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Synthesis of novel tricalcium phosphate-bioactive glass composite and functionalization with rhBMP-2

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Abstract A functionalization is required for calcium phosphate-based bone substitute materials to achieve an entire bone remodeling. In this study it was hypothesized that a tailored composite of tricalcium phosphate and a bioactive glass can be loaded sufficiently with rhBMP-2 for functionalization. A composite of 40 wt% tricalcium phosphate and 60 wt% bioactive glass resulted in two crystalline phases, wollastonite and rhenanite after sintering. SEM analysis of the composite's surface revealed a spongious bone-like morphology after treatment with different acids. RhBMP-2 was immobilized non-covalently by treating with chrome sulfuric acid (CSA) and 3-aminopropyltriethoxysilane (APS) and covalently by treating with CSA/APS, and additionally with 1,1′-carbonyldiimidazole. It was proved that samples containing non-covalently immobilized rhBMP-2 on the surface exhibit significant biological activity in contrast to the samples with covalently bound protein on the surface. We conclude that a tailored composite of tricalcium phosphate and bioactive glass can be loaded sufficiently with BMP-2.

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1 Introduction

In the field of biomaterials recent research is focused on the interface reactions and processes between implant material and the surrounding biological environment [1–4]. The understanding of these processes in detail is important for the development of tailored bone substitute implants. The most commonly used materials as bone substitute implants are calcium phosphate-based minerals, because of their chemical composition, which is similar to the natural human bone, and their osteoconductive or even biodegradable in vivo behavior. Many research groups have focused their investigations on hydroxyapatite (HAp) because of its bioactive character, and chemical similarity to the inorganic part of the human bone [5]. The calcium phosphate modification β-tricalcium phosphate (β-TCP), moreover, exhibits besides an excellent biocompatibility, resorption capabilities in vivo. However, both, HAp and β-TCP exhibit very poor mechanical properties resulting in limited application of these materials as implants. Therefore novel, innovative biomaterials, which exhibit both, good mechanical properties and resorption capabilities, are required. Many studies have reported increasing mechanical properties of the calcium-phosphate based materials through a glass phase addition because of densification via liquid phase sintering [6–9]. Besides improving of the mechanical properties, the required material should represent enhanced biodegradable ability.

Bone morphogenetic proteins (BMPs) belong to the highly conserved transforming growth factor beta superfamily of growth and differentiation factors which include

at least 15 different BMPs. BMP-2 is a homodimeric molecule with two polypeptide chains held together by a single disulfide bond. Each monomer contains in addition six other cysteine residues which are involved in three intra-chain disulfide bonds [10]. On the concave side of the protein a structural motive could be identified which was termed anthelix since a left-handed helix can be fitted into this groove [11]. The activity of BMP-2 can be tested by activation of de novo synthesis of alkaline phosphatase [12]. Self-prepared BMP-2 shows analogous biological activity in comparison to commercially available BMP-2 [13]. The immobilization of rhBMP-2 on bone replacement material is of high medical interest, because of the decisive role of BMPs in bone development and osteogenesis. Several years ago Jennissen et al. showed that titanium [14, 15] and hydroxyapatite ceramics [16] can be modified for the covalent and non-covalent immobilization of proteins such as BMP-2. These methods have been applied to bioactive glass ceramics. This is possible because glass ceramics obtain reactive OH-groups after CSA-treatment in analogy to titanium.

The hypothesis of this study was that a synthesis and subsequent surface treatment of a novel tricalcium phosphate-bioactive glass composite can enhance the amount of covalent and non-covalent immobilized rhBMP-2. The new resorbable material, obtained after the sintering process of 40 wt% β-TCP with 60 wt% bioactive glass type 45S5, was investigated. The surface of this material was activated by treating with different acids to prepare the surface conditions requested to the BMP coupling.

2 Materials and methods

2.1 Preparation of the bioactive glass ceramic material

The bioactive glass (similar to type 45S5, Hench) in the system SiO_2–Na_2O–CaO–P_2O_5 was prepared, using standard glass forming process by mixing SiO_2, Na_2CO_3, $CaCO_3$, and $CaHPO_4$ in the following weight ratio: 33.67:31.34:26.38:8.61. The powder was poured into a platinum crucible and was melted at 1350°C for 1.5 h. The melted glass was rapidly poured into water to cool down and avoid the crystallization process. After cooling, the glass was ground by a jaw crasher (BB1 A, Retsch Technology GmbH, Haan).

The β-tricalcium phosphate (β-TCP) material was derived as a commercially available powder (tricalciumphoshate, Merck, Darmstadt, Germany) and calcined at 1000°C for 1 h. The bioactive glass and β-TCP powders were mixed together in a proportion of 60:40 wt%, and subsequently granulated by spray drying. Such prepared granulate was pressed using a hydraulic press (PW40, Paul Otto Weber GmbH, Remshalden) to manufacture cylindrical specimens with 5 mm in diameter and 2 mm in thickness. Finally, the samples were sintered in a ceramic furnace at 1000°C for 5 h.

