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An ionically crosslinked hydrogel containing vancomycin coating on a porous scaffold for drug delivery and cell culture

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Abstract

The aim of this study was to prepare and characterize a scaffold with an ionically crosslinked hydrogel coating layer containing a water-soluble drug, vancomycin, via a novel drug loading method for sustained drug delivery and surface modification. The poly(D,L-lactide acid) (PDLLA)/biphasic calcium phosphate (BCP) scaffold with a highly inter-connected porous structure was fabricated by a particle-leaching/thermally induced phase separation (TIPS) method. The pre-vacuumized scaffold was immersed into an alginate/vancomycin solution. Following impregnation by the solution, the scaffold was removed and immersed in a $CaCl_2$ solution for 30 min to allow gelation of the alginate solution. In this way, the drug was not exposed to organic solvents or detrimental temperature conditions and it could avoid loss of drug during the leaching process. The water contact angles of the scaffold surface decreased after being coated with the hydrogel. The *in vitro* drug release profile showed sustained release properties which were influenced by the alginate concentration and the dissolution medium. A standardized bacterial assay showed that the drug was still active after association with the scaffold by this gentle method of drug loading. The *in vitro* osteoblast culture experiments confirmed the biocompatibility of the scaffold for attachment and proliferation of osteoblasts.

Keywords: Biodegradable polymers; Porous scaffold; Alginate hydrogel; Coating; Controlled release; Cell culture

1. Introduction

Scaffolds are designed to regenerate natural bone tissues or to create biological substitutes for defective bone tissues through the use of cells. The success of these approaches is largely dependent on the scaffold properties such as degradability, biocompatibility, highly inter-connected porosity and mechanical integrity (Tabata, 2000; Malafaya et al., 2002). PDLLA polymers have been widely used to fabricate scaffolds due to their biocompatibility and biodegradability (Heidemann et al., 2001; Hasegawa et al., 2005). The relatively low mechanical properties of the pure polymer scaffold has led to the incorporation of bioceramics such as hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) and biphasic calcium phosphate (BCP) to the polymer scaffolds to improve their mechanical properties (Shikinami and Okuno, 1999; Ignjatović et al., 1999; Sun et al., 2004).

In addition, the biodegradable scaffold is also required to have the ability to introduce growth factors to benefit the ingrowth of osteoblasts or to release drugs in a predetermined manner at a specific site of action, e.g. controlled release of antibiotics at a local site to eliminate infection accompanied by surgery (Whang et al., 2000; Gautier et al., 2001). In order to incorporate drugs (antibiotics, anti-inflammatory agents, growth factors, etc.) into the scaffold and obtain a slower sustained drug release rate, drugs were often entrapped in the scaffold by mixing them with the polymer matrix directly before the scaffold was formed (Sheridan et al., 2000; Murphy et al., 2000; Yoon et al., 2003). However, this method is not suitable for unstable drugs due to the high temperatures employed and exposure to organic solvents during the scaffold fabricating process. The technology of particle-leaching may be used for the preparation of scaffolds with larger pore sizes and relative high mechanical properties.

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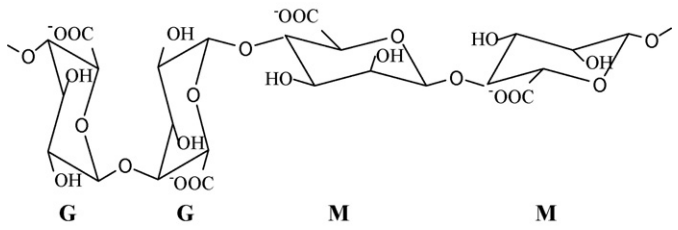

Fig. 1. Structure of alginate.

However, if the drugs are water-soluble then the method of mixing the drug with the polymer before scaffold formation is further not suitable since a premature loss of the water-soluble drug will occur during the leaching process (Sohier et al., 2003). Recently, there has been increasing interest in the coating of prefabricated scaffolds with a drug loaded polymeric film (Kim et al., 2004a,b; Ma et al., 2005). The coating layer is often designed to entrap the drug efficiently as well as to modify the scaffold surface (Young et al., 2005). Among coating polymers, the water-soluble polymers are more adaptive because many growth factors and antibiotics are hydrophilic molecules and the degradation of the drug by exposure to organic solvents can be avoided. Additionally, the wettability of the polymer scaffold surface can be improved with the hydrophilic coatings. However, water-soluble polymers lead to rapid drug release rates following polymer dissolution under aqueous conditions. In order to obtain a sustained drug release rate and maintain drug activity, the authors in this study propose the use of an ionically crosslinked hydrogel as a coating layer for entrapping a water-soluble drug, as well as for surface modification.

Hydrogels are being increasingly used as a drug and cell carrier because they are highly hydrated three-dimensional net works of polymers (Stendahl et al., 2004; Tada et al., 2005; Fujita et al., 2005; Lévesque et al., 2005). The drug entrapped in the hydrogel can be released in a sustained manner by the slow diffusion process. As cell carriers, hydrogels are structurally similar to the extra-cellular matrix of many tissues (Drury and Mooney, 2003). However, due to its low mechanical properties, the hydrogel could not be used on its own as a scaffold (Hoffmann, 2002).

Alginate is a linear polysaccharide copolymer of (1–4)-linked β-D-mannuronic acid (M) and α-D-guluronic acid (G) monomers (Fig. 1), and is derived primarily from brown seaweed and bacteria. Gels are formed when divalent cations such as Ca^{2+}, Ba^{2+}, or Sr^{2+} cooperatively interact with blocks of G monomers to form ionic bridges between different polymer chains (Tada et al., 2005). It is being widely used in tissue engineering (Cohen et al., 2003; Stevens et al., 2004) and novel drug delivery systems because of its biocompatibility and excellent gelation ability under gentle conditions. The advantages of alginate hydrogel as tissue engineering matrices include the follow properties: (a) the aqueous environment can protect cells and fragile drugs; (b) good transport of nutrient to cells and products from cells; (c) can be easily modified with cell adhesion legends. For examples, alginate hydrogel mixed with cells can be used as injectable hydrogel for cartilage reparation (Bent et al., 2001; Stevens et al., 2004); alginate beads was widely used to encapsule cells as cell carriers (Ma et al., 2003; Murtas et al., 2005; Mehlhorn et al., 2006). In addition, alginate has been widely used as drug carriers in drug delivery system (Liu et al., 2007; Sriamornsak et al., 2007; Ishak et al., 2007). However, due to its low mechanical properties, the alginate hydrogel also could not be used on its own as a bone scaffold.

Vancomycin, a water-soluble antibiotic drug, is commonly used for treating osteomyelitis and preventing osseous staphylococcal infections after surgery (Gautier et al., 2001). It was used as a model drug in this study.

To the best of the authors' knowledge, a PDLLA/BCP scaffold with an ionically crosslinked alginate hydrogel coating that incorporates a drug loading method that overcomes the limitations of premixing with the polymer prior to scaffold formation has not been reported previously for sustained release and cell culture potential. In this work, PDLLA/BCP scaffolds with highly inter-connected pores were fabricated using a method that combined particle-leaching and TIPS. This scaffold was subsequently coated with an alginate hydrogel containing vancomycin. The alginate hydrogel coated scaffolds were characterized in terms of morphology, porosity, and surface wettability. The effects of alginate concentration and type of dissolution medium on the *in vitro* drug release properties of the scaffold were also investigated. The microbiological activity of the vancomycin entrapped in the scaffold was additionally evaluated by a standardized bacterial assay. A fluorescent staining observation and MTT assay was further used to measure the proliferation of the osteoblasts cultured *in vitro*.

