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Bioactive borate glass scaffolds: in vitro and in vivo evaluation for use as a drug delivery system in the treatment of bone infection

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Abstract The objective of this work was to evaluate borate bioactive glass scaffolds (with a composition in the system Na_2O–K_2O–MgO–CaO–B_2O_3–P_2O_5) as devices for the release of the drug Vancomycin in the treatment of bone infection. A solution of ammonium phosphate, with or without dissolved Vancomycin, was used to bond borate glass particles into the shape of pellets. The in vitro degradation of the pellets and their conversion to a hydroxyapatite-type material in a simulated body fluid (SBF) were investigated using weight loss measurements, chemical analysis, X-ray diffraction, and scanning electron microscopy. The results showed that greater than 90% of the glass in the scaffolds degraded within 1 week, to form poorly crystallized hydroxyapatite (HA). Pellets loaded with Vancomycin provided controlled release of the drug over 4 days. Vancomycin-loaded scaffolds were implanted into the right tibiae of rabbits infected with osteomyelitis. The efficacy of the treatment was assessed using microbiological examination and histology. The HA formed in the scaffolds in vivo, resulting from the conversion of the glass, served as structure to support the growth of new bone and blood vessels. The results in this work indicate that bioactive borate glass could provide a promising biodegradable and bioactive material for use as both a drug delivery system and a scaffold for bone repair.

1 Introduction

Chronic bone infections like osteomyelitis are often associated with necrosis of bone and poor perfusion accompanied by infection of the surrounding tissues. As a result, only limited antibiotics administered either orally or intravenously are able to reach the site of infection [1]. Local drug delivery systems, implanted into the target sites, can release antibiotics at the desired concentrations locally, thereby avoiding the introduction of high concentrations of antibiotics to systemic circulation which might otherwise produce side effects.

Several inert and biodegradable materials are employed as vehicles for drug delivery. Commercially available poly(methyl methacrylate), PMMA, has been used clinically for more than 20 years. However, additional surgery is required to remove inert materials such as PMMA from the body, after serving their drug-release function. Biocompatible polymers, such as collagen, poly(lactic acid), and polylactate, have the advantage of being biodegradable [2, 3], but they lack the ability to bond chemically with bone. Inorganic bone grafting materials, such as calcium

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phosphate cement, hydroxyapatite (HA), and bioactive glass, have the ability to bond strongly to natural bone, and could provide an additional function as a drug release device for bone repair [4, 5].

Bioactive glasses are both osteoconductive and osteoinductive [6]. Despite of the great success of the silicate 45S5 bioactive glass (Bioglass®) in clinical applications, melt-derived silicate bioactive glasses have served mainly as a modification to polymer carriers to improve their bioactivity. Sol–gel derived silicate glasses have been the subject of investigations for use as a drug carrier for bone treatment [7, 8], but the fabrication process of these glasses is time consuming. Furthermore, the slow degradation rate remains a challenge for silicate-based bioactive glasses.

Recent in vitro studies have shown that some melt-derived borate and borosilicate glasses are bioactive as well as biodegradable, and that the degradation rate can be controlled by varying the boron content in the glass [9]. The degradation of the glass leads to the formation of a HA-type material on the surface of the glass [10], which is responsible for the formation of a strong bond with surrounding hard and soft tissue in vivo. Based on their bioactive and biodegradable properties, bioactive borate glass could be employed both as a bone grafting material and as a carrier material for drug delivery.

Although important nutritionally for developing healthy bone in humans and animals in small concentrations, borates, a major degradation product of bioactive borate glass, is toxic at large concentration [11]. As a result, concerns have been expressed about the potential systemic and local side effects of the borate-based glasses in vivo. Investigations of in vitro cell culture on bioactive borate glass substrates have shown that while some borate glasses can inhibit cell proliferation under more conventional, 'static' culture conditions, this inhibitory effect could be alleviated under more 'dynamic' culture conditions [12]. It is likely that the 'dynamic' system provided by the living body could be effective in diluting local boron concentration, and therefore alleviate the toxic effect on cells and tissues.

Our previous work has assessed the efficacy of Vancomycin-loaded borate glass scaffolds on treating osteomyelitis of rabbit model [13]. The objective of the present work was to evaluate the use of bioactive borate glass scaffolds both as a controlled release device for local delivery of the drug Vancomycin and as a bone grafting material. Scaffolds of bioactive glass loaded with Vancomycin were prepared, and the effect of the in vitro degradation of the scaffolds on the drug release behavior and the conversion to HA was evaluated. In vivo, the ability of the converted scaffold to support bone repair was assessed.

