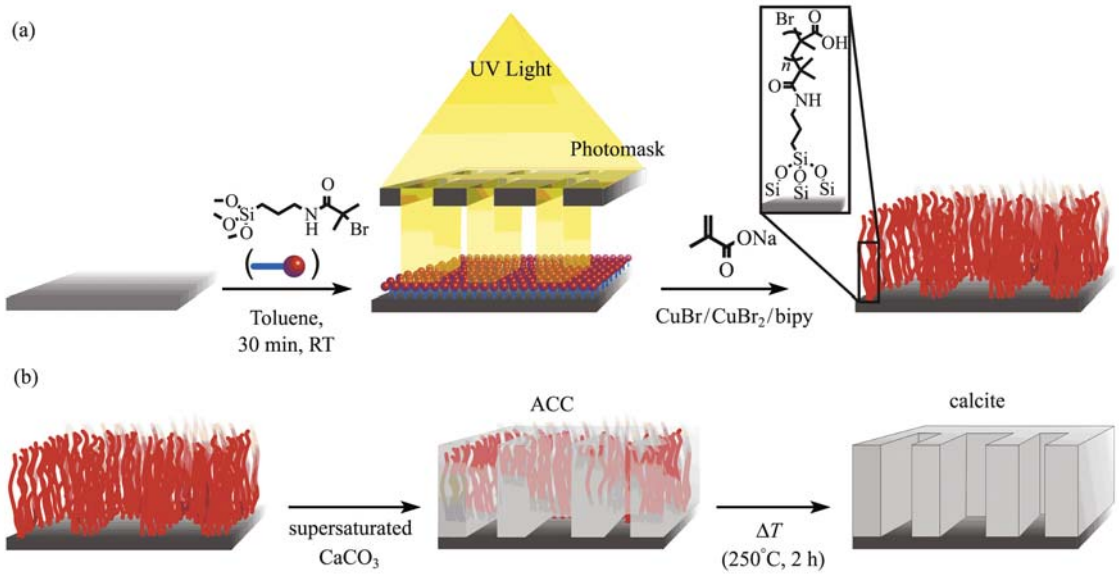


**Fig. 3** Fabrication of micropatterned calcite films: **(a)** Synthesis of poly(methacrylic acid) (PMAA) brushes via surface-initiated atom transfer radical polymerization (SI-ATRP) of photolithographically patterned substrates coated with ATRP initiator molecules; **(b)** Directed deposition of metastable amorphous calcium carbonate (ACC) and a subsequent transformation of ACC into a micropatterned calcite film by thermal treatment [62]

calcium phosphate. The aim of our studies is to produce a biomimetic composite material, which promotes bone cell growth and integrates into hard tissues by mimicking bone matter and thus combines the good mechanical properties of titanium implants with the excellent biocompatibility of hydroxyapatite.

Our approach toward fabrication of these composite materials is based on the use of patterned polymer brushes as a biomimetic, acidic macromolecular matrix, the patterns being produced by photolithographic techniques. Since carboxylate and sulfate functional groups of matrixmolecules are essential in biomineralization, we fabricate polyelectrolyte brushes, e.g., polycarboxylates and polysulfonates (Fig. 4).

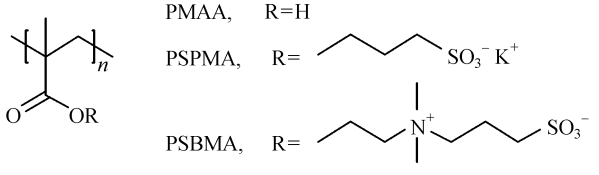


**Fig. 4** Chemical structure of the obtained polymers

Apart from investigating the influence of specific patterns formed by the polymer brushes on the growth of osteogenic cells, the height alterations of the polymer brushes provide a convenient means to test samples by scanning force microscopic techniques. A textured substrate enables us to sample the height and physical properties of the polymer brushes via atomic force microscopy (AFM). Moreover, microstructural features of polymer films could be visualized by optical light microscopy, employing differential interference contrast. Thus, the morphological response of cells and their activity and proliferation ability depend on the microstructural features imprinted in the polymer brushes.

Since this approach of using textured polymer brushes as organic template for biomimetic crystallization has been developed very recently, we will give a short introduction to surface-tethered polymers.