2. Experimental

2.1. Materials

Poly-D,L-lactide (M_η = 130 kDa) and Biphasic calcium phosphate (BCP) with particle size of 3–4 μm were obtained from Sichuan Dikang Sci&Tech Pharmaceutical Co. Ltd. (Chengdu, China). Sodium Alginate (M_w = 240 kDa,) was purchased from Chang Zheng (China). Dilute alginate solutions were dialyzed against pure water for several days to remove salt and other low-molecular weight impurities, and were thereafter freeze-dried. Vancomycin Hydrochloride was purchased from Eli Lilly (Japan). Mueller Hinton Agar and the sensitive bacteria *(Staphylococcus aureus ATCC 6538*) were obtained from The West China College of Medicine, Sichuan University (China). Dulbecco's modified Eagle's medium (DMEM) and other culture media and supplements were obtained from Invitrogen (Paisley, UK). All other regents were analytical grade and used without further purification.

2.2. Fabrication of the PDLLA/BCP porous scaffold

The PDLLA/BCP scaffold was fabricated by particle-leaching combined with a TIPS method. Briefly, BCP powder (0.5 g) was dispersed in a 1,4-dioxane (15 mL) and distilled water (1,4-dioxane/distilled water = 87/13, v/v) composite sol-

Table 1
Formulation composition of the various alginate/vancomycin coating solutions with different alginate concentrations (A1: 0.5%; A2: 1%; A3: 2%) for the PDLLA/BCP scaffold (S1)

Sample	Concentrations (w/v, %)	
	Alginate	Vancomycin
S1A1	0.5	0.8
S1A2	1	0.8
S1A3	2	0.8

vent under stirring. PDLLA (PDLLA/BCP = 9/1, w/w) (4.5 g) was then added into this suspension. By maintaining the mixtures for 30 min at 40 °C, an elastic gel was produced. The elastic polymer-BCP gel was mixed homogeneously with sieved NH_4HCO_3 salt particles (salt particle size: 300–400 μm; weight ratio of salt/PDLLA and BCP: 5/1). The mixture of polymer/BCP/salt was cast into a disk-shaped glass mould (9 mm in diameter and 5 mm in thickness), cooled rapidly in a −30 °C refrigerator and then freeze-dried using a freeze-dryer (FD-1, BIOKING). After 48 h, the solid samples were removed from the freeze-dryer and immersed in distilled water for 24 h to remove the salt by leaching. The distilled water was refreshed at 4 hourly intervals. The samples were then placed in a vacuum-dryer for 48 h at 35 °C to remove any of the remaining solvent and NH_4HCO_3 salt ($NH_4HCO_3 \rightarrow NH_3\uparrow + CO_2\uparrow + H_2O$). In this way, PDLLA/BCP scaffolds (S1) with a well-developed porous structure were prepared.

The PDLLA scaffolds without BCP particles (S2) were prepared for comparison of their mechanical properties. In addition, PDLLA/BCP scaffolds (S3) were also prepared via the above method, while using only 1,4-dioxane as solvent (scaffolds S2 were prepared using the 1,4-dioxane/distilled water (87/13, v/v) composite solvent) for a comparison of the microstructures.

2.3. Fabrication of alginate hydrogel/vancomycin coated PDLLA/BCP scaffolds

Composite solutions for coating of the scaffolds were prepared from sodium alginate and the antibiotic drug vancomycin. Various quantities (0.5, 1 and 2%, w/v) of sodium alginate were dissolved in distilled water by stirring for 3 h at room temperature. A fixed concentration (0.8%, w/v) of antibiotic drug (vancomycin) were added to the solution and stirred for 1 h.

The composite solutions were incorporated onto the PDLLA/BCP porous scaffolds by applying a vacuum. Briefly, the scaffold was placed in a glass vial; a vacuum applied and thereafter the composite solution was injected into the vial. The scaffolds were kept immersed in the composite solution for 3 h, then removed and immersed in 50 mM $CaCl_2$ solution for 1 h to allow gelation of the alginate coating. Finally, the scaffolds were removed, rinsed with distilled water to eliminate the $CaCl_2$ solution on the scaffold surface, and then dried under vacuum for 24 h. The formulation composition and designation of the various alginate hydrogel coatings are summarized in Table 1.

2.4. Characterization of the scaffold

2.4.1. SEM analysis

The porous scaffolds before and after coating with various alginate/vancomycin hydrogel formulations were dried under vacuum. The scaffolds were cut into circular slices (9 mm in diameter and 1 mm in thickness); thereafter slices from different parts of the scaffold were coated with gold and their morphologies examined by Scanning Electron Microscopy (SEM) (JSM-5900LV, Japan). The pore size was calculated from pictures taken from SEM by selecting five arbitrary areas.

2.4.2. Mechanical properties

The porous PDLLA/BCP scaffold (S1) and the PDLLA scaffold (S2) were subjected to a compression test using an electromechanical universal testing machine (SANSCMT4503, China) at a crosshead speed of 2 mm/min. The compressive offset yield stress was determined from the stress–strain curve at a 2% strain. The compressive modulus was determined from the slope at the initial stage. Five replicate determinations were undertaken for each formulation.

2.4.3. Open porosity

The open porosity can be calculated by the liquid displacement method (Karageorgiou and Kaplan, 2005). The scaffold is submerged in a known volume (V_1) of ethanol that is not a solvent for the scaffold and a series of brief evacuation repressurization cycles is conducted to force the liquid into the pores of the scaffold. After these cycles the volume of the liquid and liquid-impregnated scaffold is V_2. When the liquid-impregnated scaffold is removed, the remaining liquid volume is V_3 and open porosity is given as:

$$\pi = \frac{V_1 - V_3}{V_2 - V_3} \quad (1)$$

The above test was performed in triplicate for each kind of scaffold fabricated in this study.

2.4.4. Apparent water contact angle measurements

The apparent water-in-air contact angles of the PDLLA/BCP scaffold and the alginate hydrogel coated scaffolds were measured at room temperature using the sessile drop method by a KRVSS DSA 100 goniometer within 10 s after water dropping. Also, the water contact angles of the PDLLA/BCP films and calcium alginate films were measured by this method. Briefly, the PDLLA/BCP (9/1, w/w) composite solution (4%, w/v) were poured in to a glass Petri dish to form a PDLLA/BCP film with thickness of ∼200 μm. The calcium alginate films were fabricated by drying the alginate hydrogel under vacuum. Three samples were tested for each type of scaffold and four independent measurements at different sites of each sample were averaged.

2.4.5. In vitro release study

The alginate hydrogel/vancomycin coated scaffolds were placed in glass vials containing 5 mL of either distilled water, 1×10^{-3} mol/L of phosphate buffered saline (PBS, pH 7.4) or

1×10^{-2} mol/L of phosphate buffered saline (PBS, pH 7.4). The samples were incubated in a 37 °C incubator for up to 3 weeks under static conditions (Yoon et al., 2003). At preset time intervals, the dissolution samples (5 mL) were collected and replaced with fresh dissolution medium. The absorbance of the filtrate was determined by using an UV spectrophotometer (Shimadzu UV2450, Japan) at a wavelength of 280 nm. The amount of drug present in the filtrate was then determined from the calibration curve and the cumulative percent of drug release was calculated. The *in vitro* dissolution studies were undertaken in triplicate for each preparation.

2.4.6. Vancomycin microbiological activity

A microbiological assay was performed to estimate the active amount of vancomycin released during the *in vitro* dissolution test (Stigter et al., 2004). The alginate hydrogel/vancomycin coated scaffolds and the scaffolds coated with the drug-free alginate hydrogel were sterilized by gamma radiation (25 kGy dose, Co-60 gamma irradiator). The sterilized scaffolds were placed in glass vials containing distilled water (5 mL) at 37 °C. *In vitro* dissolution samples of the hydrogel/vancomycin scaffolds at the end of 24 h were used for the microbiological study.

