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Bioactive borosilicate glass scaffolds: in vitro degradation and bioactivity behaviors

Xin Liu · Wenhai Huang · Hailuo Fu · Aihua Yao · Deping Wang · Haobo Pan · William W. Lu · Xinquan Jiang · Xiuli Zhang

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Abstract Bioactive borosilicate glass scaffolds with the pores of several hundred micrometers and a competent compressive strength were prepared through replication method. The *in vitro* degradation and bioactivity behaviors of the scaffolds have been investigated by immersing the scaffolds statically in diluted phosphate solution at 37°C, up to 360 h. To monitor the degradation progress of the scaffolds, the amount of leaching elements from the scaffolds were determined by ICP-AES. The XRD and SEM results reveal that, during the degradation of scaffolds, the borosilicate scaffolds converted to hydroxyapatite. The compressive strength of the scaffolds decreased during degradation, in the way that can be well predicted by the degradation products, or the leachates, from the scaffolds. MTT assay results demonstrate that the degradation products have little, if any, inhibition effect on the cell proliferation, when diluted to a certain concentration ([B] <2.690 and pH value at neutral level). The study shows that borosilicate glass scaffold could be a promising candidate for bone tissue engineering material.

X. Liu · W. Huang (✉) · H. Fu · A. Yao · D. Wang
Institute of Bioengineering and Information Technology Materials, Tongji University, Shanghai 200092, China
e-mail: whhuang@tongji.edu.cn; huangwe@mst.edu

H. Pan · W. W. Lu
Faculty of Medicine, University of Hong Kong, Hong Kong 999077, China

X. Jiang · X. Zhang
Shanghai Ninth People's Hospital, Shanghai 200011, China

1 Introduction

Bone tissue engineering provides an alternative way to repair a diseased or damaged tissue and to recover its original state and function. In the tissue engineering approach, a highly porous artificial material, or scaffold, is employed as a template, to facilitate the cell attachment, proliferation and differentiation. Therefore, the scaffolds should be biocompatible, biodegradable, coupled with three-dimensional porous structure, and mechanically strong strength [1] The famous 45S5 bioactive glass developed by Hench and his coworkers, has known for its specific biological response at the interface, which is the ability to form hydroxyapatite layer in vivo and to bond with the living bone [2]. Based on this concept, other materials, which are able to introduce the formation of hydroxyapatite, arouse great interest of researchers. These materials are employed to fabricate the scaffolds for bone tissue engineering [3–5]. Interestingly, most of the bioactive glass materials are based on silicate [6]. However, despite of their excellent bioactivity, the silicate-based bioactive glasses show a relatively low biodegradability [7].

Recent studies discovered that, by employing the reactive borate (BO_3^{3-}) in the glass network, the borate-based glass provides an approach to achieve the requirements of both degradation and bioactivity for tissue engineering [8–10]. Compared with silicate-based glass, the bioactive borate-based glass shows a fast degradation behavior and yields a more rapid hydroxyapatite conversion rate in the simulated body fluid [11–13]. Besides, its degradation rate can be controlled by adjusting boron content in the glass [8].

Previous studies [14] have successfully fabricated porous scaffolds for bone tissue engineering by using the borate-based glass named as D-Alk-B glass (Double alkali borate glass) which is selected from the Na_2O–K_2O–MgO–

$CaO–SiO_2–P_2O_5–B_2O_3$ system. The study reported that the D-Alk-B glass exhibits an excellent thermal workability, and is able to be fabricated into scaffolds with desired interconnected structure through the replication method. This structure has high porosity and large pore size coupled with a competent compressive strength [14]. Therefore, this macroporous bioactive borosilicate glass scaffold has potential to be used as bone tissue engineering material.

The degradation behavior of porous scaffolds plays an important role in the process of a new tissue growth. After the implantation of the scaffold in body, the degradation of porous scaffolds affects cell vitality, cell growth, and even host response [15] and the concomitant mechanical change should match the tissue growth [1]. Thus, before further researches on the borate glass scaffold, the understanding of the scaffolds degradation behavior in vitro should be necessary. For these aims above, the in vitro degradation behavior of the D-Alk-B glass scaffolds has been studied in the present work. The degradation behavior, degradation mechanism, as well as the concomitant bioactivity of the D-Alk-B scaffolds has been discussed. The mechanical strength change of the scaffold during the degradation process and the effect of degradation products on cell proliferation are also examined.