2 Materials and methods

2.1 Preparation of scaffolds for drug delivery

Borate glass with the composition $6Na_2O$–$8K_2O$–8MgO–22CaO–$54B_2O_3$–$2P_2O_5$ (mol%), designated D-Alk-3B glass (double alkali borate glass), was used as the starting material for fabricating scaffolds with a drug delivery function. The glass was prepared using conventional laboratory glass processing techniques, as described in detail elsewhere [9]. Briefly, powders of the raw materials were melted in platinum crucible at 1100°C for 1 h, and the molten glass was quenched between two cold stainless-steel plates. Glass frits resulting from the quenching operation were crushed and sieved to obtain particles of average size 5 μm.

Scaffolds (without a drug) were prepared by binding the borate glass particles into a three-dimensional structure using a neutral buffer solution consisting 0.38 M $(NH_4)_2HPO_4$ and 0.09 M $NH_4H_2PO_4$, prepared by dissolving the chemicals in deionized water, and adjusting the pH to a value of 7.2–7.4 using ammonia. Glass particles (80 wt%) were mixed with the binding solution (20 wt%) to formed a gum-like material. The mixture was filled into cylindrical plastic molds (5.0 mm in diameter × 2.5 mm) and compressed by applying a stress of ~5 MPa. The as-prepared pellets were removed from the mold and held for 24 h at room temperature to allow setting of the binding material.

Scaffolds loaded with the drug Vancomycin (Eli Lilly, Seishin Laboratories, Japan) were prepared using the same method described above, except that the Vancomycin (concentration = 40 mg per gram of glass particles) was dissolved in the binding solution prior to mixing with the glass particles. The Vacomycin-loaded scaffolds are designated VDC, whereas scaffolds containing no Vancomycin are designated BDC (blank drug carrier).

2.2 In vitro degradation and conversion tests

The degradation of the BDC scaffolds in a simulated body fluid (SBF) at 37°C was assessed as a function of immersion time (up to 40 days) using chemical analysis, weight loss, X-ray diffraction (XRD), and scanning electron microscopy (SEM). A ratio of 1 g of scaffold to 100 ml SBF was used, and SBF was replaced every 24 h. The boron concentration in the SBF, resulting from the degradation of the borate glass scaffolds, was determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES; Optima 2100 DV; USA). Weight loss was measured after removing the scaffolds from the SBF, washing them with deionized water, and drying at 90°C. The phase composition of the scaffolds was determined using XRD (D/max 2550; Rigaku International Corp.,

USA), at a scan rate of 1.8° 2θ per minute, in the range 10–70° 2θ. The microstructures of the borate glass scaffolds before and after immersion were observed using SEM (S-2360N; Hitachi, Japan).

Release of Vancomycin from the VDC scaffolds was investigated by immersing the scaffolds in SBF at 37°C, and measuring the Vancomycin concentration in the SBF using high performance liquid chromatography (HPLC; Agilent 1100; USA). A ratio of 1 g of scaffold to 100 ml of SBF was used, and the SBF was replaced every 24 h.

2.3 In vivo implantation

The animal research protocol used in these experiments was approved by the Animal Care Committee of Shanghai Sixth People's Hospital, Shanghai Jiaotong University, China. Twenty-five 8-month old male New Zealand White rabbits (specific pathogen free) weighing between 2.11 and 3.88 kg (average weight = 2.65 kg) were used. The methicillin-resistant staphylococcus aureus (MRSA) inoculation procedures were carried out as described by Dahners and Funderburk [14]. Briefly, the rabbits were anesthetized and sequentially injected with 0.1 ml 5% sodium morrhuate (Eli Lilly, Indianapolis, IN, USA), 0.1 ml bacterial suspension containing 1×10^9 colony forming units (CFUs), and 0.1 ml sterile phosphate-buffered saline (PBS) at the medullary cavity of the right hind leg. The osteomyelitis symptom of each rabbit was examined by radiographs at 3 weeks after MRSA inoculation. Twenty-two rabbits met the criteria of osteomyelitis described by Norden et al. [15], and were selected for further studies.