2.2 Preparation of specimens for the BMP coupling process

The samples were treated in different acid solutions to obtain, through extending of the specific surface area, increased amount of reactive OH-groups on the surfaces. Five different acid solutions were tested: 5% nitric acid solution, concentrated hydrochloric acid (37%, 12 M), half concentrated hydrochloric acid solution (6 M), sulfuric acid (98%, 18 M), and chromosulfuric acid (CSA) (92% H_2SO_4, 1.3% CrO_3). Chromosulfuric acid treatment was finally chosen for producing reactive OH-groups. Bioactive glass ceramics were reacted with 50 ml chromosulfuric acid (CSA) in a Teflon holder for 60 min at 90°C. After that CSA-treated glass ceramics were washed with boiling water for 30 min.

2.3 Characterization of materials and components before coupling process

The chemical elements of the ceramic powders were determined by x-ray fluorescence analysis (PANalytical PW2400, Kassel, Germany). The specimens were characterized by x-ray powder diffractometry using copper radiation K_α (PW 3710, Philips, Eindhoven, Netherlands) to identify changes of the crystalline phases in the material before and after immersion in the various acids. The morphology and the grain sizes of the specimens before and after immersion in the various suspensions were characterized by scanning electron microscopy (type LEO 440i, Carls Zeiss NTS GmbH, Oberkochen, Germany). Moreover, the cross sections of the specimens after acid treatment were analyzed by SEM-EDX measurement to reveal the penetration of the acids, aggressiveness of these acids, and the chemical elements content.

2.4 Surface functionalization with 3-aminopropyltriethoxysilane

Surface functionalization was performed as described previously [16]. The method was developed for hydroxyapatite surfaces and was transferred on the used bioactive glass ceramics. The CSA glass ceramics were reacted with 3-aminopropyltriethoxysilane (APS) in a Teflon holder in 47.5 ml toluene to which 2.5 ml APS (i.e. 5%) had been added under inert gas. The system was then closed and heated to boiling under reflux for 3.5 h under stirring. The ceramics were then washed three times in 10 ml

trichloromethane, acetone and methanol and air dried (CSA-APS glass ceramics).

2.5 Surface activation with carbonyldiimidazole

For covalent protein coupling the CSA-APS glass ceramics were activated with 1,1′-carbonyldiimidazole (CDI, 2.5 g/50 ml) in dry acetone under inert gas at room temperature for 3.5 h under tumbling (CSA-APS-CDI glass ceramics). The CDI-activated ceramics were three times washed in 10 ml acetone and water [16].

2.6 Preparation and immobilization of rhBMP-2

Recombinant human bone morphogenetic protein 2 (rhBMP-2) was prepared as described previously [17, 18]. The biological activity of rhBMP-2 was tested with MC3T3-E1 cells by the activation of the de novo synthesis of alkaline phosphatise as described [19]. The half-activation constants ($K_{0.5}$) for self-prepared rhBMP-2 were in the same range as for commercially available rhBMP-2 (InductOs, Wyeth) [13]. Radioactive labelled rhBMP-2 (^{125}I-CT-rhBMP-2) was prepared by a modified chloramine-T method [13, 14]. For immobilization of rhBMP-2 the washed ceramic specimens (three samples of CSA glass ceramics as control, three samples of CSA-APS glass ceramic, and three samples of CSA-APS-CDI glass ceramic) were incubated for 15 h at room temperature in a solution of 20 mM sodiumacetate, pH 4.5 (buffer A) or 125 mM borate, 0.066% SDS, pH 10.0 (buffer B) [16], respectively containing rhBMP-2. The amount of bound protein was determined by using ^{125}I-CT-rhBMP-2. The specific activity was ca. 10^7 cpm/mg. After incubation the ceramics were rinsed three times in 2 ml PBS buffer (2.7 mM KCl, 1.5 mM KH_2PO_4, 136.8 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4) and counted in a γ-counter.

2.7 Desorption experiments

After adsorption of rhBMP-2 and measurement in a γ-counter the ceramics were transferred to 2 ml PBS buffer for desorption measurements. At specified times the wafers were taken out, washed three times in 2 ml PBS buffer and measured in a γ-counter. They were then resuspended in 2 ml fresh PBS buffer for the next period of desorption. This regimen was continued for 8–15 days.

2.8 Determination of the biological activity of rhBMP-2

The determination of BMP-2 activity is based on the induction of alkaline phosphatase (ALP) in MC3T3-E1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) cells [15]. 5×10^5 freshly trypsinized cells were seeded on BMP-2 coated bioactive glass ceramics placed in a well using α-modified Eagles Medium (a-MEM), supplemented with glutamine (2 mM), penicillin (50 I.U./ml), streptomycin (50 lg/ml) containing 10% FCS. After 24 h the medium of the now confluent cells was replaced by α-MEM containing 1% FCS. After 3 days the cells were fixed with 2% paraformaldehyde for 10 min. Cell membranes were permeabilized by washing with PBS/Tween 20 0.2% (v/v) and were then rinsed with phosphate buffer. The phosphatase detection kit ELF-97 (Molecular Probes, Inc., Oregon, USA) was employed in the cell culture experiment for detecting endogenous ALP-activity. The resulting product forms an intensely fluorescent yellow-green precipitate at the site of enzymatic activity. Cells were rinsed with phosphate buffer and the ALP-activity of BMP-2 stimulated cells was monitored in a fluorescence microscope (Nikon Eclipse E 400, Nikon GmbH, Düsseldorf, Germany) using the appropriate optical filters for the visualization of the fluorescent substrate (excitation wave length: 345 nm, emission wave length: 530 nm).