2 Experimental procedure

2.1 Synthesis

A melt-derived bioactive borosilicate glass with the composition of $6Na_2O–8K_2O–8MgO–22CaO–36B_2O_3–18SiO_2–2P_2O_5$ mol%, designated as D-Alk-B glass, was prepared by heating corresponding chemicals into glass melt for 2 h at 1,150°C and by quenching the melt between cold stainless-steel plates into glass frits. The glass frits were crushed into fine particles, the size of which was in the range of 1–10 μm.

The macroporous borosilicate glass-based scaffolds, or D-Alk-B glass scaffolds, were fabricated by the replication method. Commercial polyurethane foams (Shanghai No.6 Plastic Co., Ltd, China) with the open porosity of approximately 50 pores per inch were employed as the sacrificial templates. The slurry for impregnation was prepared by vigorously stirring the mixture of glass powders, ethanol solution and ethyl-cellulose at the ratio of 66.1, 31.2 and 2.6 wt%, respectively. The foams were fully immersed in the slurry, in order to be coated with a layer of glass powders. The coated foams were dried at room temperature and then underwent a programmed heat treating process to obtain the porous scaffolds. The details for fabricating the D-Alk-B glass scaffolds have been reported where else [14].

2.2 Characterizations

2.2.1 Degradation

The assessment of in vitro degradation of D-Alk-B glass scaffold was carried out by immersing statically the glass scaffolds (1 g) in 0.02 M K_2HPO_4 solution (100 ml) with the starting pH value of 7.0 at 37°C. The dilute phosphate solution employed in the experiment was described in detail by Huang et al. [16]. After the scaffold being immersed for 3, 24, 168 and 360 h, the pH value of the immersion solution was measured at 37°C, and the scaffold samples were removed from the immersion solution and dried at 90°C for 24 h respectively. The element concentrations in the immersion solution at different time were analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Optima 2100DV, USA). The compressive strengths of the removed scaffolds were then measured respectively by a tensile testing machine (CMT6104; SANS Testing Machine Inc. China) at a crosshead speed of 0.5 mm/min. The average value was taken from three parallel experiment samples.

2.2.2 Bioactivity

The microstructure of the scaffolds before and after immersing for 360 h in K_2HPO_4 solution was observed using scanning electron microscopy (SEM: Quanta 200 FEG; FEI Company, USA). The phases of powder sample from the scaffolds before and after immersion were determined by X-ray diffraction (XRD; D/max2550; Rigaku International Corp. USA).

2.2.3 MTT assay

The effect of the degradation products of D-Alk-B glass scaffolds on the cell proliferation was assessed by the colorimetric quantification of MTT (Methyl thiazolyl tetrazolium) assay using goat bone marrow stroma cells (BMSCs) from adult goat.

BMSCs were isolated by density gradient centrifugation from iliac bone marrow aspirates of adult male goats. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 IU/ml penicillin (Sigma, USA), 100 μg/ml streptomycin (Sigma,USA) and cultured at 37°C, 5% CO_2 and 95% humidity. After 5 days, the non-adherent cell fraction was removed and the culture medium was replaced with the DMEM, supplemented by 10% FBS (Hyclone, USA), 50 mg/l antiscorbic acid (Hyclone, USA), 10^{-8} mol/l dexamethasone (Sigma, USA), 2.16 g/l β-sodium glycerophosphate (Sigma, USA), 300 mg/l sodium bicarbonate

(Sigma, USA), 100 μg/ml streptomycin (Sigma,USA) and 100 IU/ml penicillin (Sigma, USA). This procedure was carried out every 3 or 4 days. After 80% confluent, adherent cells were trypsinized (0.25% trypsin). The cultured BMSCs passage 2 were collected and resuspended in the culture medium at 2.5×10^4 cells/ml plated (200 μl/well) into 96-well plates (Corning, USA).