The infected rabbits were randomly assigned into the control group and the experimental group, designated as CTL group and VDC group. A 1.5 cm × 1 cm window was made through the cortical bone to the cavity. As shown in Fig. 1, for the CTL group, the debridement of the tibial marrow cavity was carried out, followed by irrigation with normal saline. For the VDC group, after the debridement and irrigation as described for the CTL group, the VDC pellets were implanted to fill the space of the medullary cavity. The amount of pellets was 470–900 mg, depending on the severity of the osteomyelitis.

After surgery, 2.0 ml of blood was drawn from the central ear artery at 10, 24, 48, 72, and 120 h post-implantation from eight rabbits randomly selected from VDC group. The blood samples were centrifuged to leave the serum at the top layer. The blood-boron concentrations of the serum were analyzed using ICP-AES.

The rabbits were sacrificed 8 weeks after surgery. Samples of the debrided tissues from the infected sites were collected in sterile tubes for microbiological examination. The samples were inoculated on blood agar and kept for 48 h in a 37°C incubator. The coagulate tube test and the API Staph system (ATB 32 Staph. Bio-Merieux, Marcy-l' Etoile, France) were used to determine the presence of *S. aureus*. Resistance to methicillin was confirmed by detection of the mecA gene using PCR [16].

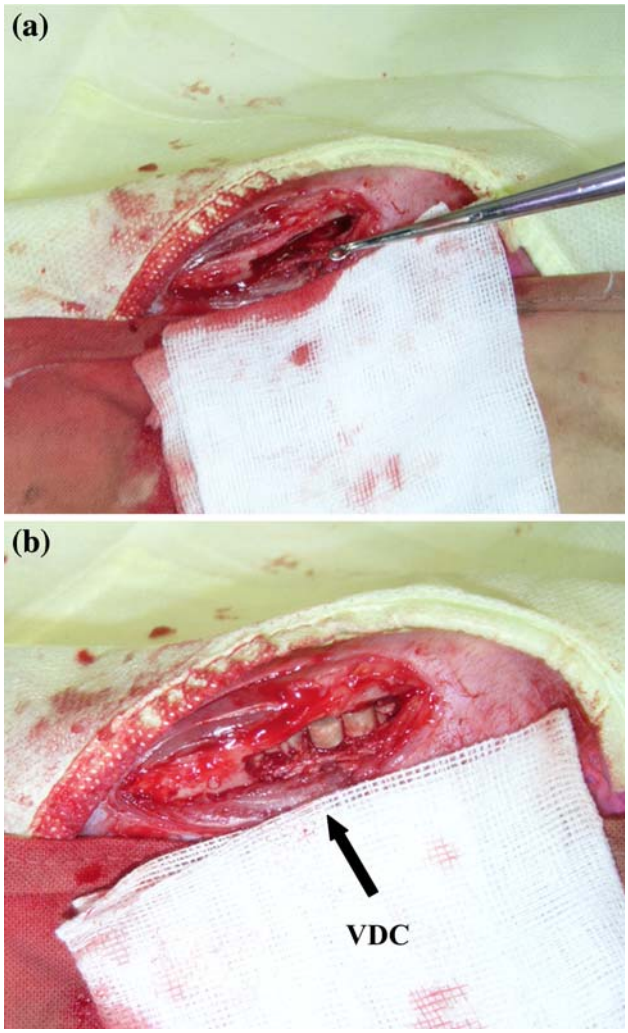

Fig. 1 Treatment of rabbits: **a** Control (CTL) group with debridement, and **b** group treated with Vancomycin-loaded bioactive borate glass (VDC) scaffolds

The excised tibia bone specimens were fixed in 10% formalin for 2 weeks, decalcified in EDTA solution for 4 weeks, and embedded in paraffin. Sections (5 μm thick) were cut along the long axis of tibia, and stained with hematoxylin and eosin (H&E), and observed using a microscope.

3 Results and discussion

3.1 In vitro degradation behavior

Degradation of bioactive borate glass in an aqueous phosphate solution is accompanied by the release of ions,

such as those of boron, and network modifiers such as Na and K, due to the degradation of the B–O bonds in the glass network, as well as a weight loss, resulting from the conversion of the glass to HA [17]. Measurement of the ionic concentrations in the solution, and the weight loss of the glass as a function of immersion time can therefore provide information about the degradation kinetics of the glass. Since elemental boron does not exist in a free state in nature, the term "boron" refers to the boron content of boric acid and inorganic borates in this paper and all the reported weights refer to boron.