The bacterial inhibition tests were performed on Mueller Hinton Agar. The sensitive bacteria (*Staphylococcus aureus ATCC 6538*) were added to a separate bottle of 50 mL sterile Mueller Hinton Broth and stirred. Each bottle was incubated in a shaking water bath at 37 °C for 2 h. The bacterial suspension was then cooled to room temperature and homogenized. The suspension (0.5 mL) was then spread onto a 10 cm diameter agar plate containing Mueller Hinton Agar. The agar plates were dried at 37 °C before applying the samples. In order to compare the sensitivity of vancomycin released from the scaffold towards *S. aureus*, the following was performed. Cellulose filter discs (0.6 cm in diameter) which were sterilized by autoclaving for 3 h at 121 °C (∼104 kPa) were placed on the Mueller Hinton Agar plate randomly. Thereafter, dissolution samples (5 μL) of the hydrogel/vancomycin scaffolds were inoculated onto the cellulose filter discs. A standard curve was prepared with vancomycin (clinical laboratory standard) at concentrations of 2, 4, 8, 16, 32, 64 and 128 μg/mL. All agar plates were incubated at 37 °C for 16 h. After incubation, the diameters of the inhibition zone were measured and averaged. Control samples coated with the drug-free alginate hydrogel were also measured by the microbiological method. All the inhibition tests were performed in triplicate.

The quantity of vancomycin released from the scaffold at the end of 24 h during the *in vitro* dissolution test was also measured using an UV spectrophotometer (Shimadzu UV2450, Japan) at a wavelength of 280 nm. Results from the microbiological assay and UV quantification were compared in order to determine the percentage of active vancomycin.

2.4.7. Osteoblastic culture

The immortalized rat osteoblastic ROS17/2.8 cell line (obtained from The West China College of Medicine, Sichuan University) was utilized in this study. These cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, UK) and supplemented with 10% foetal bovine serum (FBS, Invitrogen, Paisley, UK), 100 mg/mL streptomycin and 100 U/mL penicillin. The culture media was changed every alternate day.

2.4.7.1. Analysis of the ROS17/2.8 cells attachment on the scaffold. The sterilized (25 kGy dose, Co-60 gamma irradiator) alginate hydrogel/vancomycin coated scaffolds (S1A2) were cut into circular slices (9 mm in diameter and 1 mm in thickness), placed into a 24-well plate and cells were seeded into the well plates at a density of 1×10^5 cells/well. All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. After culturing for 5 h, Cells numbers were counted after rinsing by PBS in order to remove the unattached cells and digestion by trypsin. The morphology of the attached cells after 24 h were observed by SEM. The ROS17/2.8 cells on the scaffolds were washed twice with PBS for 10 min. The cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (PBS) at 4 °C for 2 h, and then dehydrated by increasing the concentration of alcohol (50%, 70%, 80%, 90%, 95%, 99% and $2 \times 100\%$). The critical point drying of specimens was undertaken with liquid CO_2. The critical point drying often make the PDLLA/BCP scaffold shrinked and distortional, the cells only dehydrated with alcohol was also observed as the control.

2.4.7.2. Analysis of cell cytotoxicity and proliferation of the ROS17/2.8cells. The sterilized alginate hydrogel/vancomycin coated scaffolds (S1A2) were cut into circular slices (9 mm in diameter and 1 mm in thickness), placed into a 24-well plate and cells were seeded into the well plates at a density of 2×10^4 cells/well. All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. The medium was changed every 3 days. For each time point, six replicate determinations were undertaken (three for the fluorescent staining observation and three for the MTT assay).

Fluorescent staining and observation: After each time point, the samples were stained with 0.01% AO (acridine orange) for 5 min; washed with PBS and examined under a fluorescence microscope (OLYMPUS B × 60). For each time point, the test was performed in triplicate.

MTT assay: A MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay was used to assess cell proliferation by measuring mitochondrial succinate dehydrogenase activity (Stevens et al., 2004). After 1, 3 and 6 days, MTT solution (200 μL) (5 mg/mL, Sigma) was added to each well. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 for 4 h to allow the formation of formazan crystals. After the culture medium was aspirated off, the sample was washed with PBS and DMSO (dimethylsulfoxide) (1 mL) was then added into each well. The well plate was left on a shaking platform for 10 min. Thereafter, the solution (200 μL) was collected and pipetted into a 96-well plate. The plates were read on a Microplate reader (BIO-RAD, model 550), using a test wavelength of 570 nm. The cells without a scaffold served as the negative control in this study. Using the above similar culture

Table 2
Mechanical properties of the PDLLA/BCP scaffold S1and the PDLLA scaffold S2

Sample scaffold	Compressive offset yield strength at 2% of strain (MPa ± S.D., $n=5$)	Compressive modulus (MPa ± S.D., $n=5$)
S1 (PDLLA/BCP)	1.12 ± 0.05	10.47 ± 0.12
S2 (PDLLA)	0.58 ± 0.07	6.41 ± 0.09

Table 3
Effect of the alginate hydrogel concentration on porosity of the PDLLA/BCP scaffolds

Sample	Alginate concentration (w/v, %)	Porosity (% ± S.D., $n=3$)
S1	0	84.5 ± 2.1
S1A1	0.5	82.3 ± 3.0
S1A2	1	78.6 ± 2.7
S1A3	2	52.5 ± 4.3

conditions, results from the PDLLA/BCP scaffold S1 (i.e. without the hydrogel coating) was compared to the hydrogel coated scaffold (S1A2).

2.5. Statistical analysis

The data were analyzed statistically using a Tukey multiple range post hoc test with SPSS for windows (SPSSInc. Version 11.5 Chicago, USA). Statistical tests were performed at a 95% significance level ($p<0.05$).

3. Results

3.1. Morphology

Fig. 2(A–C) show the morphologies of the porous PDLLA/BCP scaffold S1 fabricated by the particle-leaching/TIPS method. The expected 3D inter-connected porous structure was evident. From the SEM pictures, it was observed that the pore size of the pores were about 300–400 μm. In addition, the small pores of 20–50 μm formed in the phase separation process and smaller pores of 5 μm induced by solvent vaporization in the pore walls could be observed. Fig. 2(D–F) show the morphologies of the porous PDLLA/BCP scaffold S3 fabricated by the particle-leaching method only. Compared to the scaffold S1, the small pores with a pore size of 20–50 μm could not be observed on scaffold S3.

Fig. 3 shows the morphologies of the porous scaffolds after coating with various concentrations of alginate hydrogel/vancomycin solution. At a concentration of 0.5% (S1A1), the scaffold surface was not coated homogeneously by the hydrogel layer (Fig. 3(A)). An increase in the coating concentration (S1A2, 1%) led to a more homogeneous coating layer with some changes in the scaffold structure: the pore size decreased from 300–400 μm to approximately 250–350 μm (Fig. 3(B)). At a much higher concentration of 2% (S1A3), the pores became much smaller and some pores were fully clogged (Fig. 3(D)).

Fig. 4 shows the thickness of the coating layer at various concentrations of coating solutions. It is observed that the coating layer increased in thickness with an increase in the concentration of the coating solution. At a concentration of 0.5 (S1A1), 1 (S1A2) and 2% (S1A3), the thickness of the coating layer was approximately 0.2–0.5 μm, 1–1.5 μm and 2–3 μm, respectively.

3.2. Mechanical properties

Table 2 represents the mechanical properties of the scaffolds. Compared to the pure PDLLA scaffold, the PDLLA/BCP scaffold exhibited a higher compressive offset yield strength at 2% of strain. The compressive modulus behaved in a similar manner to the compressive offset yield strength. The results indicate that the mechanical properties of the scaffold were enhanced obviously by the BCP particles.

3.3. Porosity

Table 3 shows the open porosity of the PDLLA/BCP scaffold S1 and the scaffolds after coating with the alginate hydrogel (S1A1, S2A2 and S1A3). The porosity (calculated from Eq. (1)) of the scaffold S1, S1A1, S1A2 and S1A3 were 84.5 ± 2.1, 82.3 ± 3.0, 78.6 ± 2.7, and 52.5 ± 4.3%, respectively. Clearly, the porosity of the scaffold decreased significantly ($p<0.05$) with an increase in the concentration of the coating solution.

3.4. The wettability of the scaffold surface

The water contact angle of the PDLLA/BCP film was 87.2 ± 3.4°. The water drops can spread completely on calcium alginate films, hence the water contact angle of it could be regarded as 0°. This means that alginates are highly hydrophilic materials. The data in Table 4 indicate that the apparent contact angles were significantly reduced after being coated with the alginate hydrogel. The average water contact angle of the uncoated scaffold was 116.2 ± 7.1°. The water contact angles of the hydrogel coated scaffold S1A1, S1A2 and S1A3 were 54.3 ± 8.3°, 39.3 ± 9.2° and 16.92 ± 5.4°, respectively. There was therefore a decrease in the contact angles with an increase in the alginate concentration.