Interest in surface-attached polyelectrolytes has increased enormously within the last decade, because most of them are water-soluble and therefore interesting for biochemical and medical applications [63], as well as in the extensive field of biotechnology [64]. The advantage of polymer brushes over other surface modification methods, such as self-assembled monolayers, is their mechanical and chemical robustness. Especially the use of polyelectrolyte structures — i.e., brushes carrying charges — shows special and unique properties, such as swelling behavior, complex formation, capacity of ion exchange, autophobic behavior, etc. [65]. Concise reviews about the synthesis and characterization have been published recently [66,67].

Surface-attached polymers can adapt different folding states: “pancake”, “mushroom” and “brush”. When the chains interact strongly with the substrate surface and they are given sufficient space, they typically fold into flat “pancake”-like morphologies [68]. A so-called “mushroom regime” exists at low grafting densities, if the polymer chains have only weak interactions to the surface and form a random coil [69]. However, at higher grafting

densities the polymer chains interact with each other and stretch away from the interface to avoid overlapping, thus forming a polymer "brush" [68].

Probably the most frequently investigated type of surface-attached polymers is the "brush". Polymer brushes are defined as assemblies of macromolecules chemically tethered at one end to a substrate or interface [69].

Two methods to fabricate polymer brushes have become widely accepted: "grafting to" and "grafting from." These techniques comprise four different approaches to synthesize polymer brushes. Ruehe summarized them in the book *Polymer Brushes* [68]. In the first approach, amphiphilic block copolymers with a water-soluble block and a water-insoluble block are spread at the air/water interface of a Langmuir trough [70]. After compression of the polymer monolayer, it can be transferred to solid substrates by dipping these through the monolayer/water interface.

A second approach describes the physisorption of block copolymers or end-functionalized polymers from solution onto a solid surface [71]. One part of the block copolymer adsorbs strongly at the surface and acts an anchor for the polymer chains. The other block has only weak interactions with the surface because the interactions of the polymer with the solvent are stronger.

A third and rather unusual way is the chemisorption of polymer chains, in which the chains are covalently attached to the surface. Nevertheless, the polymers still have to diffuse and become attached to the substrate surface.

Thus, all three "grafting to" approaches yield surface-tethered polymer coatings of low densities. Therefore the "grafting from" method is the most common technique to covalently attach polymer chains to a surface [72]. It is based on the diffusion of a small monomer molecule to activated initiator sites or growing polymer chains which are covalently attached to the surface [73,74].

Initiator molecules such as silanes on glass or silicon [75] and thiols on gold [76] are anchored to the surface and form a self-assembled monolayer. As a result, a so-called surface-initiated (SI) polymerization appears, which means the polymer chains grow from the surface-attached initiator molecules. This approach is very popular due to the variety of functionalized vinyl monomers available, such as styrene [77], (meth)acrylates [75,78], acrylamides [63], and acrylonitrile [79], which can be employed in a chain growth reaction such as free or controlled radical polymerization [80], carbocationic [81] and anionic polymerization [82], and ring-opening metathesis polymerization [83,84]. Describing all polymerization techniques would clearly go beyond the scope of this article, which is the reason why we concentrate on the living radical polymerization.

The most commonly used polymerization method via the "grafting from" technique is the atom transfer radical polymerization (ATRP). This method uses an alkyl halide as an initiator and a Cu(I)/bipy complex as a catalyst. The monomer is polymerized by repetitive atom transfer radical additions to yield well-defined high-molecular-weight polymers with narrow molecular weight distributions [85]. The polymerization cycle begins with the transfer of a halide radical to the catalyst, i.e., the Cu(I) complex, thus transforming the initiator/growing chain to a radical species itself. This enables the newly formed radical site to react with a monomer molecule of the polymerization solution. The cycle is closed by the capping of the radical chain end which then switches to the dormant state. This mechanism assures that the radical seldom appears at the chain end, thus minimalizing the probability of side reactions or chain termination, which happens in an uncontrolled free polymerization. In these controlled polymerizations, we therefore find less homogeneously built polymers in solution.

Compared with other "living" radical systems, ATRP represents a simple, inexpensive, and widely applicable method for controlled radical polymerization.