Figure 2 shows the boron concentration released from the BDC scaffolds (without Vancomycin) into the SBF after each day of immersion, as well as the cumulative amount of boron released. The cumulative boron concentration increased rapidly initially, but then decreased markedly at longer times. Based on the total boron present in the as-prepared (starting) glass scaffold, ~35 wt% of the boron content of the scaffold was released after only 1 day, increasing to ~80 wt% released after 3 days, and reaching 90 wt% released after 1 week. The degradation rate of this borate glass is markedly faster than silicate bioactive glasses such as 45S5 and 13–93 [18].

The weight loss of the BDC scaffolds (Fig. 3) showed trends similar to those of the boron release data described above. After a rapid increase at shorter times, the weight loss slowed, and reached a steady, limiting value of ~73% after 7–10 days. Assuming that all the calcium present in the as-prepared (starting) glass scaffold converted completely to HA with the stoichiometric composition, $Ca_{10}(PO_4)_6(OH)_2$, the 'theoretical' weigh loss is estimated to be 74% (shown as dashed line in Fig. 3). The almost identical values for the limiting weight loss and the theoretical weight loss indicate that the borate glass in the BDC scaffolds was almost completely converted to HA. Furthermore, the similar trend in the cumulative boron concentration in the SBF and the weight loss data is an indication that the degradation of the scaffold and the conversion to HA occur in closely parallel steps.

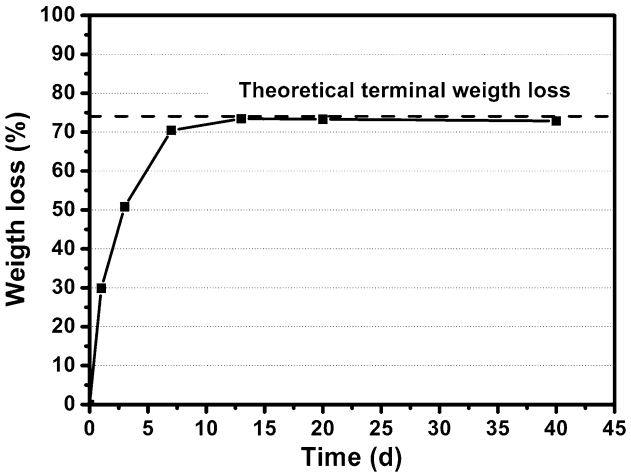

Fig. 3 Weight loss of bioactive borate glass scaffolds as a function of immersion time in a SBF. The 'theoretical' weight loss of the scaffolds is shown by the *dashed line*

As the SBF penetrated into the VDC scaffolds (with Vancomycin) and corroded the borate glass particles and the binding phosphate material, the drug was dissolved into the surrounding solution and then diffused through the pores between the glass particles. The release of Vancomycin from the scaffolds (Fig. 4) increased rapidly initially, and reached a nearly constant value after 3–4 days, which was almost equal to 100% of the drug in the as-prepared scaffold.

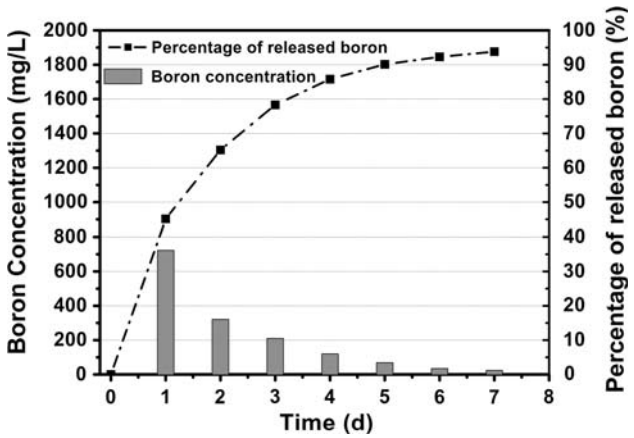

Fig. 2 Concentration of boron released each day from the scaffolds into a simulated body fluid (SBF), and the cumulated concentration of boron released (as a fraction of the boron concentration in the as-prepared scaffold)