3.5. In vitro vancomycin release

The amounts of vancomycin released *in vitro* were measured using an UV spectrophotometer and the cumulative release of

Table 4
The apparent water contact angles of the PDLLA/BCP scaffold S1and alginate hydrogel coated scaffolds (S1A1, S1A2 and S1A3) and the water contact angles of the PDLLA/BCP film

Sample	Concentrations of alginate (w/v, %) coating solution	Contact angle (±S.D., $n=3$)
PDLLA/BCP film		87.2 ± 3.4
S1	0	116.2 ± 7.1
S1A1	0.5	54.3 ± 8.3
S1A2	1	39.3 ± 9.2
S1A3	2	16.9 ± 5.4

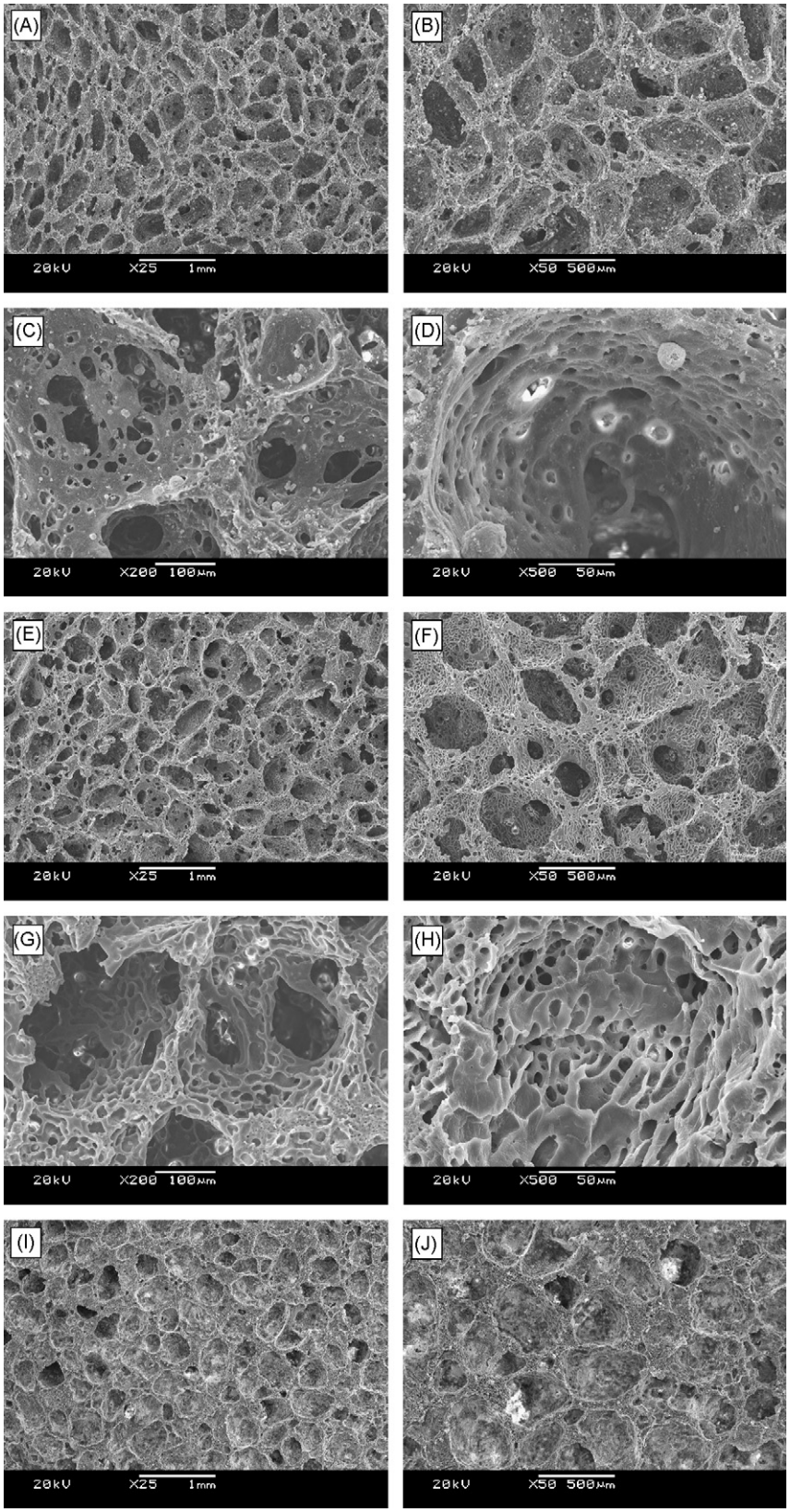

Fig. 2. Morphology of the PDLLA/BCP scaffold S1 (A–D) and PDLLA scaffold S2 (E–H) and the PDLLA/BCP scaffold S3 (I–L) fabricated by the particle-leaching method only).

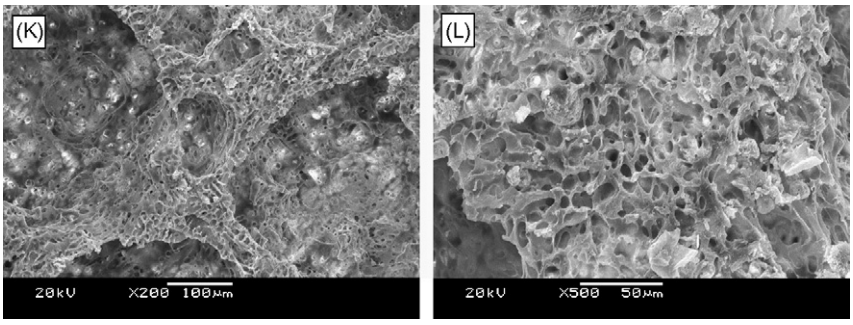

Fig. 2. (*Continued*).

vancomycin are shown in Figs. 5 and 6. The total amount of loaded vancomycin per scaffold was calculated via the drug release test itself. In this study, after the time (18 days) shown in Figs. 5 and 6, the drug release test was continued until the absorbance of the filtrate was close to the control sample (without drug). Finally, the scaffold was broken to ensure that all the loaded drug was released. The sum of all the released drug was the total amount of loaded drug for every scaffold. The average amount of loaded vancomycin in scaffold S1A1, S1A2 and S1A3 was 2.8 ± 0.3 mg, 3.1 ± 0.4 mg and 4.4 ± 0.6 mg, respectively. The average drug loading efficiency is $82.7 \pm 1.3\%$. For all samples, vancomycin was released in a slow sustained manner from the scaffolds.

Fig. 5 illustrates the effect of the concentration of alginate coating solution on the release behavior in distilled water at 37 °C. On day 6, approximately 94.0, 81.7 and 62.7% of vancomycin was released with an alginate concentration of 0.5, 1 and 2% (w/v), respectively. The amount of drug released showed a statistically significant high level ($p < 0.05$) with the high alginate concentration. Clearly, higher concentrations of coating solutions led to more retarded drug release rates.

Fig. 6 shows the influence of the dissolution media on drug release rates of vancomycin. A significant ($p < 0.05$) faster drug release was also demonstrated at higher concentrations of phosphate ion. For example, on day 6 approximately 62.7, 73.6 and 79.5% of vancomycin was released in a dissolution medium of distilled water, 1×10^{-3} mol/L of PBS and 1×10^{-2} mol/L of PBS, respectively, The results indicate that the drug release rate was influenced by the salt concentration of the dissolution medium.