2.9 Curve fitting and statistic

For curve fitting of the kinetic data the PC program PRISM 4 (Graph Pad Software Inc., San Diego, CA) was employed.

3 Results

The results of the chemical analysis of the manufactured glass are summarized in Table 1 and are comparable to the theoretical elements content of the so-called Hench glass type 45S5 (Bioglass®). Some impurities derived from starting materials were found in the chemical composition of obtained glass; principally Al_2O_3 was present in amount of 0.08 wt% and MgO in amount of 0.07 wt%. The XRD analysis confirmed that the obtained bioactive glass contained only the amorphous phase (Fig. 1). The glass transition temperature T_g of the bioactive glass was approximately 550°C and the exothermal peak which corresponds to the temperature of the crystallization T_c was around 770°C. XRD analysis revealed that the primary phases in the composite were changed after the thermal treatment. Two new phases were detected by the XRD measurement after thermal treatment of the composite samples: $NaCaPO_4$ (rhenanite) and $CaSiO_3$ (wollastonite) (Fig. 2). The SEM-EDX measurements of cross-section of the sample have confirmed the presence of a third, new amorphous phase in the system which was undetectable by XRD measurement. Treatment with acid solutions resulted in a very strong reaction on the material surfaces and caused the dissolving process of crystalline phases. Owing to this procedure a

Table 1 Comparison of the chemical composition of the Hench glass (type 45S5) with the bioactive glass synthesized in this study, measured by X-ray fluorescence (XRF)

Oxide	Hench glass type 45S5 (wt%)	Synthesized glass (wt%)
SiO_2	45.0	45.41
Na_2O	24.5	23.1
CaO	24.5	25.0
P_2O_5	6.0	6.2
Al_2O_3	–	0.08
MgO	–	0.07
TiO	–	0.03

It is shown that the oxides content of the laboratory obtained glass is comparable to the theoretical content of bioactive glass 45S5. Impurities are presents owing to the manufacturing process

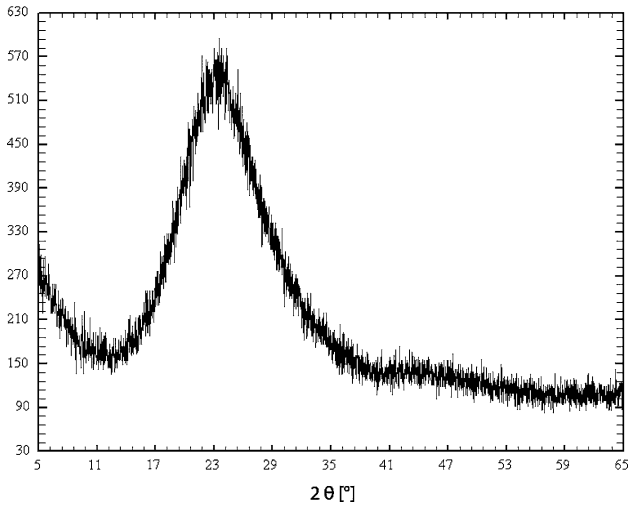

Fig. 1 XRD diagram of the obtained 45S5 bioactive glass showing the pattern shape typical for the amorphous phase. The diagram confirms the vitreous character of the specimen, as no crystalline peaks occur

spongious-like structure was created. The same effect was observed on the surface of the samples treated with each acid (sulphuric acid, chrome-sulphuric acid, nitric acid, and hydrochloric acid) (Fig. 3). Hydrochloric acid was the most aggressive and partially dissolved outer-layer of the specimens as shown in Fig. 4. XRD measurements of the samples after hydrochloric acid treatment confirmed aggressive character of this acid. The XRD diagram showed no crystalline peaks of the samples after treatment with half concentrated hydrochloric acid solution (6 M) and after concentrated hydrochloric acid (37%, 12 M) (Fig. 5). The second most aggressive acid was sulphuric acid (98%, 18 M), which penetrated the samples in app. 0.5 mm depth (Fig. 4). Chromosulphuric acid (92% H_2SO_4, 1.3% CrO_3) and 5% nitric acid induced similar results. The penetration of those both acids in the samples was rather low and did not