The solution with the degradation products of D-Alk-B scaffolds were prepared by putting 2 g D-Alk-B glass scaffolds at 10 ml DMEM solution at 37°C for 24 h with constant shaking. The extract solution was then filtrated (0.22 μm pore size) to remove the possible solid particles of the scaffolds, and diluted with the culture medium, leaving the dilutions with the initial extract solution of 100%, 50%, 25% and 12.5% volume percentage respectively. The hydroxybenzene (64 g/l) was used as the positive control, while the culture medium without any extract solution was used as the negative control. The boron concentrations of the dilutions were determined by ICP-AES, and the pH values were also measured.

The plates with the cells were incubated at 37°C in humidified atmosphere of 5% CO_2 in air for 24 h. After the incubation, the medium in the well was replaced by the prepared dilutions and the control solutions respectively. At the time point of 1, 3, 6 and 9 d, the cell culture was treated with 20 μl/well of MTT solution at 5 mg/ml and incubated for 4 h at 37°C in humidified atmosphere of 5% of CO_2 in air. The dehydrogenase enzymes secreted from the mitochondria of metabolically active cells reduced the MTT and form non-water-soluble purple formazan crystals within the cells. The crystals amount indicates the number of living cells. The MTT solution was removed and 200 μl dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The absorbance measurements of the solution were performed on a microplate reader (Elx800, Bio-Tek, USA) at 490 nm. The optical density (OD) from the reader represents the number of viable cells.

3 Results and discussion

3.1 Degradation

During the immersion in phosphate solution, the D-Alk-B glass scaffolds were attacked by the surrounding environment, and the elements were leached from the scaffolds. As a result, the scaffolds gradually degraded themselves, causing ion changes in the soaking solution. Weight loss was widely used to monitor the degradation of soaking samples [17, 18]. However, in this experiment, some precipitate might form on the scaffolds and disturb the weight loss measurement. Thus, the ion changes in the solution would be more sensitive to detect the degradation. The concentrations of the glass modifier Na, K, Ca and Mg, as well as the major glass network former B and Si in the solution at different immersing time points are determined by ICP. The accumulated amount of these elements released from the 1 g glass scaffold can be then calculated from the measurement, according to the immersion ratio of 1 g per 100 ml K_2HPO_4 solution (0.02 M). The consumption of P for 1 g scaffold in the solution was determined from the decrease of P concentration also detected by ICP. The results were shown in Fig. 1. All glass elements show an increase with the soaking time, except P, indicating the degradation progress of the scaffolds. The hydrolysis of the glass leads to the corrosion of the borate and silicate network, and makes the progressive increase of leaching boron and silicon. The increasing alkalis (Na and K) released from the glass cause the increase of pH value of solution, due to the inter-diffusion of H^+ and alkalis. It is noticeable that, although the curves of pH value and the element release of K, Na as well as B show a constant increase, the increasing rates are gradually alleviated with time, especially after the first 24 h. This means the rate of the degradation process is slowing down.

The leaching of elements from the glass, which was traditionally considered as the corrosion of glass, was regarded as the degradation property of the bioactive borosilicate glass in present study. At high boron oxide content, the corrosion of borosilicate glass is mainly controlled by the boron content [19]. In the case of D-Alk-B glass, the proportion of boron p_B in the covalent network of D-Alk-B glass is as high as 0.8, calculated from the glass composition as:

$$p_B = \frac{2[B_2O_3]}{[SiO_2] + 2[B_2O_3]}, \quad (1)$$

where $[B_2O_3]$ and $[SiO_2]$ are the molar contents of B_2O_3 and SiO_2 in the glass composition respectively. Therefore,

