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In vitro sustained release of recombinant human bone morphogenetic protein-2 microspheres embedded in thermosensitive hydrogels

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Received July 29, 2011, accepted August 26, 2011

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Pharmazie 67: 299–303 (2012) *doi: 10.1691/ph.2012.1649*

Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a critical regulator of osteogenic capacity that is commonly used in bone grafts. The effectiveness of rhBMP-2 may be reduced as it can become unstable and degraded after injection into the body. Microspheres are considered appropriate vehicles for the sustained release of proteins *in vivo*. In this study, rhBMP-2 microspheres were manufactured using the water-in-oil-in-water (W/O/W) double-emulsion solvent-extraction technique by encapsulation in poly(lactic-co-glycolic) acid (PLGA). The microspheres were then embedded in two hydrogels made of either poloxamer 407 hydrogel or chitosan thioglycolic acid (CS-TA). The encapsulation efficiency and *in vitro* release of rhBMP-2 were examined and compared with the control release system (rhBMP-2 microspheres alone). The rhBMP-2 microspheres in the CS-TA hydrogel showed the lowest burst release (about 40% in the first 8 h) among the three groups. The mechanisms may be the high viscosity of CS-TA hydrogel and the sustained release characteristics of CS-TA itself. The CS-TA hydrogel combined with PLGA microspheres can efficiently encapsulate rhBMP-2, control the burst release at early time points, and provide sustained release *in vitro*. It may be an appropriate rhBMP-2 vehicle for bone regeneration.

1. Introduction

An increasing number of orthopaedic surgeries require bone grafts. Bone grafts are required to repair bone defects caused by trauma and defects created during the removal of large tumours of the bone; they are also required during vertebral fusion surgery and long bone non-unions in tibiae, respectively (Bessa et al. 2008). Most clinical trials have focused on members of the bone morphogenetic protein (BMP) family due to their osteogenic capacity (Wozney et al. 1988). Among the BMP family, BMP-2 shows the most powerful and rapid bone induction (Bostrom et al. 1996; Sciadini and Johnson 2000; Toriumi et al. 1991), and it has been reported to induce differentiation of mesenchymal cells into osteoblasts and chondrocytes (Ducy and Karsenty 2000; Utida et al. 1990). Clinically, however, BMP-2 promotion of bone formation is markedly limited due to its rapid diffusion and absorption when it is injected into the body (Takaoka et al. 1991). To maximise BMP-2-induced bone formation, it is necessary to find an ideal sustained delivery method. It is reported that sustained growth factor delivery can be established through different non-covalent retention mechanisms, including physical entrapment, absorption and complexation (Luginbuehl et al. 2004). Apart from entrapping it in a gelatine sponge, BMP-2 is usually encapsulated in poly(lactic-*co*-glycolic acid) (PLGA) microspheres. These microspheres can prolong the sustained release of BMP-2, maximising its ability to induce bone formation.

rhBMP-2 microspheres were reported to have an initial burst release in an *in vitro* study (Kempen et al. 2008). To decrease the burst release of BMP-2 microspheres and maintain their biological activity *in vivo*, a scaffold made of an ideal vehicle is urgently needed. Such a vehicle must possess enough power adhered to the surface of three-dimensional porous titanium, which has good osteogenic conduction and has been proven to accelerate osteogenesis (Zhong-li et al. 2007). Additionally, the ideal BMP-2 vehicle should possess the following properties: (1) good biocompatibility; (2) controlled release of BMP-2, which will prevent it from being absorbed rapidly after being embedded into the vehicle; (3) good conductibility; (4) enough strength to support the broken ends of fractured bone; (5) good biodegradability; (6) a three dimensional and porous structure that is required for adsorbing BMP-2 microspheres; (7) sterile; and be easily obtainable; (8) good moldability; (9) easy storage (Qifeng Zhang 2005). Although many vehicles for BMP-2 have been reported, none meet all of the requirements mentioned above. Most researchers still focus on finding an ideal vehicle for rhBMP-2.

In this study, two novel hydrogels were investigated; they were fabricated as sustained-release drug-delivery systems. The encapsulation efficiency and release of rhBMP-2 from these drug-delivery systems *in vitro* were evaluated using an rhBMP-2 ELISA test. Our studies demonstrated that chitosan thioglycolic acid (CS-TA) hydrogel efficiently decreased the burst release of rhBMP-2 compared with the other two release systems.