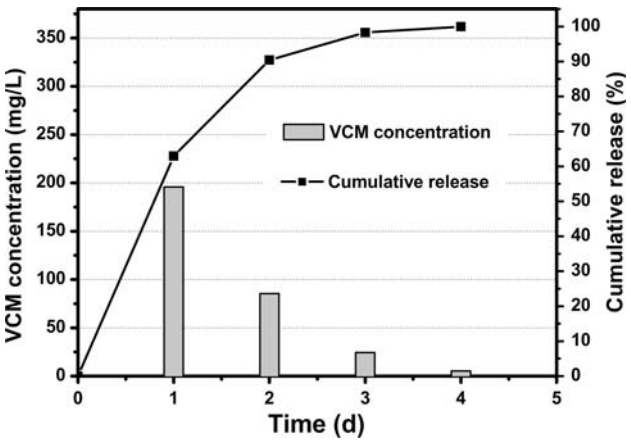

Fig. 4 Concentration of Vancomycin released from the scaffolds after each day of immersion in a SBF, and the cumulative amount released (as a fraction of the amount of Vancomycin in the as-prepared scaffolds)

3.2 Conversion of scaffolds to hydroxyapatite

XRD showed that the glass particles had a pattern typical of an amorphous material (Fig. 5). However, the pattern of the as-prepared BDC scaffold showed peaks corresponding to those of a ammonium magnesium phosphate binding phase, $NH_4MgPO_4{\cdot}6H_2O$ (JCPDS 71-2089), which resulted from the binding phase. This ammonium magnesium phosphate binding phase, which is biocompatible and degradable in vivo, forms the major hydration product of fast-setting, magnesium phosphate cement for bone repair [19]. In the present work, when mixed with the binding solution, the highly reactive borate glass released magnesium ions which presumably reacted with the ammonium and phosphate ions in the solution to form the ammonium magnesium phosphate phase. When the scaffold was immersed in the SBF, the peaks corresponding to the binding phase disappeared within 7 days, and the amorphous diffraction pattern of the original glass shifted, giving rise to minor peaks corresponding to those of a standard HA (JCPDS 72-1243). The broad peaks with low intensity in XRD pattern (7 days) indicate that the as-formed HA was poorly crystallized or had a nanoscale size. Similar results were found in previous work, when borate glass was soaked in dilute potassium phosphate solution [9]. After the scaffolds were soaked for 40 days in the SBF, the formation of crystalline HA improved significantly, as revealed by the presence of several strong peaks of HA in the XRD pattern. This phase transformation from amorphous or poorly crystallized HA to crystalline HA proceeded without noticeable composition changes in the scaffold, as implied by the almost constant weight loss data after 7–10 days (Fig. 3).

SEM images showed that prior to immersion in the SBF (Fig. 6a), the borate glass particles in the scaffolds were bonded together, and had smooth surfaces typical of a glass. After immersion for 7 days, the binding phase presumably dissolved, and the particles had a rougher surface, presumably caused by degradation of the glass and conversion to a poorly crystallized HA (Fig. 6b). With longer increased immersion time (40 days), the scaffold consisted

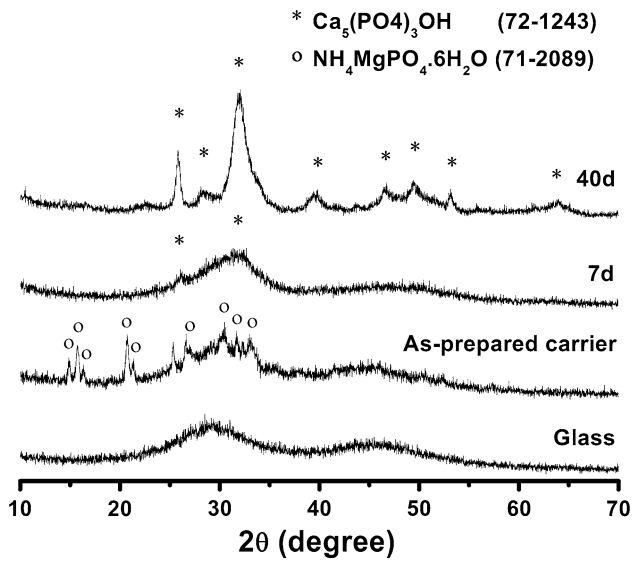

Fig. 5 XRD patterns of the starting borate glass powder, the as-prepared borate glass scaffold, and the borate glass scaffold after immersion for 7 and 40 days in a SBF