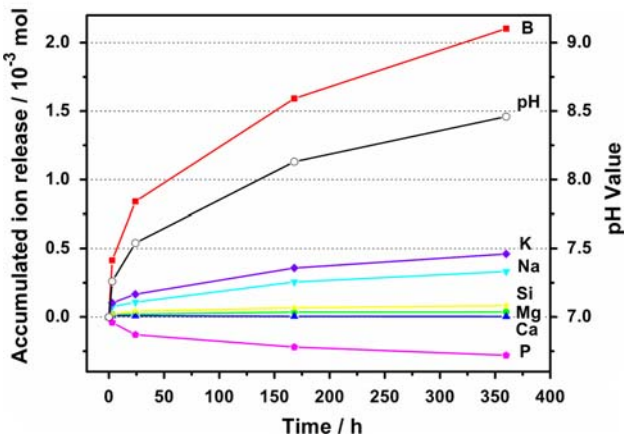

Fig. 1 Accumulative amount of different elements leaching from 1 g glass scaffolds and the pH value of the corresponding immersing solution

Table 1 Percentages of the leaching elements of the scaffolds to the total element amounts in the scaffolds

Elements	B	Na	K
Total amount in 1 g D-Alk-B scaffolds (10^{-3} mol)	11.06	1.84	2.46
Percentage of the leaching amount of the elements in scaffold (%) after 360 h immersion	19.01	18.01	18.73

for D-Alk-B glass scaffolds, the degradation behavior is mainly controlled by the breakage of B-containing bonds within the glass. Figure 1 indicates that, compared with silicon, boron shows a relatively obvious increase, due to the facile breakage of borate network and the easy diffusion of borate. As shown in Table 1, after 360 h immersion, about 20% of the boron in the glass network was released from the scaffolds, so were the elements of Na and K. The fact that the ions release easily from glass indicates that the facile breakage of the borate network is the major contributor to the relatively fast degradation property of D-Alk-B scaffolds.

When the borate network in the glass was broken, the network modifiers can be released from the broken boron network. As shown in Fig. 2, both the amount of K and Na released from glass show a well linear relationship with the boron amount in the solution. The alkaline earth elements (Mg and Ca) are also supposed to be released from the broken boron network. However, despite their higher molar portions in the D-Alk-B glass composition than the alkalis, their amounts in the immersion solution change slightly, keeping at a relative low level. The amount of magnesium is $<0.2 \times 10^{-3}$ mol and that of calcium is even lower, $<0.01 \times 10^{-3}$ mol. It is known that both the magnesium and calcium phosphate salt are insoluble in water. As a result, instead of easily diffusing to the soaking solution, the magnesium and calcium ions were captured by the phosphate ions in the soaking solution, and formed precipitate on the surface of the scaffolds. The consumption of the P in Fig. 1 is consistent with the precipitate formation.

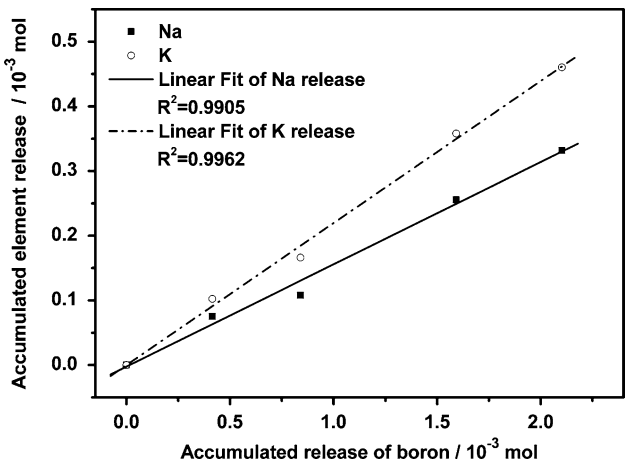

Fig. 2 The relationship between the amount of alkalis and the boron in the immersion solution