2. Investigations, results and discussion

2.1. Production of the microspheres and the stability of rhBMP-2

rhBMP-2 is an alkyl protein that is extremely unstable both *in vitro* and *in vivo*. Therefore, factors that may affect the stability of rhBMP-2 must be considered, including dissolubility of the rhBMP-2, storage containers, vortex speed, storage temperature, pH, and so on. Polyethylene glycol (PEG) was reported to stabilise growth factors upon microencapsulation (AL-Azzam et al. 2005); however, PEG has been shown to decrease the pH within microspheres (Mäder et al. 1998), which is not suitable for rhBMP-2 (Lochmann et al. 2010). In our previous experiments, when PEG4000 was added as the inner phase of the microspheres, the stability of rhBMP-2 decreased to the point at which the protein could not be detected with an rhBMP-2 ELISA kit.

As rhBMP-2 is an alkyl protein with an isoelectric point of approximately 9.0, it can interact with the carboxyl group of PLGA-COOH and increase both the adsorption and encapsulation of the protein in the microspheres, thereby decreasing the burst release (Duggirala et al. 1996). Moreover, it has been hypothesised that the higher molecular weight of PLGA may contribute to the lower and incomplete release of encapsulated proteins (Ruhé et al. 2005). Therefore, the lower molecular weight PLGA-COOH (with a lactic to glycolic acid ratio of 75:25 and a weight-average molecular weight of 10000) was used as the outer oil phase during the fabrication of rhBMP-2 microspheres. The whole process was conducted in a glass container in an ice-bath. The resulting rhBMP-2 microspheres were vacuum-dried and stored at 4 °C, a temperature at which rhBMP-2 is stable.

Then the two kinds of CS-TA hydrogels were prepared as follows. CS-TA hydrogels were prepared with 40 mg CS-TA, 960 µl β-sodium glycerophosphate (0.6 mmol/l)and 10 µl Poly (ethylene glycol) diacrylate (PEGDA). The mixture was then homogenized with ultrasound and stirred using a vortex. The resulting yellow fluid hydrogel was stored at room temperature. Poloxamer hydrogels were fabricated by the homogenisation of 15 g poloxamer 407, 15 g poloxamer 188 and 100 ml H_2O, resulting in transparent fluid hydrogels. Then the rhBMP-2 microspheres were embedded into the formed hydrogels mentioned above.

2.2. Size distribution and particle morphology

It is not possible to observe every particle with light microscopy or SEM; however, this can be done with dynamic light diffraction. Size evaluation was accomplished using the Mie theory, which is regarded as accurate, especially in low absorbing samples. The size distribution demonstrated that the microspheres fabricated with the water-in-oil-in water (W/O/W) double-emulsion-solvent-extraction technique were well distributed in size, with more than 98% of particles having a diameter of approximately 1 µm.

Globular-shaped microspheres were obtained (Fig. 1). The surface of the particles was smooth. Among the investigated samples, the size was uniform, and no obvious broken particles were observed.

2.3. Hydrogel morphology

SEM pictures show that there are many beehive-like porosities on the CS-TA hydrogel. These porosities could adhere to and contain more rhBMP-2 microspheres and therefore may be a more suitable vehicle for rhBMP-2 microspheres (Fig. 2A). In contrast, only a few pores were observed on the surface of the poloxamer hydrogel (Fig. 2B).

