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Biomimetic *in situ* nucleation of calcium phosphates by protein immobilization on high strength ceramic materials

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ABSTRACT

Zirconia has been commonly used in the dental industry because of its excellent biological, mechanical and aesthetic properties. This material, however, is classified as nearly inert. To bioactivate the ceramic surface, biomimetic depositions of calcium phosphate coatings have been developed. We demonstrate an accelerated biomimetic coating method on zirconia using a specific pre-treatment with biological agents. We have chosen bovine serum albumin as a standard protein. Through the pre-treatment of the zirconia using a hydroxylation or additionally immobilizing the bovine serum albumin on the surface, we could influence the CaP deposition rate. Immunohistochemical analyses verified the presence of BSA on the zirconia surfaces. After immersion in simulated body fluid at 40 °C, the samples were analyzed by scanning electron microscopy and X-ray diffraction to visualize the CaP formation. Here we could show as proof-of-principle that it is possible to accelerate biomimetic coating processes on zirconia implants containing BSA on their surface.

1. Introduction

High strength ceramic materials are commonly used in the orthopedic and dental industry because of their excellent mechanical properties and high compatibility with the physiological environment. This material group is classified as nearly inert. To achieve additional functionalities, such as bioactivity on the surface of the inert oxide ceramics, various techniques were developed. The *in vivo* reaction at the interface between the implanted material and the surrounding tissue is influenced by two crucial factors: chemical and topographical properties of the material surface. Thus, on one hand, tailored structured samples were produced using different techniques to influence the cell response, such as cell adhesion, migration, proliferation or differentiation [1–3]. On the other hand, a chemical composition of the ceramic surface was modified by immobilizing functional groups [4–8].

A further alternative to modifying biological properties of the implant surfaces is coating with bioactive materials, such as calcium phosphates (CaP), which change both chemical and morphological properties. CaP coatings have been widely used on Ti-based dental and orthopedic implants to improve their osseointegration. The most common methods for CaP coatings are plasma spraying, sputtering deposition, sol-gel coating, electrochemical deposition, and biomimetic deposition [9–12]. The last-mentioned method, biomimetic deposition, is very promising, since the chemical and phase composition can be adjusted to those of natural hard tissue. Moreover, this is the process carried out at room temperature which does not require any thermal post-treatment. Thus, biological components, such as proteins, drugs or bactericide, could be easily incorporated into the CaP coating system while depositing [13,14]. This method is based on the precipitation of CaP crystals on the substrate surface by soaking in a supersaturated solution. The first successful trials to precipitate CaP crystals on a material surface as a CaP coating were introduced by Tanahashi et al. [15]. To accelerate the coating process, a modified 1.5 times concentrated SBF was developed. Since then, several scientific groups have been working on the enhancement of the CaP coating using a variety of modifications and concentrations of SBF [16–21]. It has been proven that the higher the concentration of SBF, the more rapid CaP crystals nucleation occurs on the substrate surface [18–20]. Barrere et al. showed that it is possible to achieve complete CaP coatings on titanium alloy substrate within only 24 h of soaking in 5 times concentrated SBF [18]. SBF solutions concentrated more than 1.5 times are, however, unstable and have to be additionally stabilized by adjusting its pH value, e. g., by using CO_2 gas [18,19].

The nucleation of CaP crystals depends not only on the SBF composition and concentration but also on the surface functional groups, such as hydroxyl (–OH), carboxyl (–COOH) or phosphate ($–OPO_3H_2$)

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groups obtained by acid or salt treatments or by the immobilization of self-assembled monolayers exhibiting active functional end-groups [22–26]. Thus, the substrate surfaces were chemically modified to accelerate the CaP deposition by soaking in supersaturated SBF solution. Metals or polymers are more susceptible for this kind of treatment than ceramic materials, which exhibit inert character and chemical resistance [8]. Uchida et al. compared different pre-treatments of alumina/zirconia nanocomposite substrates varying concentrations of acids and alkaline solutions and proved their influence on CaP nucleation after soaking in the SBF solution [27]. They observed the formation of CaP coatings on each pre-treated substrate after 7 days of SBF incubation and in the case of 5M H_3PO_4 activation after just 3 days of SBF soaking time. This is due to the presence of –OH-functional groups on the substrate surface. Kalterborn et al. obtained similar results; they reported the formation of CaP coatings on alumina surfaces treated with concentrated H_3PO_4 solution after 2 weeks of SBF soaking time [26]. The presence of –COOH-groups on the surface determines much stronger CaP nucleation than the other functional groups. This could be proven using an example of biomimetic CaP nucleation on sericin, the outer part of a silk thread produced by spiders or silk worms [28]. The authors hypothesize that acidic proteins play a crucial role in bone mineralization. This is due to the carboxyl or amino groups present in the protein structure. In their study, they have proposed the mechanism of hydroxyapatite (HA) nucleation.

The proteins are strongly involved in the bone growth process *in vivo*. Their immobilization to the substrate surface could result in the acceleration of the apatite growth and the formation of a biomimetic bone-like coating. Thus, in the present study we propose a pre-treatment technique using protein immobilization to the inert ceramic surface to accelerate the bone-like apatite formation as a biomimetic coating exhibiting bioactive and osteoinductive properties.