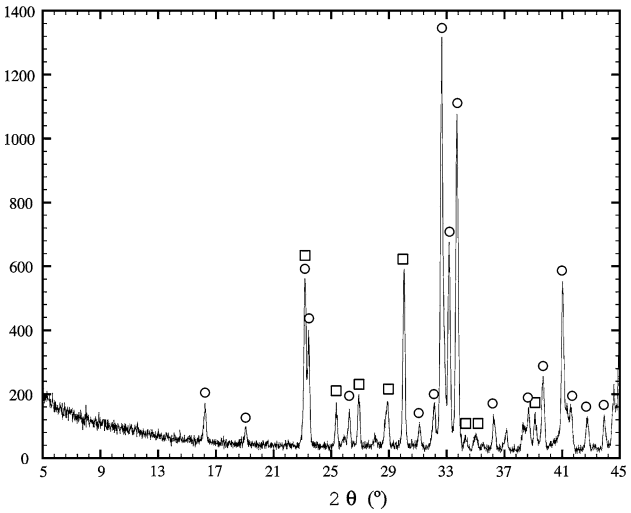

Fig. 2 XRD diagram of the β-tricalcium phosphate/bioactive glass composite after thermal treatment at 1000°C for 1 h. The initial phases, β-tricalcium phosphate and bioactive glass reacted during sintering, creating the two new crystalline phases—wollastonite/$CaSiO_3$ (marked as *circle*) and rhenanite/$NaCaPO_4$ (marked as quadrangle)

destroy the sample structures. In further experiments for BMP-2 coupling chromosulphuric acid was chosen as a pretreatment.

RhBMP-2 could be immobilized on bioactive glass ceramics non-covalently via hydrophilic interactions after CSA treatment of the glass ceramics and via hydrophobic interactions after modification of the surface with 3-aminopropyltriethoxysilane (APS) or covalently after activation with 1,1′-carbonyldiimidazole (CDI). Without CSA treatment only small amounts of rhBMP-2 were bound on glass ceramics (1.9–5.1 µg/cm^2, $c_0 = 0.1$ mg/ml). It was possible to bind covalently 32 µg rhBMP-2/cm^2 on CSA-treated glass ceramics using buffer A (pH 4.5). By incubation at pH 10.0 (buffer B) up to 15 µg protein/cm^2 could be immobilized (Table 2). In desorption experiments it was shown that release of the bound protein occurs in the form of a two-phase exponential decay function (Fig. 6a, b). An initial burst process phase was followed by a prolonged second process phase. By comparing half-lives a clear difference in the release of immobilized rhBMP-2 in dependence of the way of immobilisation could be observed (Table 3). Covalently bound protein (CSA-APS-CDI glass ceramic) was released from the surface with a half-life of nearly 100 days for the second slow phase in comparison to ca. 30 days for non-covalently bound rhBMP-2 (CSA glass ceramic, CSA-APS glass ceramic). At pH 10.0 (buffer B) non-covalently immobilized rhBMP-2 (Fig. 6b) showed a pronounced burst phase. Over 60% of the rhBMP-2 load was released in the first 48 h of desorption. In contrast only a small amount of covalently bound protein was released during the initial phase. Both