However, ATRP is not possible for a couple of monomers such as (meth)acrylic acid, as the acid functionality will poison the ATRP catalyst [86]. This shortcoming might be alleviated by the use of charged monomers of (meth)acrylic acid such as sodium (meth)-acrylate [78] or by using protected monomers such as tert butylacrylate which can be subsequently hydrolyzed using HCl [87].

To obtain 3D patterned polymer brushes, a host of diverse patterning methods exist, which limit the subsequent polymer growth to defined areas of the substrate. The most popular techniques are microcontact printing [88], photolithography [89], and direct writing approaches such as electron-beam or ion-beam lithography [90,91]. In contrast to most patterning methods, the direct writing techniques are able to fabricate patterns in the nano-scale.

As our main interest lies in producing implant coatings that should integrate well in hard tissue, we are investigating the biocompatibility of every part of the coating, including the polymer brushes alone. Therefore, we tested different polymer brushes on human MSCs (hMSCs) and observed the viability, morphology, and adhesion of these cells.

hMSCs are adult stem cells. They can be isolated from the bone marrow or even the adipose tissue [92–94]. hMSCs are pluripotent and thus able to differentiate into osteoblasts, adipocytes, and chondroblasts, or even to myocytes, stromal cells, fibroblasts, and neuronal and endocrine cells [92–96]. Therefore, they are interesting subjects of research to regenerative medicine and tissue engineering.

Our interest in the hMSCs is founded on their good proliferation abilities while still maintaining their physiological phenotype [94]. Thus, they have advantages over cell lines that are transformed by oncogenes or viruses and over primary cultures with inferior proliferation performance [94]. However, they still have the drawback of

aging after some passages, so that fresh cells have to be isolated anew [94].

## 2 Experimental

First, the cleaned substrates (e.g., silicon wafers, glass slides) were coated with the synthesized initiator 3-(2-bromoisobutyramido)propyl(trimethoxy)silane [30]. Afterwards, these initiator-attached substrates were irradiated by deep UV-light using a photomask to obtain a subsequent structuring. The non-exposed areas were polymerized with different electrolyte monomers [sodium methacrylate (NaMA; Aldrich), 3-sulfopropylmethacrylate (SPMA; Aldrich), 2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammoniumhydroxide (SBMA; Merck)] in a following step by transferring the irradiated substrate to a vessel containing the polymerization solution [78]. The polymerization conditions are modified versions of Tugulu et al. [78] for NaMA, Masci and co-workers [97] for SPMA, and the Huck group [98] for SBMA, and were optimized at room temperature with a Cu(I)/Cu(II)/bipy catalyst system and under nitrogen or argon atmosphere. The obtained patterned polymer brushes were analyzed with optical microscopy employing differential interference contrast (DIC; Olympus IX70) and AFM (Agilent 5500). The homogeneity of the polymerization and the sharpness of the pattern could be clearly observed with the optical microscope in the DIC mode. The thickness of the polymer brushes in air, water, and various salt solutions and their mechanical properties like elasticity, adhesion, and rupture forces can be determined by AFM measurements. As polymer brushes at certain ion concentrations, we aim to examine their state under mineralization conditions to ensure their swollen conformation and optimize the mineralization parameters.

In air the brushes can simply be scanned in the intermittent contact mode (silicon cantilever 42 N/m), whereas the swollen brushes in water are very deformable. Thus, very soft cantilevers (SiNi 0.32 N/m) and tips with a large diameter ($SiO_2$ spheres with $d = 1$ μm) are necessary to determine the brush heights in solution. To compare the height profiles in air with the ones in water, the brushes were scanned in contact mode with a force of 8 nN. The samples were incubated in water for 20 min prior to measurement.

The hMSCs were obtained from the proximal tibia of two different healthy donors. The cells were isolated by the Ignatius group from the harvested bone marrow by Ficoll density gradient (Histopaque®-1077; Sigma-Aldrich) and adhesion capability to cell culture plates with medium changes twice a week following the procedure by Pittenger et al. [95]. The hMSCs were cultivated in Dulbecco's Modified Eagle Medium (Biochrom) with 10% fetal calf serum (BioWhittaker Cambrex), 1% L-glutamine, 1% penicillin/streptomycine, and 0.5% fungizon at 8.5% $CO_2$, 37°C and 95% humidity. At 50%–80% confluence the cells were passaged by 0.05% trypsin/0.02% EDTA treatment. For the biocompatibility experiments passage 2–4 cells were used at a density of 10000 or 20000 cells/cm$^2$ and seeded on the glass substrates in 12-well plates. The substrates were lying on PTFE seals with a diameter of 15 mm, to make handling and transferring the substrates easier.