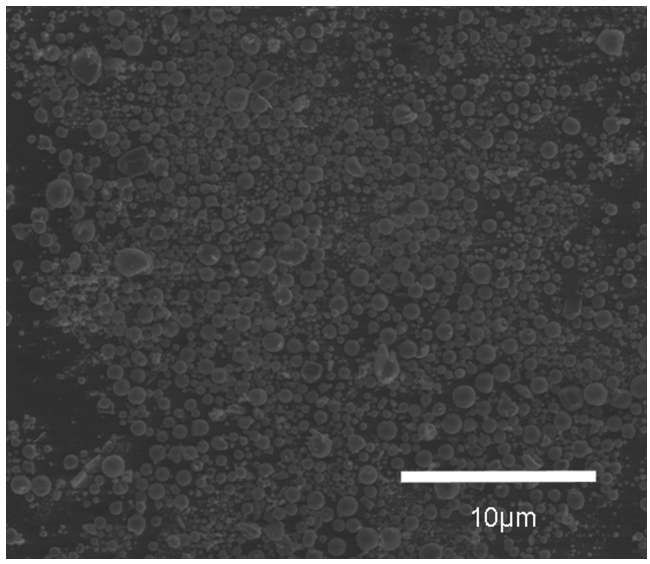


Fig. 1: Scanning electron micrographs of rhBMP2 microspheres

2.4. Thermosensitivity and viscosity of hydrogel

The two hydrogels can flow and molded when initially constituted, but after heating in a 37 °C water bath in less than 10 min, they changed from mobile fluid to solidified gels (Fig. 3). This process is often called "curing" and involves the formation of covalent crosslinks between polymer chains to form a macromolecular network (Hatefi and Amsden 2002). Meanwhile, the viscosities of the two hydrogels were measured (Fig. 4). The viscosity of 4% CS-TA hydrogel was higher than 25% poloxamer hydrogel.

2.5. Safety evaluation by haemolysis testing

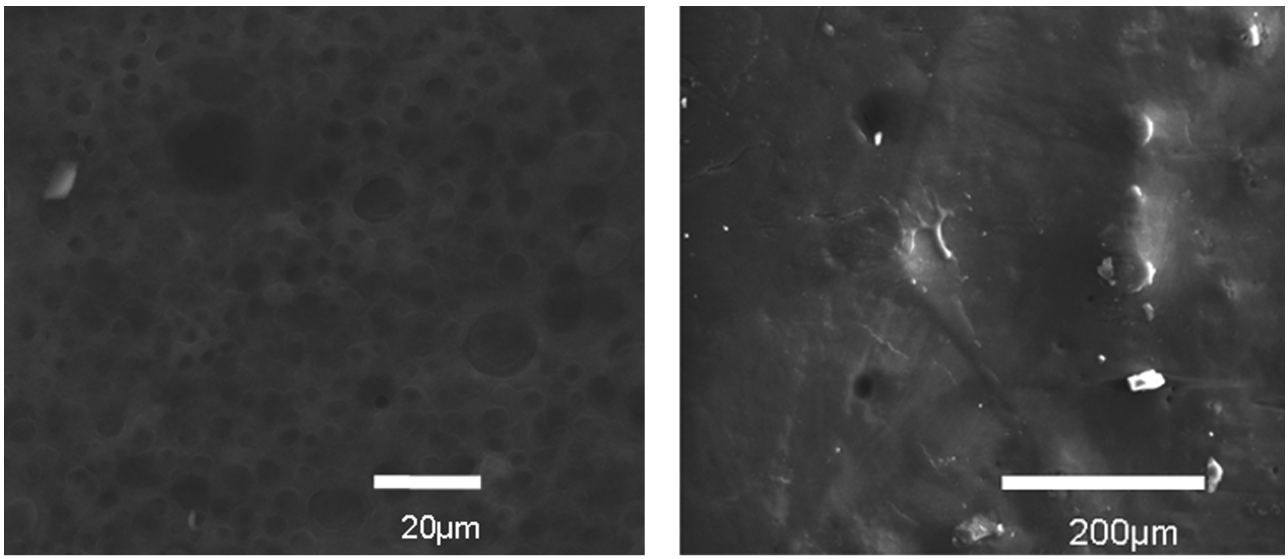
Poloxamer and CS-TA hydrogels were evaluated in an *in vitro* haemolysis test using fresh whole rabbit blood (Rekha and Sharma 2009). The poloxamer hydrogels still appeared red after centrifugation, which demonstrated the hydrogel-induced haemolysis. In contrast, the CS-TA hydrogels were transparent, with the blood cells sinking to the bottom of the test tube. These results demonstrated that the CS-TA hydrogel does not induce haemolysis. The results showed no significant difference between the CS-TA hydrogel and the negative control group (physiological saline) ($P > 0.05$) (Table). Thus, it can be concluded that CS-TA hydrogel does not cause haemolysis.