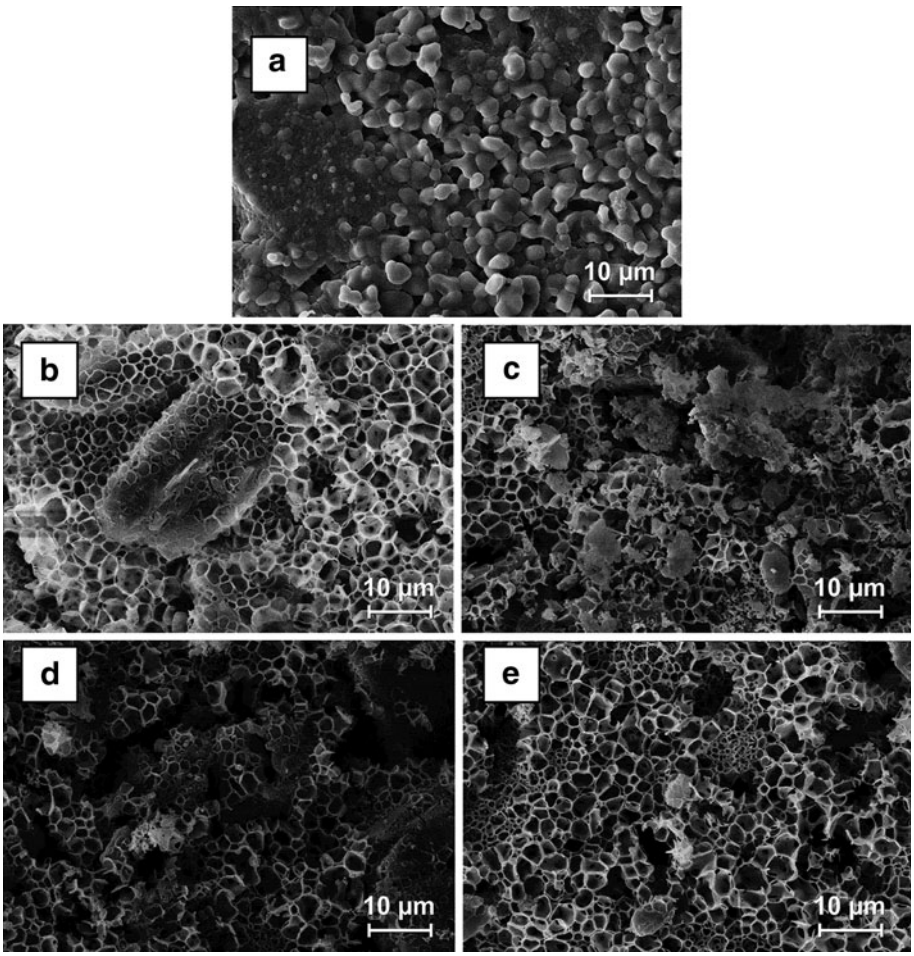

Fig. 3 SEM micrographs of the surface of β-tricalcium phosphate/bioactive glass composite after sintering at 1000°C: **a** before the pre-treatment process; and after pre-treatment with different solutions: **b** chrome-sulfuric acid (92% H_2SO_4, 1.3% CrO_3); **c** half concentrated hydrochloric acid solution (6 M); **d** sulfuric acid (98%, 18 M); and **e** 5% nitric acid solution. The micrographs show, that after each acid treatment, the surface morphology of the specimen was strongly changed, creating the porous, spongy bone like structure

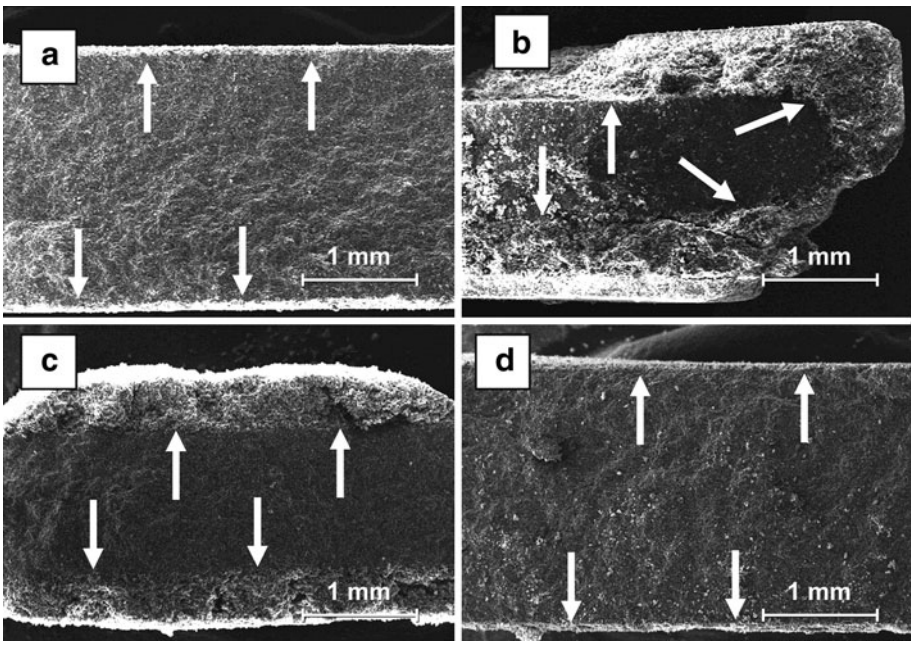

Fig. 4 SEM micrographs of the cross sections of the sintered β-tricalcium phosphate/bioactive glass composite after pre-treatment with: **a** chrome-sulfuric acid (92% H_2SO_4, 1.3% CrO_3); **b** half concentrated hydrochloric acid solution (6 M); **c** sulfuric acid (98%, 18 M); and **d** 5% nitric acid. The *arrows* indicate the penetration depth of the acids, respectively

CSA-APS glass ceramic and CSA glass ceramic loaded with non-covalently bound BMP-2 at pH 10.0 (buffer B) showed biological activity comparable to the positive control (soluble BMP-2; 20 nM) as demonstrated by the intensely fluorescent yellow-green precipitate. In contrast CSA-APS-CDI glass ceramic with covalently bound

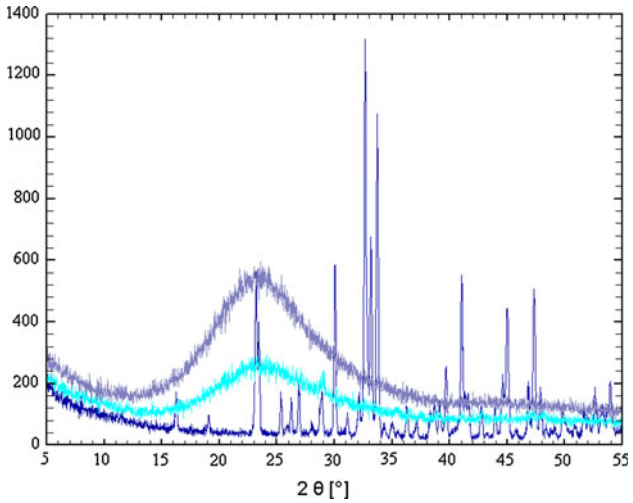

Fig. 5 XRD diagram of: lower curve—sample before acid treatment, which shows the two crystalline phases wollastonite/$CaSiO_3$ and rhenanite/$NaCaPO_4$ (see fig. 2); middle curve—sample after treatment with half concentrated hydrochloric acid solution (6 M), which shows the partial dissolving process of the crystalline phases after acid treatment; upper curve—sample after concentrated hydrochloric acid (37%, 12 M) treatment, which shows completely dissolve crystalline structure on the material surface (no crystalline peaks occur)