3.6. Microbiological activity

Experimental results of vancomycin concentration obtained with UV spectrophotometric and microbiological measurements of the vancomycin dissolution samples allowed the determination of the percentage of active vancomycin released from the scaffold. Table 5 shows that the percentage of active vancomycin

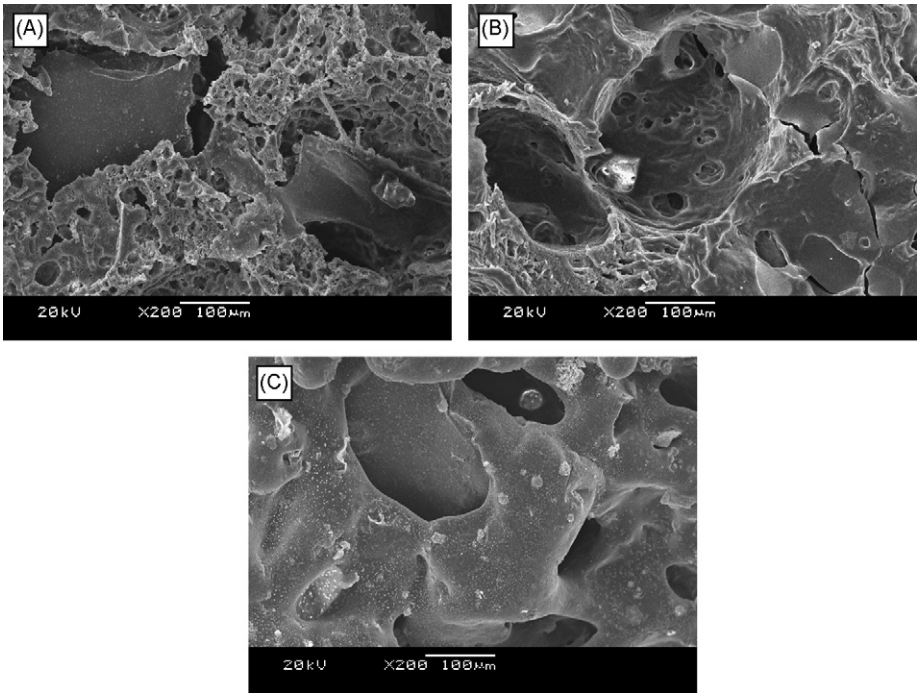

Fig. 3. Morphology of the PDLLA/BCP scaffolds S1 coated with various concentrations of alginate coating solution. (A) S1A1: 0.5%; (B) S1A2: 1%; (C) S1A3: 2%.

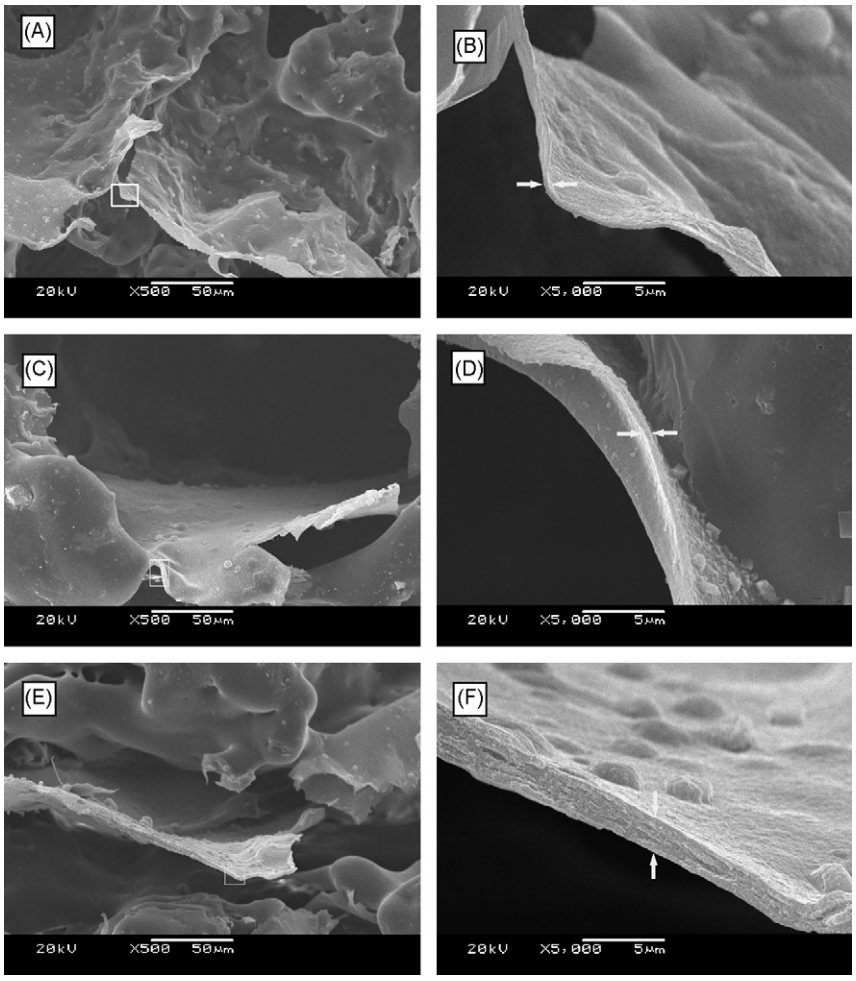

Fig. 4. Thickness of the coating layer of the alginate hydrogel/vancomycin coated PDLLA/BCP scaffolds S1 at various concentrations of alginate coating solutions. (A and B): S1A1, 0.5%; (C and D): S1A2, 1%; (E and F): S1A3, 2%.

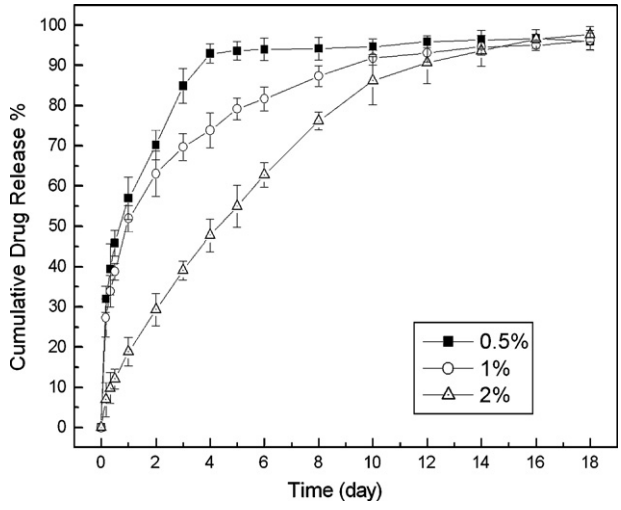

Fig. 5. Effect of the alginate hydrogel concentration on the drug release properties of vancomycin.

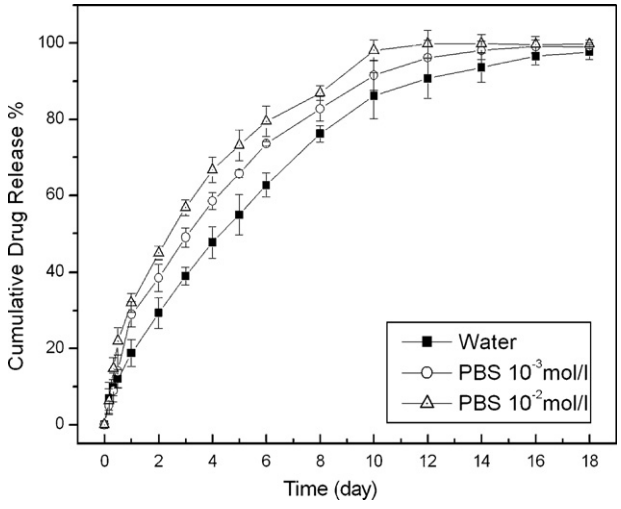

Fig. 6. Effect of the dissolution media on the cumulative release of vancomycin from the alginate hydrogel/vancomycin coated-scaffolds.

Table 5
Percentage of microbiologically active vancomycin released from the scaffolds after 24 h

The eluted vancomycin amount (mg ± S.D.)		Active vancomycin (%)
UV assay	Microbiological assay	
1.46 ± 0.08	1.31 ± 0.05	89.7

was 89.7%. These results may confirm that vancomycin was almost completely active after association with the scaffold by this coating method.