2.6. Encapsulation efficiency

One advantage of double emulsion techniques is the high encapsulation efficiency into PLGA (Park et al. 2005). In this study, the encapsulation efficiency was as high as 98.86%. The high efficiency prevented reduced waste rhBMP-2.

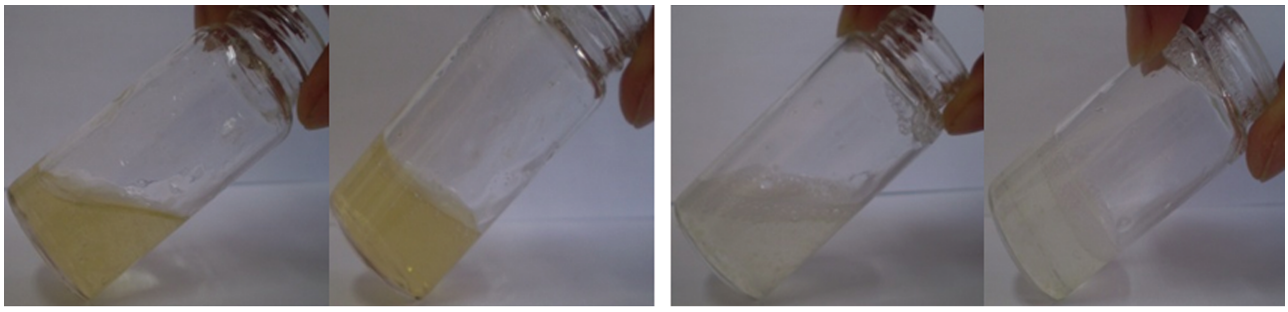
2.7. Determination of in vitro release

In vitro release profiles were assessed for a period of 21 days (Fig. 5). The burst release of the CS-TA hydrogel release system was approximately 40% within the first 8 hours, compared with



(A) (B)

Fig. 2: Scanning electron and light micrographs showing the morphology of the CS-TA hydrogel (A) and the poloxamer hydrogel (B). The CS-TA hydrogel has many beehive-like pores on the surface, which could adhere to rhBMP-2 microspheres, making it a suitable vehicle. In contrast, there were only a few pores observed on the surface of the poloxamer hydrogel



(A) (B)

Fig. 3: Thermosensitivity of CS-TA hydrogel (A) and poloxamer hydrogel (B)

65% for the poloxamer hydrogel release system and approximately 80% for the rhBMP-2 microspheres alone. This may be due to the higher viscosity and porosity of the CS-TA hydrogel compared with the poloxamer hydrogel. Therefore, the CS-TA hydrogel was considered to be a more suitable vehicle for decreasing the burst release of rhBMP-2.

2.8. Conclusions

In this study, rhBMP-2 microspheres with high encapsulation efficiency were prepared using W/O/W double-emulsion-solvent-extraction. To carry more rhBMP-2 microspheres in the three-dimensional porous titanium (3-D porous Ti) in the next step, the size of the microparticles was 1 μm in this paper com-