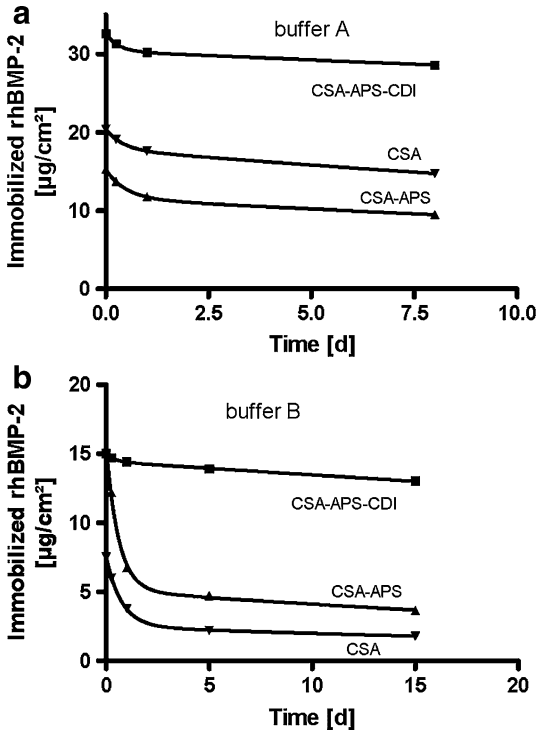

Fig. 6 Two-phase exponential release of rhBMP-2 from bioactive glass ceramics. After adsorption of rhBMP-2 in **a** 20 mM sodiumacetate, pH 4.5 (buffer A), **b** 125 mM borate, 0.066% SDS, pH 10.0 (buffer B) the glass ceramics were transferred to 2 ml PBS buffer. At specified times the wafers were taken out, washed three times in 2 ml PBS and measured in a γ-counter. The release of immobilized rhBMP-2 occurs in the form of a two-phase exponential decay function. An initial burst process phase is followed by a prolonged second process phase

rhBMP-2 showed no fluorescence at the site of enzymatic activity (Fig. 7) though the amount of released protein should be high enough to induce alkaline phosphatase (ca. 40 nM).

4 Discussion

It was shown that the sintering process of the innovative β-TCP/bioactive glass composite results in the same phase contents ($NaCaPO_4$/rhenanite, $CaSiO_3$/wollastonite and glassy matrix) compared to analogous composites made of hydroxyapatite/bioactive glass which are already reported in the literature [20, 21]. In contrast to Hesaraki et al., who report no β-TCP phase changes after mixing with the magnesia-containing glass and sintering, the two new crystalline phases, $NaCaPO_4$ (rhenanite), and $CaSiO_3$ (wollastonite) were formed as a result of the reaction between the bioactive glass phase and calcium phosphate [20]. Moreover, a third phase was detected in the system by the SEM-EDX measurement. By SEM-EDX measurement it was found in one phase Ca, Si and O elements ($CaSiO_3$, wollastonite), in another phase Na, Ca, P and O ($NaCaPO_4$, rhenanite) and in third phase it was found Ca, Na, Si and O elements. The latter phase was amorphous (similar to the initial bioactive glass) as it could not be detected by the XRD-measurements (Fig. 2). W. Gong et al. have suggested the mechanism of formation of rhenanite,

Table 2 Immobilization of rhBMP-2 on glass ceramics

Buffer type	Immobilized rhBMP-2		
	Hydrophilic (CSA) (µg/cm^2)	Hydrophobic (CSA-APS) (µg/cm^2)	Covalent (CSA-APS-CDI) (µg/cm^2)
Buffer A (c_0 = 0.2 mg/ml)	20.44 ± 4.46	15.25 ± 0.82	32.62 ± 3.09
Buffer B (c_0 = 0.3 mg/ml)	7.54 ± 1.84	15.32 ± 2.59	15.03 ± 0.87

For immobilization of rhBMP-2 modified and non-modified CSA-treated glass ceramics were incubated for 15 h at room temperature in a solution of 20 mM sodium acetate, pH 4.5 (buffer A) or 125 mM borate, 0.066% SDS, pH 10.0 (buffer B) respectively containing rhBMP-2 (0.2–0.3 mg/ml). The amount of bound protein was determined by using ^{125}I-rhBMP-2. After incubation the glass ceramics were washed three times in 2 ml PBS buffer and counted in a γ-counter