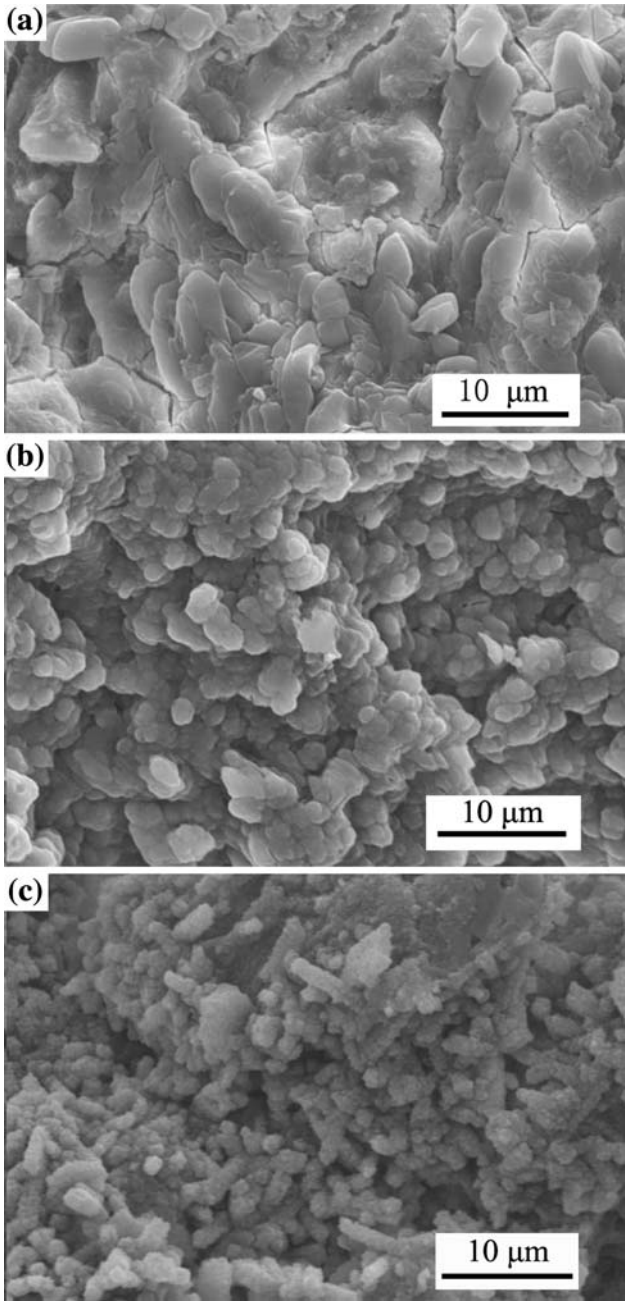

Fig. 6 Microstructure of the as-prepared scaffold (**a**), and the scaffold after immersion for 7 days (**b**) and 40 days (**c**) in a SBF

of fine rod-like particles (Fig. 6c), identified by XRD to be HA. The rod-like HA crystals were arranged randomly to form a porous structure, with pores of size equal to several microns. This in situ formed structure, consisting of porous HA, could serve as a scaffold for bone repair.

3.3 In vivo bone repair

Boron, which is widely present in small concentrations in water and food, is known to be important nutritionally for developing healthy bone in humans and animals [20]. The boron level in bone is found to be appreciably higher than in other tissues [21]. Boron can quickly distribute throughout the body fluid, and be excreted through urine. It has been reported that after a single intravenous dose, 99% of the boric acid was excreted unchanged in the urine over 120 h, while no tendency for boron to accumulate was observed [11]. Based on these findings, investigations into the potential application of borate glasses in bone repair and tissue engineering are warranted.

At sufficiently high concentrations, boron is toxic. The most sensitive end point of boron toxicity appears to be chronic toxicity, including the developmental and reproductive toxicity [21]. For rabbits, a daily boron dose of 22 mg per kg of rabbit body weight per day for 14 consecutive days was reported to cause no observed adverse effect of developmental toxicity [22], which indicates a wide safety margin for borate-based glass implantation. A single oral dose of boric acid causing acute toxicity for dogs, rabbits and cats is even higher, varying from 250 to 350 mg boron per kg of animal body weight [23]. As found in the present in vitro degradation results (Fig. 2), boron release from scaffolds into SBF showed a rapid increase at the very beginning of the immersion process (during the first day). However, degradation of the pellets and the boron release rate can be retarded by the HA-type layer formed on the surface of the glass. This indicates that the chronic and acute adverse effects caused by boron might be avoided by controlling the rapid release of boron during the first day to a concentration lower than that needed to cause the acute toxicity and maintaining the long-term boron release rate lower than that of the chronic toxicity threshold.