3.7. Cell growth on the scaffold

3.7.1. Effect of alginate coating on ROS17/2.8 attachment

Fig. 7 shows the numbers of cells attached on the uncoated PDLLA/BCP scaffold (S1) and the alginate hydrogel/vancomycin coated PDLLA/BCP scaffold (S1A2) after culturing for 5 h. There were $5.38 \pm 0.88 \times 10^4$ cells and $4.87 \pm 0.18 \times 10^4$ cells attached on the scaffold S1 and S1A2, respectively. The number of cells attached on the scaffold S1 was significant higher ($p < 0.05$) than the cells attached on the scaffold S1A2.

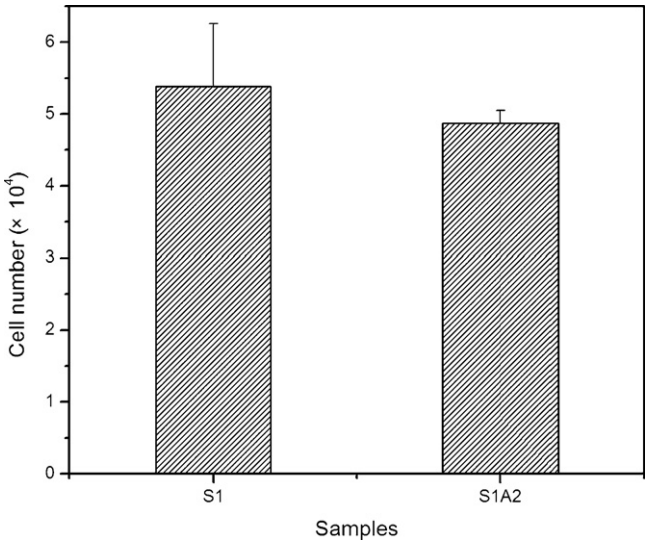

Fig. 7. Comparison of the number of cells attached on the uncoated PDLLA/BCP scaffold (S1) and the alginate hydrogel/vancomycin coated-scaffold (S1A2) for 5 h culturing.

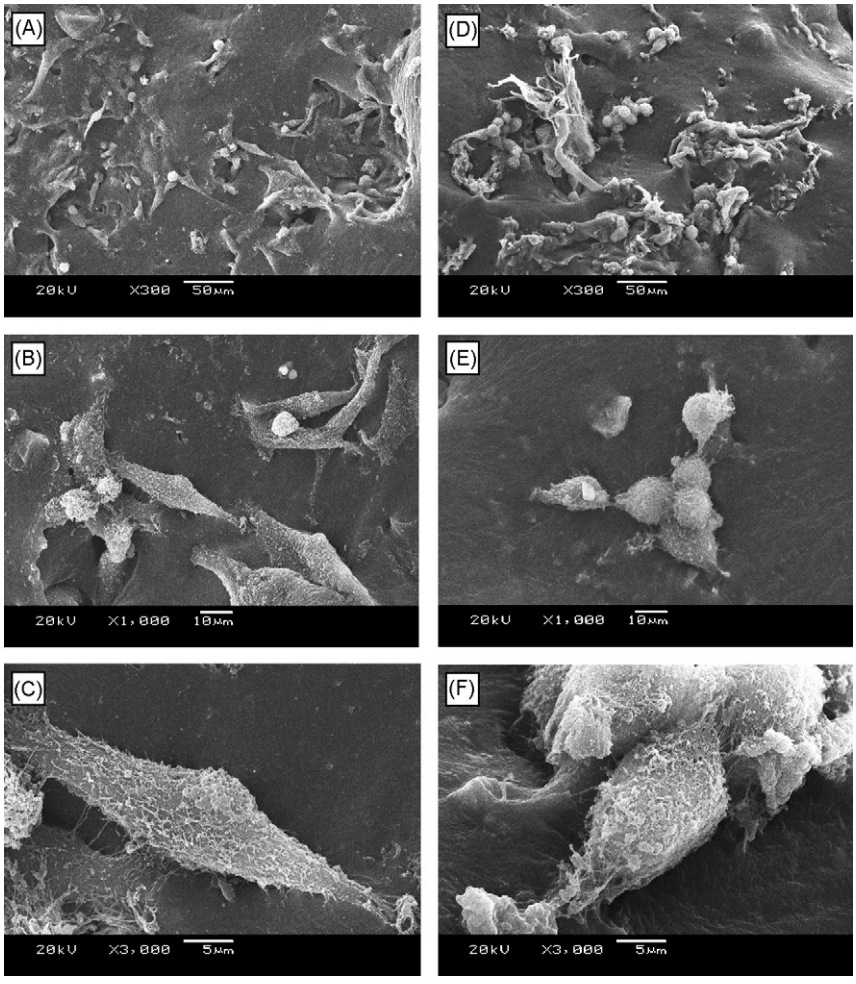

Fig. 8. SEM photographs showing morphology of ROS17/2.8 cells (dehydrated by the increasing concentration of alcohol and the critical point drying method) attached on the uncoated PDLLA/BCP scaffold S1 (A–C) and the alginate hydrogel/vancomycin coated-scaffold S1A2 (D–F) for 24 h culturing.

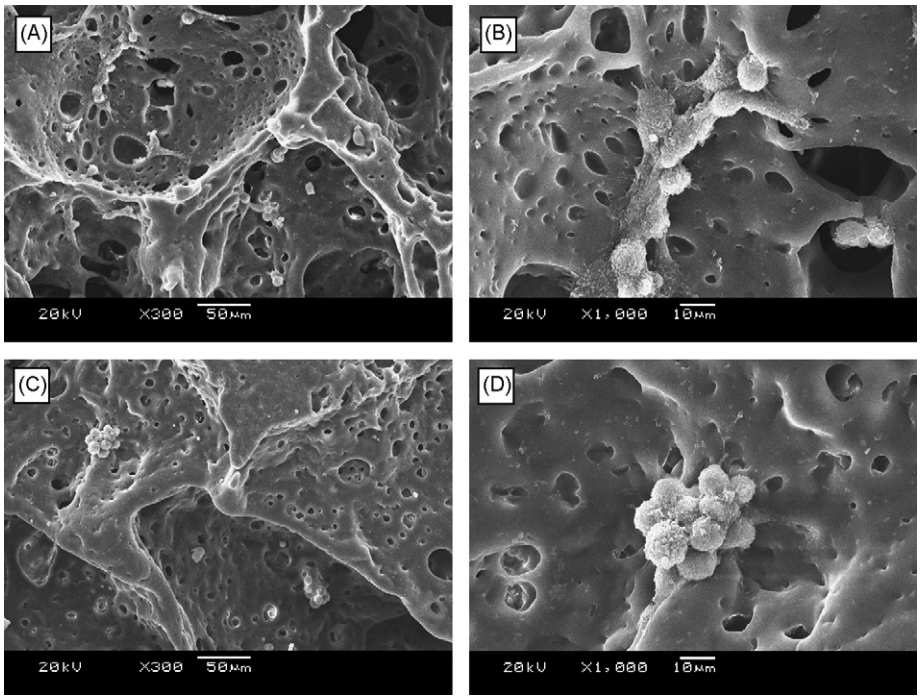

Fig. 9. SEM photographs showing morphology of ROS17/2.8 cells (dehydrated only by the increasing concentration of alcohol) attached on the uncoated PDLLA/BCP scaffold S1 (A and B) and the alginate hydrogel/vancomycin coated-scaffold S1A2 (C and D) for 24 h culturing.

Figs. 8 and 9 show the SEM photographs of ROS17/2.8 cells attached on the uncoated PDLLA/BCP scaffold S1 and the alginate hydrogel/vancomycin coated-scaffold S1A2 for 24 h culturing. The samples in Fig. 8 were dehydrated by the increasing concentration of alcohol and the critical point drying method. It can be seen that the morphologies of both scaffolds changed, because the scaffold shrinked and distorted heavily in the critical point drying process. The samples in Fig. 9 were dehydrated only with the increasing concentration of alcohol. From Figs. 8 and 9, the cells adhered on the uncoated PDLLA/BCP scaffold S1 were more than those on the calcium alginate hydrogel coated PDLLA/BCP scaffold S1A2. Also, SEM examination of cells cultured on the S1 (Fig. 8(A–C)) revealed most osteoblasts to have a spindle shape, while the osteoblast adhered on the scaffold S1A2 (Fig. 8(D–F)) have a round shape. The result indicates that the alginate coated scaffold show lower cell attachment than the uncoated scaffold.