Table 3 Half-lives for the release of rhBMP-2 from modified glass ceramics

Buffer type	Modification	Initial load (μg/cm^2)	Half-life 1 (days)	Half-life 2 (days)	R^2
Buffer A	CSA	20.44	0.3	29.0	1.000
	CSA-APS	15.25	0.3	28.6	1.000
	CSA-APS-CDI	32.62	0.2	93.6	1.000
Buffer B	CSA	7.54	0.5	31.3	0.9998
	CSA-APS	15.32	0.4	31.7	0.9984
	CSA-APS-CDI	15.03	0.3	99.4	0.9985

After adsorption of rhBMP-2 and measurement in a γ-counter the ceramics were transferred to 2 ml PBS buffer for desorption measurements. At specified times the glass ceramics were taken out, washed three times in 2 ml PBS and measured again. They were then resuspended in 2 ml fresh PBS buffer for the next period of desorption. Data were fitted to a two-phase exponential decay and the half-lives were calculated

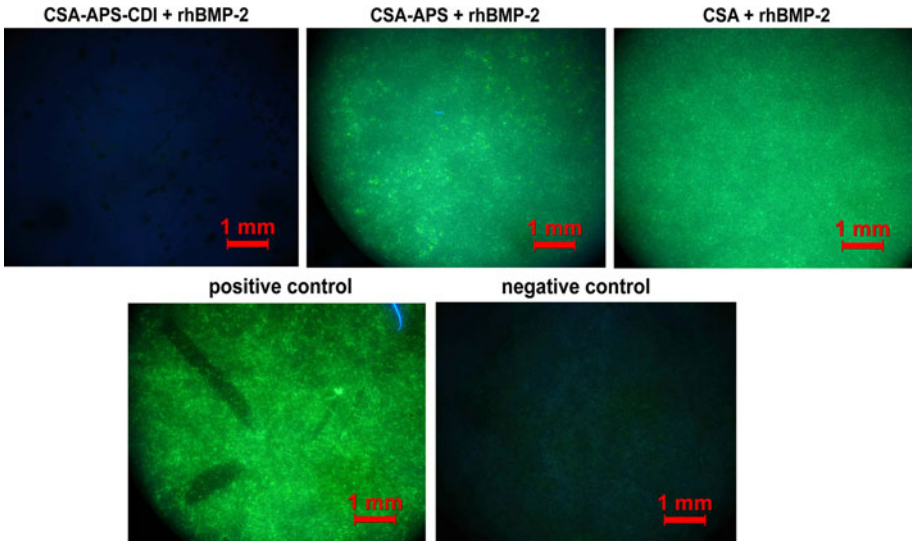

Fig. 7 Biological activity of rhBMP-2 immobilized on differently modified glass ceramics (magnification 1:2). 5×10^5 freshly trypsinized cells were seeded on BMP-2 coated bioactive glass ceramics using α-MEM containing 10% FCS. After 24 h the medium of the now confluent cells was replaced by α-MEM containing 1% FCS. After 3 days the cells were fixed with 2% paraformaldehyde. Determination of BMP-2 activity using the phosphatase detection kit ELF-97 is based on the induction of alkaline phosphatase (ALP) in MC3T3-E1 cells. The resulting product forms an intensely fluorescent yellow-green precipitate at the site of enzymatic activity (positive control: soluble rhBMP-2)

wollastonite and glassy matrix using the analogical example of hydroxyapatite/bioglass composite [20]. According to this mechanism, during the sintering process occur following two reactions:

$$\left[2Ca_5(PO_4)_3(OH)\right]_{cryst.} + \left[xNa_2O \cdot ySiO_2\right]_{glass} \rightarrow \left[6NaCaPO_4\right]_{cryst.} + \left[(x-3)Na_2O \cdot 4CaO \cdot ySiO_2\right]_{glass} + H_2O \quad (1)$$

$$\left[(x-3)Na_2O \cdot 4CaO \cdot ySiO_2\right]_{glass} \rightarrow \left[4CaSiO_3\right]_{cryst.} + \left[(x-3)Na_2O \cdot (y-4)SiO_2\right]_{glass} \quad (2)$$

They suggest that the calcium phosphate phase converts completely into rhenanite phase because of diffusion of Na into hydroxyapatite phase (Eq. 1) and additionally the CaO/SiO_2 ratio increases and wollastonite crystallizes due to supersaturation (Eq. 2) [22]. Different research groups have proved that wollastonite and rhenanite have the potential to enhance osteogenesis and exhibit bioactive and even bioresorbable in vivo behavior [23–26]. Moreover some studies have already reported the bioactive properties of wollastonite/rhenanite in amorphous matrix in various phase concentration contents, which has been obtained from different than in the present study basic raw materials (glass–ceramic materials) [20, 21]. Therefore, the synthesized composite ceramic material should be suitable as bone substitute material. In order to allow the coupling of BMP-2 the samples were treated with various acid solutions to obtain the requested active groups (e.g. hydroxyl groups) on the material surfaces. The morphology of the samples surfaces was strongly changed by the acid treatments

(Fig. 3). Initially all samples contained three phases: wollastonite ($CaSiO_3$), rhenanite ($NaCaPO_4$) and amorphous phase. The grains that consisted of rhenanite and wollastonite phases were coated with an amorphous layer. After contact of those samples with the different acid solutions, the rhenanite and wollastonite phases were dissolved, whereas the amorphous phase endured the acid treatment, creating a very porous, spongious-like structure (Figs. 3, 5).