2. Material and methods

2.1. SBF manufacturing

The 1.5 times concentrated SBF solution was prepared according to Tanahashi et al. [15]. Briefly, chemicals were added to 37 °C warm ultra-pure water and constantly stirred step-by-step as follows: NaCl, $NaHCO_3$, KCl, K_2HPO_4, $MgCl_2{\cdot}6H_2O$, 1.0 M HCl, $CaCl_2{\cdot}2H_2O$, Na_2SO_4, and TRIS. The 1.5 times SBF was stored at 5 °C before use. Table 1 summarizes the chemical composition of conventional SBF, 1.5 times concentrated SBF and human blood plasma.

2.2. Substrate preparation and treatment

To produce specimens for the CaP coating, commercially available press-appropriated zirconia granulates (TZ-3YS-E, Tosoh, Japan) were uniaxially pressed (p = 100 MPa) and subsequently sintered at 1450 °C for 2 h. The samples prepared in this way were then ground and polished using SiC-abrasive paper and diamond paste with a last polishing step of 15 µm grit site. The polishing process was stopped at this stage to obtain sharp edges on the substrate surface.

Before soaking in the 1.5 times concentrated SBF, the ZrO_2-samples were pre-treated. The first batch was used as polished. The second batch was activated by 5 M H_3PO_4 for 15 h at room temperature to achieve the active –OH-functional groups on the zirconia surface. The samples were then gently rinsed 3 times using ultra-pure water and air-dried or placed directly into the BSA solution for the preparation of the third batch. The third batch was treated with 5 M H_3PO_4-solution for 15 h at room temperature and subsequently immersed in 1% (w/w) bovine serum albumin for 2 h under shaking to avoid sedimentation of the proteins. This was done to immobilize the BSA proteins on the –OH-activated ZrO_2-surfaces. 1% (w/w) BSA was prepared using commercially available protein powder (BSA, Sigma-Aldrich, Steinheim, Germany) dissolved in ultra-pure water. The samples were gently rinsed 3 times using ultra-pure water to removed residual uncoupled proteins and were then immediately covered with SBF solution.

BSA was chosen as a model protein, which is well-standardized and established to evaluate proof of principle. For future tests, more application-appropriated proteins, such as BMP, fibronectin, HGF or RGD, are considered, since BSA could induce an allergic reaction *in vivo* [29].

The samples prepared in this manner were soaked in the 1.5 times concentrated SBF at 40 °C for 3 and 10 days. Each sample was placed in a lockable glass flask and covered with 5 ml of SBF solution.

Table 1
Theoretical ion concentrations of human blood plasma compared with conventional simulated body fluid and 1.5× concentrated simulated body fluid.

Solution	Concentrations (mol/m^3)							
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
Human blood plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5
SBF	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5
1.5 × SBF	213.0	7.5	2.3	3.8	221.7	6.3	1.5	0.8

2.3. Verification of immobilized proteins

The visualization process of immobilized BSA on substrate surfaces using immunofluorescence was established and described in our previous study [8]. To summarize in brief, the immobilized proteins were fixed with 4% paraformaldehyde in PBS for 15 min and subsequently blocked with horse serum dissolved in PBS for 30 min at room temperature. Afterwards, the samples were incubated for 12 h with PBS solution containing a primary antibody against BSA (concentration 1:200 BSA antibody out of rabbit, antibodies-online GmbH, Aachen), which reacts specifically with BSA. Then, the samples were rinsed 3 times with PBS and incubated for 2 h with a fluorescein-labeled secondary antibody (Alexa Fluor 488 goat antirabbit IgG, Life Technologies) dissolved in PBS. The samples were gently rinsed with PBS, and immunofluorescence staining was analyzed with a fluorescence microscope (AXIO Imager M2m, Zeiss, Wetzlar, Germany). Control samples were treated in the same way as the functionalized samples to exclude any non-specific binding of the secondary antibody.

2.4. Substrate characterization

To determine the morphological changes on the substrate surfaces after soaking in SBF, scanning electron microscopy (Leo 440i, Carl Zeiss, Jena, Germany) was applied. It was possible to visualize the CaP crystals on the substrate surface. Moreover, the thickness of the CaP coatings could be measured by imaging a cross-section of ZrO_2 substrates. X-ray diffraction measurements (PW 3710, Philips, Eindhoven, the Netherlands) were performed to analyze crystalline phases of substrate and CaP coatings.

3. Results

The presence of stable immobilized BSA proteins on the zirconia surface has been proven using immunohistochemistry. Fig. 1 shows two representative samples. The green fluorescent signal was only detectable on the sample pre-treated with 5 M H_3PO_4 and subsequently with 1% BSA solution (Fig. 1, left). It proves that the protein immobilization process was successful. The control sample (Fig. 1, right) shows no fluorescent signal which confirms no unspecific protein-binding during the immunostaining process.

Subsequently, the CaP coating was manufactured on untreated surface, surface after activation by H_3PO_4 and surface after BSA loading by soaking the samples in SBF solution for 3 and 10 days. The pH values of the SBF after each process stage were evaluated and are presented in