Substrates for the biocompatibility studies were PSPMA, PSBMA, and PMAA brush-coated glass slides with grooves of different sizes varying from 2.5 μm to 160 μm. In those grooves the surface should correspond to the UV-irradiated initiator SAM; the rest of the substrate presents the respective polymer brush to the cell. Substrates with either of those surfaces (unpatterned polymer brush coating and irradiated initiator SAM) were used for comparison. Control surfaces were cleaned glass slides and cell culture plates. All samples were sterilized with 70% ethanol and dried overnight.

During culture the cell morphology was examined at regular time steps with an Olympus IX70 phase contrast microscope.

## 3 Results

To survey the quality of the polymerized samples, the cover slides were analyzed through an optical light microscope in DIC mode (Fig. 5). In this mode the gradient of optical density of the sample in focus is transformed into a grayscale image contrast. Therefore, a homogenous, sharp, and defined stripe pattern can be observed, although the refraction indices of the polymer and the glass substrate have similar values.

A further analytical technique for the quality screening is AFM as mentioned above. With AFM the exact height can be measured both in air and in water, as shown in Fig. 6 for the height of dry and annealed PSBMA, PSPMA, and PMAA.

The swelling behavior of PMAA brushes in water is enormous: they swell by a factor of 5, while PSBMA and PSPMA reach "only" twice the height of the original dry state (Fig. 7).

To test the polymer coatings' biocompatibility, many questions have to be answered, like the toxicity of the polymer materials, cell adhesion on the substrate, and the influence of the pattern on cell growth. MSCs were cultivated on PSBMA-, PSPMA-, and PMAA-coated substrates for one week and the morphology was examined with a phase contrast microscope (Fig. 8). The plastic surface of the 12-well cell culture plate used, cleaned cover slides, and irradiated and non-irradiated slides covered with initiator molecules served as control for the cell growth. Besides the patterned samples, non-patterned ones were also employed.

The images, taken on days 1 and 4 after the cell seeding,

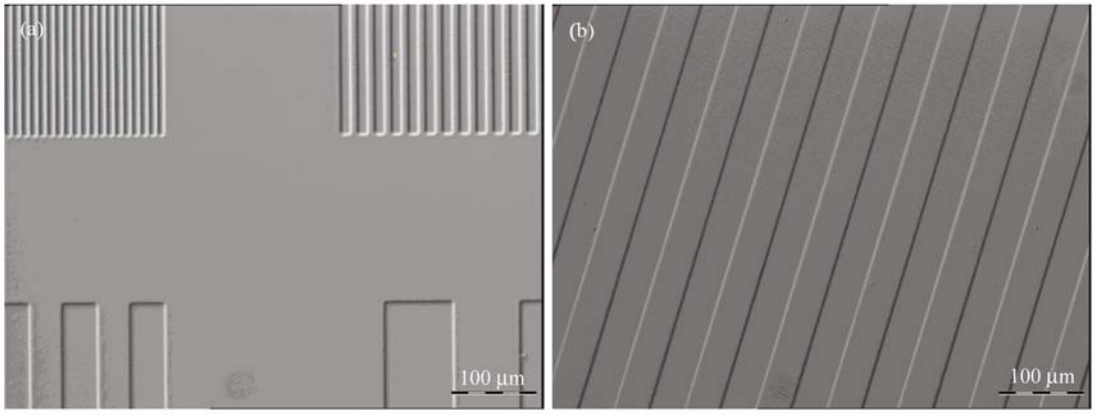


**Fig. 5** Differential interference contrast micrographs of photolithographically patterned PMAA brushes: **(a)** image of four different pattern sizes; **(b)** stripe pattern