3.2 Bioactivity

The formed precipitation was analyzed by SEM and XRD methods. Figure 3a and b shows the microstructure of scaffolds, before and after immersion respectively. The as-received D-Alk-B glass scaffold exhibits a three-dimensional interconnection with the pore size of around 200 $\sim$ 500 μm, and the surface of the scaffold appears smooth on the whole. After being immersed for 360 h, the macroporous structure of the scaffold remained, however, the surface of the scaffold lost the glass smoothness. The large scale image, shown in Fig. 3c, indicates that nano-scale particles were nucleated on the surface of the scaffold. The SEM-EDXS analysis reveals the presence of Ca, Mg, P, C and O, as shown in the inlet of Fig. 3c. Carbon was absorbed from the air and might substitute some lattices in the hydroxyapatite crystal, which is supposed to be closer to the structure of bone [20]. The XRD patterns, shown in Fig. 4, exhibit several peaks growing from the original amorphous spectrum, corresponding to those of hydroxyapatite. Therefore, the degradation of the D-Alk-B scaffolds leads to the formation of hydroxyapatite, which is considered to be responsible for bone bonding and a crucial criterion to judge the bioactivity of biomaterials.

3.3 Mechanical strength change during degradation

The mechanical property is one of the requirements of an ideal scaffold for tissue engineering and the mechanical strength change during the degradation should match the tissue growth [1]. Thus, the study on the strength change of the D-Alk-B scaffold during the immersion is necessary. The basic load-bearing units of the D-Alk-B scaffolds are glass struts and walls [14]. Figure 5 shows the schematic change of the cross-section of the basic units in the scaffolds during the immersion. With the progress of the reaction between the scaffolds and phosphate solution, the glass gradually converted to hydroxyapatite. The loosely precipitated hydroxyapatite layer on the scaffold surface

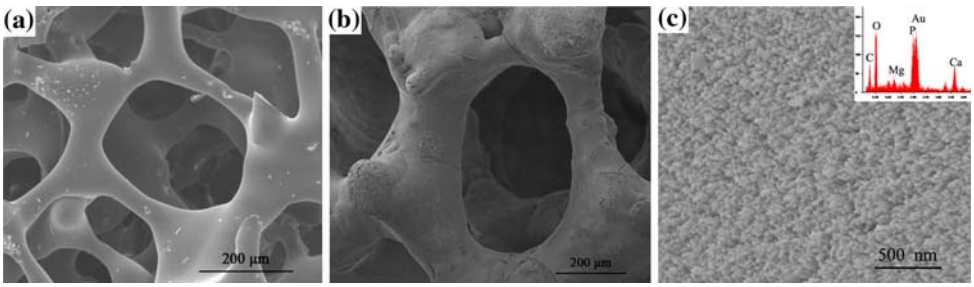

Fig. 3 SEM images of the D-Alk-B glass scaffold before **a** and after **b** immersion and **c** the surface product and the corresponding EDXS analysis

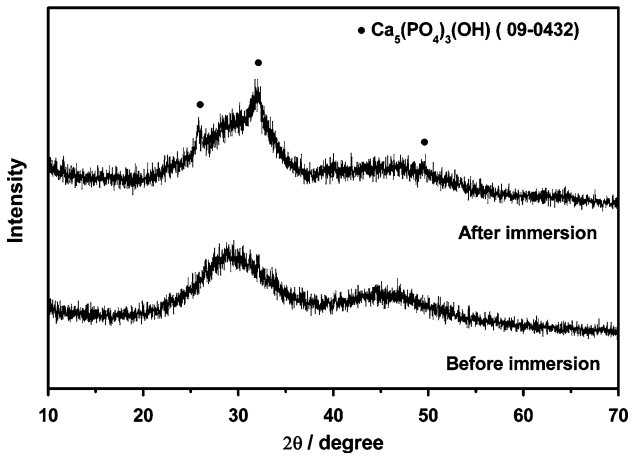

Fig. 4 XRD results of the scaffolds before and after immersion

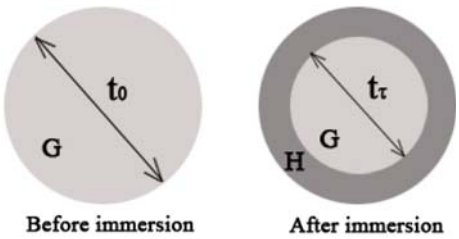

Fig. 5 Schematic of the glass thickness change after immersion, where G represents the glass and H represents the hydroxyapatite layer

shows no obvious strength, compared with the unconverted glass. Therefore, the scaffold strength will decrease with the degradation progress of scaffolds.