To investigate, which of the acids exhibit the most aggressive behavior, cross sections of the samples after acid treatment were prepared and analyzed (Fig. 4). The most aggressive, from those four acids was the hydrochloric acid solution. During preparation the cross-section of this sample, the outer layer was already partially damaged (Fig. 4b). This result correlates with the XRD measurement which revealed that after treatment with the hydrochloric acid solution, only the amorphous phase remained (Fig. 5). This indicates that the original crystal structure of the specimen was completely destroyed. The other, very aggressive solution was the sulphuric acid (Fig. 4c). The penetration area of this acid was approximately 0.5 mm deep. It can be concluded that neither hydrochloric acid nor sulphuric acid treatments are adequate for this composite material because of aggressive etching reaction which destroys construction of samples, and decrease its mechanical properties. The infiltration depth of chromosulphuric acid and nitric acid were comparably shallow (approximately 0.1–0.2 mm). Those results showed that the chrome-sulphuric acid and the nitric acid did not destroy the whole material construction and the samples after treatment are stable. Moreover the specific surface area significantly increased through appearing of spongious-like structure (Fig. 3b, e). There were no significant differences between the results of chrome-sulfuric acid and nitric acid treatment. Chromosulfuric acid treatment was chosen for further experiments because experimental data from previous work had shown good results as pre-treatment for BMP-2 coupling [15].

Depending on the method of surface modification and the used incubation buffer 7–32 μg rhBMP-2 per cm^2 could be immobilized on glass ceramics. Von Walter et al. used APS modification of a TiO_2/glass composite for BMP-2 immobilization resulting in a BMP-2 loading of ca. 0.5 μg/cm^2 [27]. However concentration of the BMP-2 solution for incubation was much lower (1–5 μg/ml). Under hydrophilic conditions the ca. threefold amount of BMP-2 adsorbed the surface at pH 4.5 (buffer A) compared to pH 10 (buffer B) (20.4 vs. 7.5 μg/cm^2). This may be due to the fact that the isoelectric point (pI) of *E. coli* BMP-2 is at pH 8.5 [28]. Therefore lowering the pH will result in an increase of positively charged amino acid side groups facilitating enhanced binding to negatively charged groups on the surface of glass ceramics. There was no difference in BMP-2 loading under hydrophobic conditions at different pH values (ca. 15 μg/cm^2). As BMP-2 is highly stabilized by disulfide bridges no change in overall three dimensional structure and therefore distribution of hydrophobic patches responsible for binding is to be expected. Covalent binding of BMP-2 at pH 4.5 resulted in a ca. twofold increase in the amount of bound BMP-2 compared to binding at pH 10 (32.6 vs. 15.0 μg/cm^2). As shown for bovine serum albumin there is a broad maximum for optimal coupling conditions in the range from pH 4–5 with a second peak at pH 9 [29]. Until now it is unclear, which characteristics of BMP-2 release are necessary for the formation of new bone. BMP-2 is released from the body during the whole period of fracture healing [30]. In this publication we show that the amount of released protein can be steered by variation of half-lives and characteristic of the burst phase. Half-lives for the release of BMP-2 were in the range of ca 30 days for non-covalently bound BMP-2 and ca. 90–100 days for covalently bound protein emphasizing different mechanisms of binding. Over 60% of non-covalently rhBMP-2 bound at pH 10 is released in the first 2 days of desorption in a pronounced burst-phase in contrast to the release of only ca. 10–15% at pH 4.5. By using both variants in future in vivo experiments it can be determined which kinetic of rhBMP-2 release results in stronger stimulation of bone formation.

The determination of biological activity in vitro shows that non-covalently immobilized rhBMP-2 is biologically highly active (Fig. 7). In contrast covalently bound protein does not show any biological activity although juxtracrine stimulation is supposable. A reason for absent activity could be the insufficient length of the APS spacer molecule thus preventing binding of cell-membrane BMP-2 receptors to surface-bound BMP-2. However even under conditions for covalent binding a small amount of BMP-2 is released which should be sufficient for inducing alkaline phosphatase activity. Missing bioactivity of released BMP-2 which was bound under conditions for covalent binding may be due to inactivation of the bound protein. Further work is needed to analyze the reasons of this effect.

As a next step, bone formation by BMP-2 coated tricalcium phosphate glass composites has to be shown in vivo. As has been demonstrated in previous experiments using titanium surfaces and BMP-2 the amount of protein released from tricalcium phosphate glass composites should be sufficient for obtaining a biological effect in vivo [31].

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