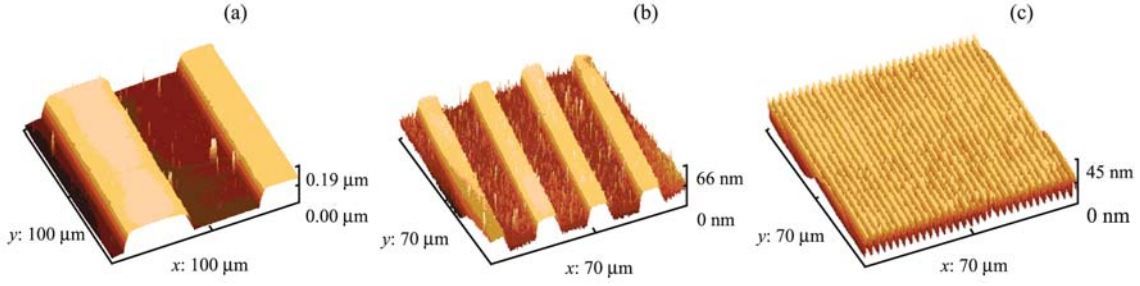


**Fig. 6** 3D topographic AFM images of patterned polymer brushes with different width of lines and polymers: **(a)** PSBMA with 40 μm width; **(b)** PSPMA with 10 μm width; **(c)** PMAA with 2.5 μm width

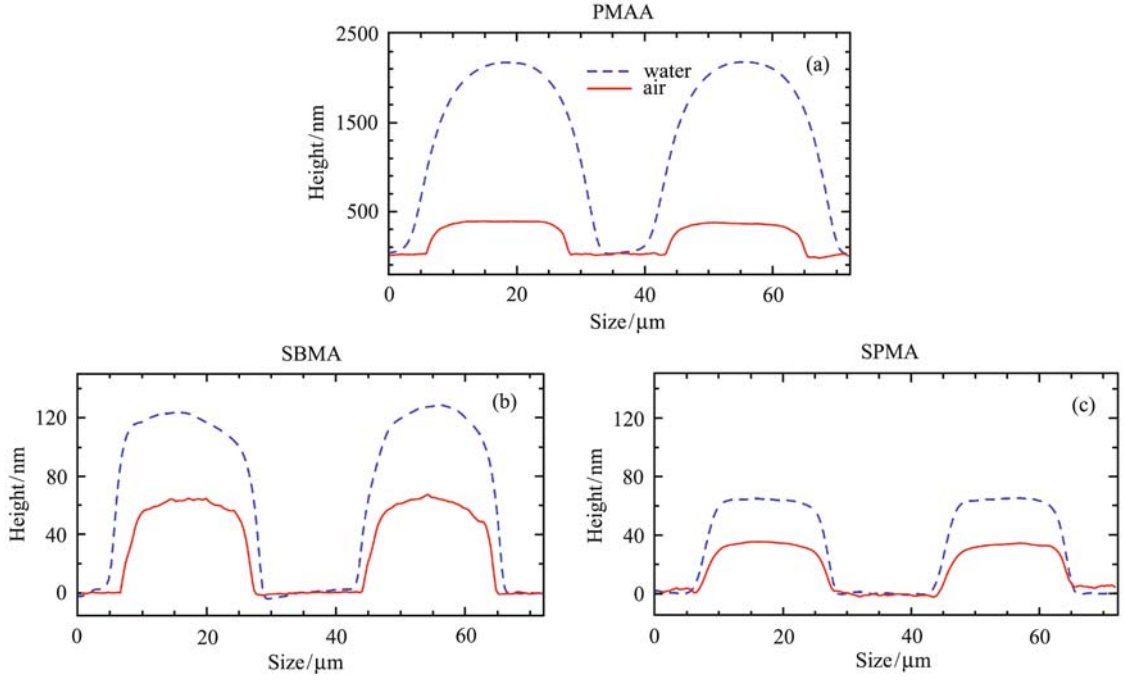


**Fig. 7** Height profiles of patterned **(a)** PMAA, **(b)** PSBMA, and **(c)** PSPMA brushes in air (solid line) and in water (dashed line) determined by AFM