3.7.2. Effect of alginate coating on viability of the ROS17/2.8

The fluorescent micrographs of the osteoblasts cultured on the scaffolds after day 1, 3 and 6 are shown in Fig. 10. It can be seen that the osteoblasts are all attached to the pore surface of the two groups of scaffolds and proliferate along the pore walls.

A MTT assay was also performed after day 1, 3 and 6 to determine cell proliferation on the scaffold. After treatment of the cell-scaffold constructs with the MTT solution, dark blue crystals of formazan were seen, indicating the presence of metabolically active cells. The MTT absorption was measured at 570 nm with a background subtraction at 630 nm. A higher absorbance indicates that there are either more cells or that they are metabolizing. Fig. 11 shows that the MTT absorption of all the samples increased over time indicating cell proliferation on the scaffold. There was no statistically significant difference ($p > 0.05$) between the uncoated scaffold S1 and the alginate hydrogel coated scaffold S1A2 after 1 and 3 days. On day 6, the alginate coated scaffold S1A2 showed a little lower cell proliferation than the PDLLA scaffold without alginate coatings (S1).

4. Discussion

In this study, a PDLLA/BCP scaffold was fabricated by a particle-leaching method combined with TIPS and then coated with an alginate hydrogel containing an antibiotic drug, vancomycin. In this formulation design, each component had a specific function. PDLLA has been widely used as three-dimensional scaffolds for its biodegradability and biocompatibility (Nam and Park, 1999; Zhu et al., 2003; Kim et al., 2004a,b). The relatively low mechanical properties of the pure polymer scaffold has led to the incorporation of bioceramics to the polymer scaffolds to improve their mechanical properties. In this study, the ceramics BCP was incorporated with the PDLLA scaffold to enhance the mechanical properties of the scaffold. The hydrogel coating was expected to entrap the drug successfully in the scaffold and to also promote a sustained drug release behavior. Additionally, the hydrogel coating was expected to improve wettability of the scaffold to facilitate the culture medium infiltrate throughout the scaffold.

As a scaffold and drug carrier in tissue engineering, high mechanical properties and a highly inter-connected porous structure were required. Compared to the bioceramics scaffold, the polymer only scaffold possesses relatively low mechanical

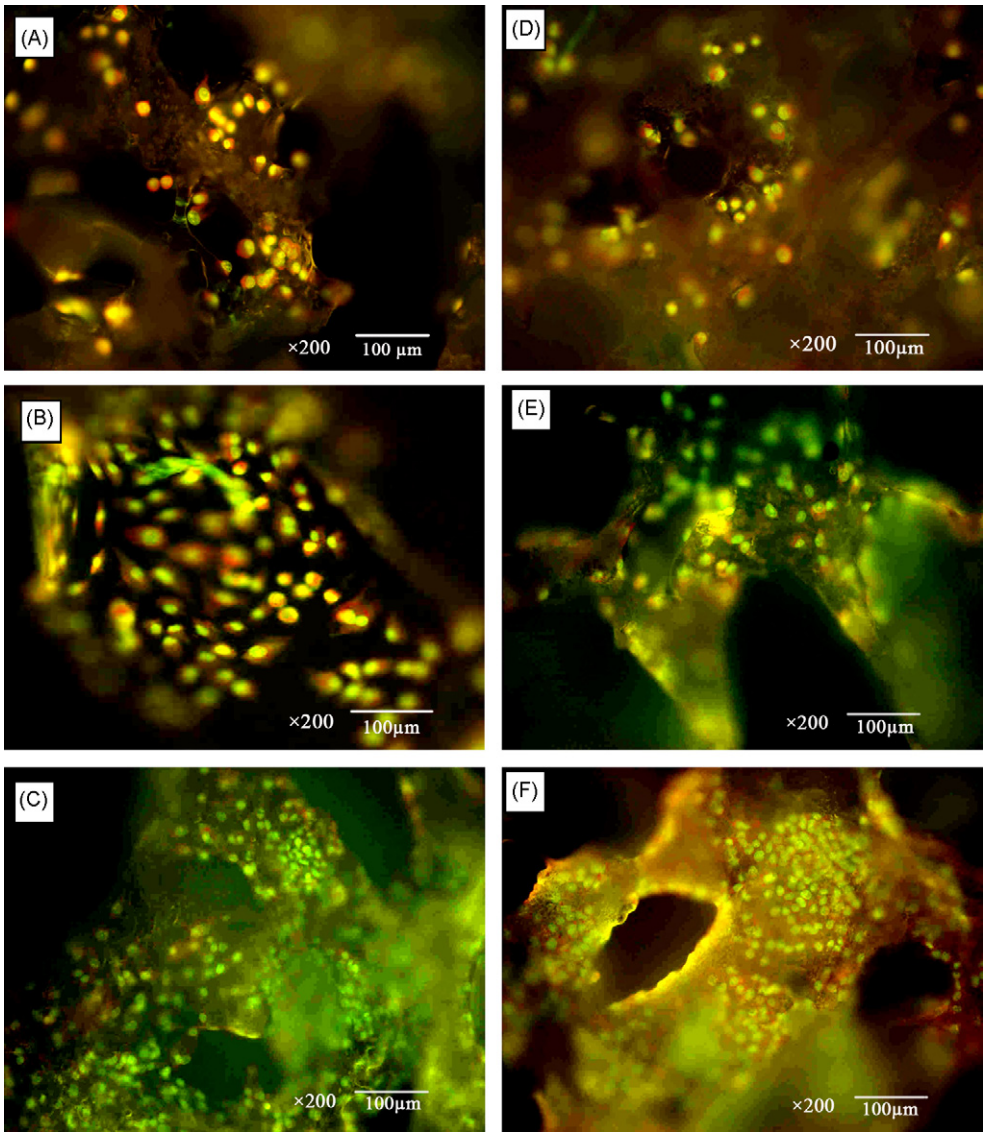

Fig. 10. Fluorescent micrographs of the osteoblasts cultured on PDLLA/BCP scaffolds S1 (A: day 1; B: day 3; C: day 6) and alginate hydrogel coated-scaffolds S1A2 (D: day 1; E: day 3; F: day 6).

properties, but with controllable degradation rates. Therefore, bioceramics (HA, β-TCP, etc.) were generally incorporated with the polymer scaffolds to improve their mechanical properties (Shikinami and Okuno, 1999; Ignjatović et al., 1999). PDLLA/β-TCP scaffolds with improved mechanical properties were fabricated in our previous work and have been reported (Sun et al., 2004). BCP is more effective in bone repairment or regeneration than pure HA or pure β-TCP, and has a controllable degradation rate to a certain degree (Ramay and Zhang, 2004). The mechanical properties of the scaffold in this study were also significantly enhanced by the BCP particles (Table 2). The compressive offset yield strength of the PDLLA/BCP scaffold S1 (1.12 MPa) was about two times higher than the pure PDLLA scaffold S2 (0.58 MPa). Also, the compressive modulus was also increased from 6.41 to 10.47 MPa.

The porous structure can provide both sufficient space for blood circulation and also a large surface area for the entrapment of large amounts of drugs (Kim et al., 2004a,b). In this study, the particle-leaching/TIPS method was used to fabricate a porous scaffold. Compared to the TIPS method, the particle-leaching method can provide the porous scaffold with larger pore sizes and higher mechanical properties for the relatively high ratio of solid PDLLA material and solvent. The general concentration of polymer used in the phase separation method is lower than 15% (Nam and Park, 1999; Hua et al., 2002; Tu et al., 2003) while the ratio of PDLLA and the solvent was about 33% (w/v) in this study. However, by addition of water to the solvent with a ratio of 87:13 (v/v), the small pores with pore sizes of 20–50 μm in the pore (300–400 μm) walls were successfully formed in the phase separation process (Fig. 2). These small pores of 20–50 μm can make the scaffold highly inter-connective.