The degradation ratio of scaffold at τ time was defined as the unconverted glass percentage of the scaffold, designated as D_τ. As shown in Fig. 5, the degradation ratio D_τ quantitatively reveals the thickness change of the load-bearing units, given by

$$D_\tau = \frac{S_\tau}{S_0} = \frac{\pi(t_\tau/2)^2}{\pi(t_0/2)^2} = \frac{t_\tau^2}{t_0^2}, \tag{2}$$

where S_0 and S_τ were the areas of the cross-section of the strut before and after immersion for τ time, t_0 is the initial thickness and t_τ is the thickness after immersing for τ time.

The degradation ratio D_τ can be calculated from the degradation products, the ions leached from scaffolds, in the soaking solution. Since the boron network is the main structure of the D-Alk-B glass and boron can easily diffuse to the solution, it is reasonable to assume that the accumulated boron amount in the solution has a linear relation with the degradation progress of scaffolds, leading to

$$D_\tau = 1 - \frac{M_\tau}{M_B}, \tag{3}$$

where M_τ is the accumulative mol amount of the element boron leaching from 1 g glass scaffolds at the immersion time point τ, and M_B is the total mol amount of the element boron in 1 g glass scaffolds. Replacing the D_τ in Eq. 2 with that in Eq. 3, the change of load-bearing units can be then estimated by the boron concentration in the solution, as followed:

$$\frac{t_\tau}{t_0} = \left(1 - \frac{M_\tau}{M_B}\right)^{1/2}. \tag{4}$$

The compressive strength has approximately an exponential dependence on the thickness of load-bearing units [14]. Therefore, if the original compressive strength of scaffolds σ_{tc_o} is known, the compressive strength of the scaffolds after immersing for τ time, designated as σ_{tc_τ}, can be then predicted by

$$\sigma_{tc\_\tau} = \sigma_{tc\_o}\left(1 - \frac{M_\tau}{M_B}\right)^n, \tag{5}$$

where n is a constant. In the present study, it is found that when $n = 3$, the theoretical strengths calculated from Eq. 5 are close to the experimental ones. As shown in Fig. 6, the original experimental compressive strength of scaffolds before immersion is about 6.2 MPa, which is larger than that of the reported scaffolds based on silicate glass [21]. The degradation of scaffolds brings a significant drop of scaffold strength. By using the leaching boron concentration, the decreased strengths during degradation can be well predicted on the whole except a minor deviation of the one at 360 h. This might be caused by certain absorption of the boron by the formed porous hydroxyapatite layer, thus led a lower leaching boron concentration measured.

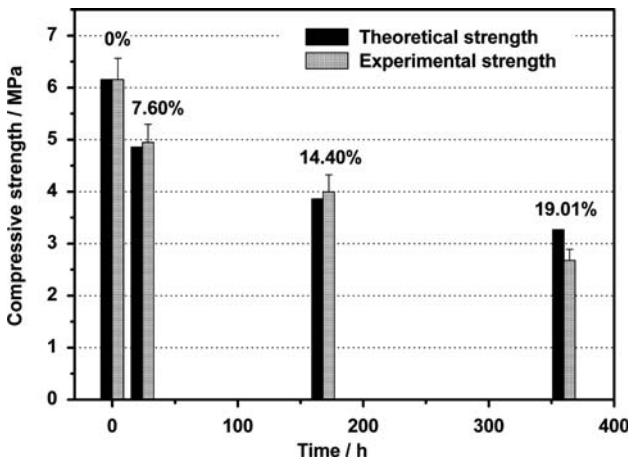

Fig. 6 Experimental compressive strengths of scaffolds immersed for different time duration and the corresponding calculated values. Inside numbers are the percentage of the release amount of boron to the total one, also known as M_τ/M_B