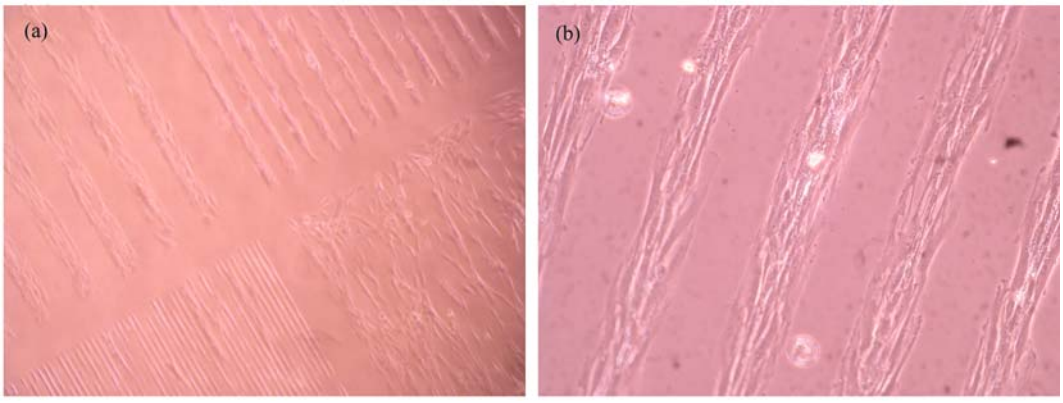


**Fig. 8** Phase contrast light microscopy images of cell adhesion of patterned PSBMA brushes: **(a)** hMSC cell culture between the patterned PSBMA brushes (day 4 after seeding), 40-fold magnification; **(b)** hMSC cells between the PSBMA stripes (day 1 after seeding), 200-fold magnification

respectively, show clearly the cell adhesion and growth between the polymer brush patterns (Fig. 8).

As a general observation we can state that cell growth on the plastic and glass control substrate was similar as qualitatively observed through the light microscope. The patterned polymer brush samples show a faster cell growth compared with non-patterned substrates. But on the latter ones, proliferation only occurs in the grooves between the polymer brush regions. It could also be observed that the cells on the PSBMA- and PSPMA-patterned coated samples show a better cell proliferation than on the PMAA-coated ones.

## 4 Discussion

The polymer brushes alone are known for their anti-fouling abilities [99]. Widely known are the polyethylene glycol-based systems to suppress cell adhesion [29,100,101], but also sulfobetaine and other zwitterionic mostly choline-based brushes were used for studies on the control of the attachment or detachment of cells on the surfaces using a patterned polymer substrate [102–107].

Iwata and co-workers [108] investigated the behavior of L-929 mouse fibroblasts on patterned poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) brushes of high density. The up to 15-nm-thick coating prevented protein adsorption and cell adhesion. Fibroblasts were only observed between the PMPC brushes. The same behavior of bovine aortic endothelial cells on polysulfobetaine methacrylate (polySBMA) brushes was observed by the Jiang group [109].

Osteoblasts and MSCs are known to prefer hydrophilic surfaces over hydrophobic ones [5]. Hydrophilicity causes higher alkaline phosphatase activity and osteocalcin levels, thus a better osteogenic environment and differentiation [110]. Nevertheless, the hMSCs in our experiments preferred the hydrophobic grooves while growth on the hydrophilic polymer brushes was inhibited.

The high chain mobility and excluded volume of the polymer brushes are probably the reasons for their ability to diminish protein and cell adhesion [100].

Mendelsohn et al. [111] described the large effect that swelling behavior of surface coatings could have on cell adhesion. With the same chemical composition the coatings with higher swelling ability exhibited cytophobic properties, whereas cells grew on those surfaces with more compact structure. Considering the capability of polymer brushes in good solvents to exhibit a height about twice to five times of that in dry state, this could also be a reason for their anti-adhesive properties.

Thus, the exciting question of how the properties of the polymer brushes change after calcification with calcium phosphate remains, just as the question of how the cells would react to the altered properties of these modified substrates.

## 5 Conclusions

As shown above, polymer brushes are suitable organic matrices for calcium carbonate mineralization. We were able to produce a wide range of surface-tethered polymers, which were functionalized with biomineralization relevant groups. hMSCs were growing in the grooves between the areas with polymer coating. These results, however, are only the beginning, and there is potential for further promising investigations with calcium phosphate. Thereby we enter the complex, yet highly interesting field of biomaterials science with its intricate interface between biological tissue and material surfaces. Some parameters of cell-biomaterial interactions, such as roughness, hydrophilicity, partly (bio)chemistry and topography, have already been elucidated, but the exact mechanisms are still unclear. There is also not much known about the interactions between the polymers and inorganic crystals, although many publications deal with the work and investigations on polymer-controlled mineralization.

Future studies on this complex topic may help to get a better and more complete understanding of the correlation between the minerals and their organic matrices as well as between the minerals and cells.

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