In the present study, the maximum amount of borate glass scaffold implanted into the rabbits is calculated to be ~430 mg of glass per kg of rabbit body weight. This is equivalent to a total boron concentration of ~75 mg per kg of rabbit body weight, which is far below the acute toxicity level (250–350 mg boron per kg of body weight), and is also insufficient to cause a daily boron intake of 22 mg per kg of body weight for more than 4 days. This indicates that both the acute and chronic toxicity of boron can be avoided with the borate glass implantation used in the present work.

The boron concentrations in blood and in urine are widely used as measures of boron exposure [24]. Figure 7 shows the boron concentrations in the serum from eight rabbits for times of 10–120 h post implantation. The trend of the concentration changes is consistent with the in vitro boron release (Fig. 2). The degradation of the scaffold began immediately after implantation, and after 10 h, the serum boron concentration reached its highest level (~7 μg/ml), indicating the most rapid degradation rate soon after implantation. The serum boron concentration decreased with time, indicating that the releasing rate of boron from the pellets had become slower. Unlike the case of a single dose of boron, the serum boron concentration at 120 h post implantation was still at a relatively high level, instead of dropping back to the value of the CTL group which was lower than the detectable value of 0.3 μg/ml. This was considered to be the result of the continuing degradation of the scaffolds in vivo.

The efficacy of the borate glass delivery system in treating osteomyelitis was evaluated by microbiological examination. After 8 weeks of surgery, 7 out of 11 rabbits in the CTL group and 2 out of 11 animals in VDC group exhibited MRSA infection. The negative rate of the VDC group was 81.8% for the VDC group, significantly higher than that for the CTL group (36.4%).

After treated with the VDC scaffolds for 8 weeks, no histological evidence of abscess formation, bone destruction, and inflammation were found in the of MRSA negative section, indicating the recovery from osteomyelitis. The section was decalcified before staining, so apatite in the bone or in the converted glass was completely removed. However, as shown in the H&E-stained section (Fig. 8), instead of showing a blank area, the area formerly occupied by the scaffold (denoted S) was stained bright

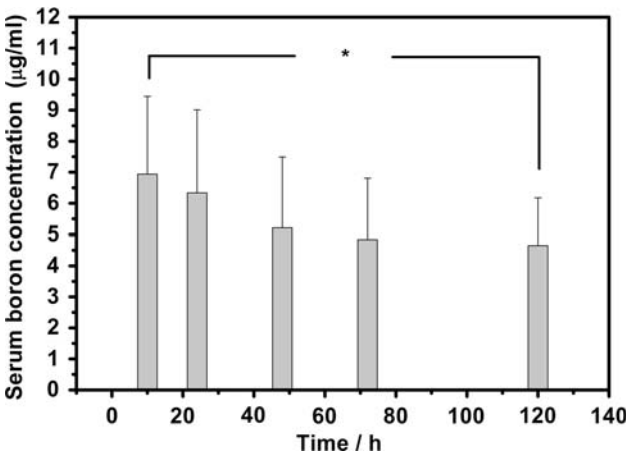

Fig. 7 Boron concentration in the serum for various times after implantation of borate glass scaffolds into the tibiae of rabbits. Mean ± SD; $n = 8$. * Significant decrease of boron content in serum ($P < 0.05$, one-way ANOVA)