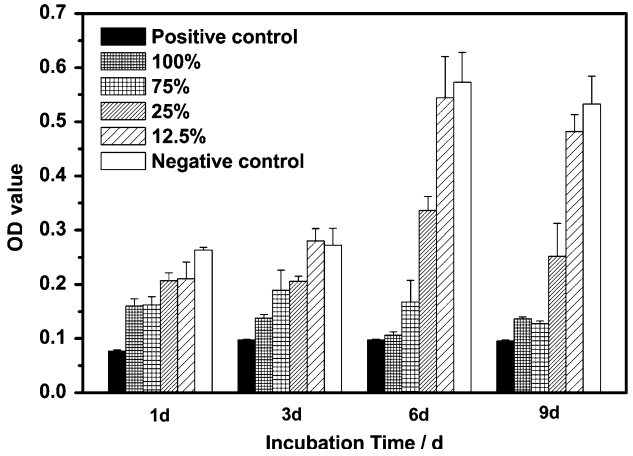

Fig. 7 Cell growths after 1d, 3d, 6d and 9d with solutions containing different percentages of initial extract solution, 100%, 50%, 25% and 12.5% respectively, compared with positive and negative controls. Error bars represent means ± SD ($n = 3$)

3.4 Cytocompatibility of degradation products

The influence of the leaching ions on the cell growth is evaluated by MTT assay. The corresponding boron concentration and the pH value of the dilutions are listed in Table 2. The OD value provides an indication of cell growth and proliferation under different concentrations of leachates. As shown from the OD value in Fig. 7, the 100% initial extract solution shows minor better cell viability than the positive control on the whole (student's *t*-test $P < 0.05$ for 1d, 3d and 9d). However, the value is significant lower than that of the negative control, indicates certain inhibition of boron and alkalic solution on the BMSCs cell proliferation. With the increased dilution ratios, boron concentrations decreased and the alkalic pH values lowered, which lead to an alleviated inhibition effect on cell proliferation. As shown in Fig. 7, from 3d to 6d, both of the 25% and 12.5% dilutions shows an increase of viable cells ($P < 0.05$). Moreover, the OD value of the 12.5% dilution, which has a boron concentration of 2.960 mM and a nearly neutral pH value, shows no significant statistically difference to the negative control ($P > 0.05$). Therefore, degradation products of D-Alk-B scaffold under certain concentration have little, if any, inhibition effect on BMSCs cell growth. As discussed above, the leaching rate of ions can be alleviated during the immersion, owing much to the formed hydroxyapatite layer which retards the ion diffusion. It could be expected that the scaffold might have some inhibition effect on cell growth at the very beginning of the degradation progress, however, as the degradation progress continues, the inhibition effect will be alleviated and finally disappear. More experiments on the cytocomparibility of the scaffolds are going on.

Table 2 Boron concentration and pH value of different dilutions

Percentage of the initial extract solution	100%	50%	25%	12.5%
Boron concentration/mM	23.680	11.839	5.920	2.960
pH value	9.12	8.53	8.04	7.76

4 Conclusions

Macroporous bioactive borosilicate glass scaffolds with competent mechanical strength were synthesized by replication method, using the glass from Na_2O–K_2O–MgO–CaO–SiO_2–P_2O_5–B_2O_3 system. The mechanism for degradation and bioactivity behavior of the scaffolds were assessed by immersing the scaffolds in dilute phosphate solution. The degradation of borosilicate glass with high boron content is contributed to the breakage of the boron network. Network modifier ions, like Na, K, Mg and Ca, were leaching from the scaffold during the degradation. The leaching calcium ions were captured by phosphate ions and thus led to the formation of hydroxyapatite precipitate on the surface of the scaffolds, which is considered as the signal of in vitro bioactivity. By this way, the degradation behavior leads to the bioactivity of the scaffolds. With the progress of degradation, the compressive strength of the scaffolds decreased, in a way that can be well predicted by the degradation products. MTT results indicate that the degradation products of certain concentration have certain inhabitation effect on the cell growth. However, when the extracts are diluted to neutral pH value with the boron concentration <2.960 mM, no inhabitation or even certain stimulation effect of the cell proliferation appears. It is

reasonable to expect that the acceptable or even favorable alkali and boron concentrations for the cell growth can be obtained through the alleviation effect of the formed hydroxyapatite layer.

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