The morphology and porosity of the scaffolds with the alginate hydrogel coating could be tailored via the alginate solution

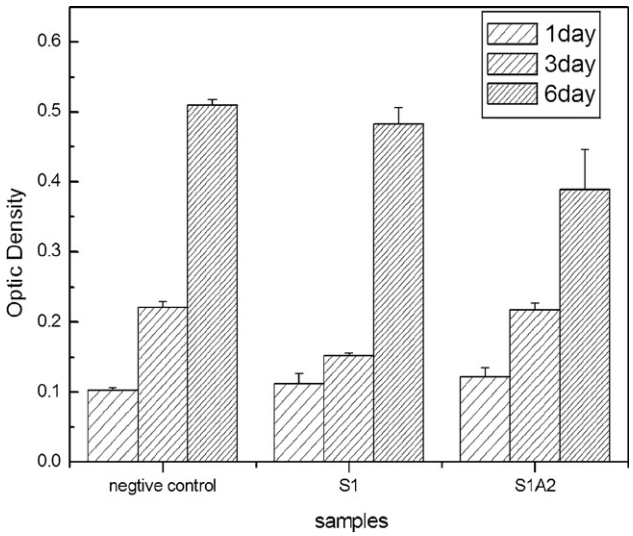

Fig. 11. MTT absorbance at 570 nm of the osteoblasts cultured on PDLLA/BCP scaffolds S1, alginate hydrogel coated-scaffolds S1A2 and the negative control group (osteoblasts cultured on the 24-well plate) after day 1, day 3 and day 6.

concentration employed. The coating solution with a concentration of 1% was most suitable to be used for coating the scaffold. At a low concentration of 0.5%, the scaffold surface was not covered completely by the alginate coating layer (Fig. 3(A)). At a concentration of 1% (S1A2, Fig. 3(B)), the alginate layer coated the scaffold surface nearly completely. The pore walls were all covered by the dried hydrogel layers and the pore size of pores decreased from 300–400 to 250–350 μm. However, there were still some small pores and cracks in the coating layers which can contribute to keeping the scaffolds highly inter-connected. In addition, when the hydrogel layer absorbs water, the highly hydrated three-dimensional net works of the hydrogel can still facilitate permeation of nutrients in and cell products out of the gel (Kim et al., 2004a,b; Ma et al., 2005). Therefore, an increase in the alginate concentration from 0.5 to 1% revealed that the alginate coating became more integrated while the thickness increased (Fig. 4). At a much higher concentration of 2%, the coating layer covered the scaffold completely, but some pores were fully clogged (Fig. 3(C)) and the porosity of the scaffold was also reduced to a low value ($52.5 \pm 4.3\%$). Under the wet condition (cell condition), after the hydrogel absorb water, the coating layer will become more thicker, therefore the cell infiltration into the scaffolds would be affected by the reduced pore size and the porosity resulted by the too thicker coating layer.

In this study, the antibiotic vancomycin was successfully entrapped in the scaffold with almost complete activity by mixing it with the sodium alginate coating solution. The vancomycin, a water-soluble antibiotic drug, is commonly used for treating osteomyelitis and preventing osseous staphylococcal infections after surgery (Gautier et al., 2001). It was used as a model drug in this study. This gentle method avoided various potential harmful effects to the drug as with other methods, e.g. exposure to organic solvents in a hydrophobic polymer coating method, negative effects of high temperatures, and premature loss of drug during the leaching process. The result of the standardized bacterial assay (Table 4) proved that the vancomycin was not denatured by this loading method. Much of the growth factors are also water-soluble and unstable, thereby indicating that this hydrogel coating method has the potential to be used for loading of these drugs as well.

The release of drugs from a hydrogel delivery system involves absorption of water into the polymer matrix and subsequent diffusion of the drugs as determined by Fick's Law. The *in vitro* release profiles of vancomycin from the scaffolds exhibited linear release patterns at an early stage and slower release patterns at a later stage, and were similar to a first order release kinetic rate (especially for the S1A3 scaffold coated with a relatively high concentration of alginate solution). The drug release rate was influenced by the alginate concentration of coating solution and the phosphate concentration of the release medium solution. The release profiles (Fig. 5) indicate that the higher concentration of the coating solution resulted in the slower release rate. The thicker coating layer and high density of the network at higher alginate concentrations (Fig. 4) may have contributed to the slower drug release observed with increasing alginate concentrations. The profile (Fig. 6) show that an increase in phosphate concentration of the release medium solution resulted in faster drug release rates. This may be due to the existence of the HPO_4^{2-} and $H_2PO_4^-$ ions in the PBS medium that can facilitate the separation of Ca^{2+} out from the ionically crosslinked alginate hydrogel thereby enhancing issolution of the alginate hydrogel.

The antibiotic, vancomycin, was therefore successfully entrapped in the scaffold by the alginate hydrogel coating with complete activity and sustained controllable release behavior. The *in vitro* osteoblast culture experiment showed that the uncoated PDLLA scaffold and the alginate coated scaffold both possessed good biocompatibility for osteoblast growth. It could be seen that the MTT absorption of all the samples increased over time thereby indicating cell proliferation on both scaffold. The fluorescent micrographs of the osteoblasts (Fig. 10) and the MTT assay results (Fig. 11) also confirmed that the hydrogel coating layer containing the antibiotic drug had no cell cytotoxicity.

However, the alginate coated scaffold showed lower cell attachment compared with the uncoated scaffold (Figs. 7–9). According to the literatures, the hydrophobic PDLLA surface does not facilitate cell adherence and growth (Yang et al., 2002). The surface wettability is one of the factors which influence the cell attachment on the materials. The improvements of the surface wettability of the hydrophobic polymers surface often enhance the cell adhesion on the materials (Webb et al., 1998; Yang et al., 2002). In this study, the wettability of the scaffold surface was improved significantly by the alginate coating. The results (Fig. 11) in this revision showed there are no significant differences between the PDLLA/BCP samples and the alginate coated scaffold after 1 and 3 days. It was reasonable to think that, at the initial times, though the alginate coated surface showed lower cell attachment, the alginate coated scaffold could be easier infiltrated by the cell medium than the uncoated scaffold due to its improved wettability. After 6 days, the PDLLA scaffold without alginate coatings (S1) could be also infiltrate well by the cell medium, so the alginate coated scaffold S1A2 showed a little lower cell proliferation due to its poor cell attachment. The

result indicates that the alginate does not facilitate cell adherence either, and the cell adhesion on the materials will not always improved by the enhancement of surface wettability. The cell adhesion on the materials was also influenced by the surface chemistry (charged functional groups, natural recognition sites, etc.) and surface roughness. Most cell types are able to bind to the peptide sequence RGD (Arg-Gly-Asp), and the alginate may be easily modified with this peptide to promote cell adhesion (Rowley et al., 1999; Drury and Mooney, 2003). The cell adhesion of this drug loaded scaffold in this study remained to be improved by modifying the alginate coating layer.

5. Conclusions

A well-developed porous PDLLA/BCP scaffold with enhanced mechanical properties was fabricated by a particle-leaching/TIPS method. In order to incorporate the antibiotic vancomycin into the scaffold and maintain its biological activity, the scaffold was coated with an ionically crosslinked alginate hydrogel containing vancomycin. The *in vitro* vancomycin release profiles demonstrated sustained release properties; and the release rate was highly dependent on the coating solution concentration and the dissolution medium. The *in vitro* biological activity test showed that the drug was successfully entrapped in the scaffold with complete activity by this method. In addition, the *in vitro* cell culture experiment proved that the alginate hydrogel-coated scaffold had suitable biocompatibility for the growth of osteoblasts. Although the cell adhesion of the alginate coating layer remained to be improved, the drug loaded alginate coated scaffold designed in this study has the potential to be utilized in bone defect regeneration due to its sustained drug release properties, maintenance of activity and appropriate biocompatibility.

Acknowledgements

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