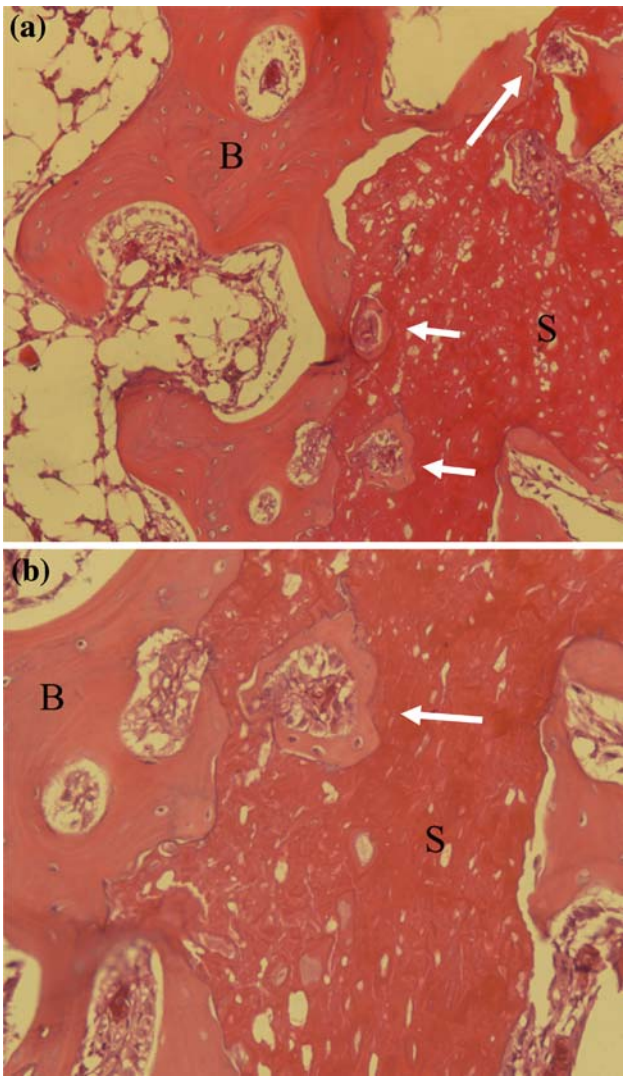

Fig. 8 Optical micrographs of tissue response around scaffolds at 8 weeks after implantion: **a** magnification ×100, **b** magnification ×200. S represents the area formerly occupied by the scaffold, and B represents bone

pink, which is the result of eosin stain. The eosinophilic structures are generally known to be composed of intracellular or extracellular protein. This presumably resulted from the ability of proteins to adsorb to nano-sized hydroxyapatite [25, 26]. Pores of size equal to tens of microns scattered within the area S, as shown in the section, are thought to be useful for mass transportation.

In Fig. 8a, new bone formation (denoted B) was intimately adjacent to the area S, indicating the ability HA scaffold and adsorbed proteins to stimulate bone formation. A larger magnification of the interface between the new bone (B) and the scaffold (S) (Fig. 8b) showed a well connected interface, without any transition area. Blood vessels, from the nearby bone (arrowed) were able to grow into the outer region of the scaffold and new bone was formed around the blood vessels. Presumably, osteoblastic cells, supported by the nutrients from the blood vessels, are able to gradually transform the surrounding protein/HA scaffold into new bone tissue.

Whereas in vitro studies have showed that high local boron concentration and pH value produced by borate glass scaffolds inhibit the proliferation of cells, the present in vivo investigation showed no tissue damage or inhibition of bone growth after implantation of borate glass scaffolds for 8 weeks. Despite the rapid degradation rate of the bioactive glass scaffolds used in the present work, no tissue damage or inhibition of bone growth was observed. This might be attributed to the fast metabolism of boron in vivo, as well the pH and ion buffer capacity of the body solution.

4 Conclusions

Particles of a borate glass, with a composition in the Na_2O–K_2O–MgO–CaO–B_2O_3–P_2O_5 system, were bonded into porous three dimensional scaffolds using an ammonium phosphate solution, for use as a delivery device for Vancomycin in the treatment of chronic bone infection. Immersion of the scaffolds in a simulated body fluid (SBF) resulted in rapid degradation of the borate glass, and the almost complete conversion to a poorly crystallized hydroxyapatite (HA) within 7 days which transformed to crystalline HA at longer immersion time. Vancomycin was almost completely released from scaffolds during the first 4 days of the degradation process in the SBF. Vancomycin-loaded scaffolds implanted for 8 weeks into the medullary cavity in the right tibiae of rabbits infected with osteomyelitis resulted in no observable tissue damage or inhibition of bone growth. Microbiological examination showed that 81.8% of the group treated with the Vancomycin-loaded scaffolds had negative MRSA. Blood vessels were found to grow into the outer region of the scaffolds and participate in the bone regeneration. The results indicate that bioactive borate glass is a promising biodegradable and bioactive material for use as a scaffold as well as local drug delivery device for treating bone infection.

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