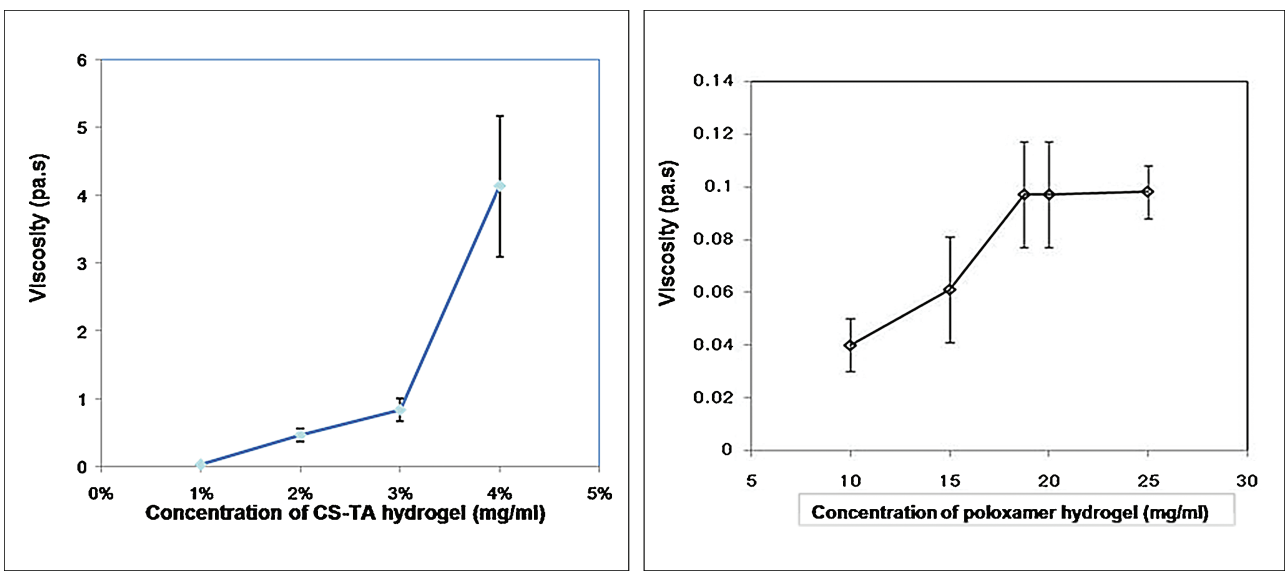


Fig. 4: Viscosity of different concentration of CS-TA hydrogel (A) and poloxamer hydrogel (B)

Table: Haemolysis of CS-TA hydrogel with and without PEGDA

Major components	Abs* (λ=545 nm)	p
Water	-7.358 ± 0.00749	0.00**
100% CS-TA	-0.115 ± 0.00749	0.138
50% CS-TA	-0.0064 ± 0.00749	0.403
25% CS-TA	-0.0071 ± 0.00749	0.352
100%CS-TA + PEGDA	-0.0139 ± 0.00749	0.074
50%CS-TA + PEGDA	-0.0091 ± 0.00749	0.237
25% CS-TA + PEGDA	-0.0062 ± 0.00749	0.418

** $p < 0.05$, All groups were compared with a negative control (physiologic saline group). The statistic result showed no significant difference from the negative control group ($P > 0.05$). Thus, it can be concluded that CS-TA hydrogel does not induce haemolysis.
* Mean ± SD.

pared with the size of the rhBMP-2 microspheres reported in previous studies (about 60 μm) (Lochmann et al. 2010). While small microspheres are more easily embedded in scaffolding materials, they also have a higher burst release. We therefore investigated the ability of two different thermosensitive hydrogels to decrease the burst release of small rhBMP-2 microspheres. Our data demonstrate that CS-TA is a novel and safe hydrogel that decreases the burst release or rhBMP-2 microspheres. We hypothesise that the high viscosity and porous surface of the CS-TA hydrogel contributes to its ability to control burst release. Additionally, the CS-TA hydrogel could firmly adsorb rhBMP-2 microparticles on the surface of a 3-D porous Ti. Thus, rhBMP-2 microspheres could be combined with 3-D porous Ti to form a composite that has good osteogenetic induction and conduction for the treatment of large scale bone defects *in vivo*. Further, the CS-TA hydrogel is fluid at room temperature but becomes solid at 37 °C in only a few minutes, which is convenient for the surgery. Future studies being carried out at our institution will evaluate the *in vivo* ossific activity of the composite (rhBMP-2 microspheres combined with 3-D porous Ti using CS-TA hydrogel).

3. Experimental

3.1. Materials

The following machines and instruments were purchased from manufacturers as noted: GF-1 high speed disperser (Haimen Kylin-Bell Lab Instruments Co., Ltd, Jiangsu Province, China); THZ-D constant-temperature oscillator (Taicang Lab Instruments Co., Ltd, Jiangsu Province, China); ultrasonic disintegration instrument (Tianjin Henggao Technology Development Co., LTD, China.); environmental scanning electron microscopy (FEI-QUANTA600, FEI, USA); Laser Particle Sizer LS800 (OMEC Company, Zhuhai, China); NDJ-9S digital display viscosity instrument (Tianping Lab Instrument Co., Ltd, Shanghai, China). Reagents were purchased from manufacturers as noted: recombinant human bone morphogenetic protein-2 (rhBMP-2, Sino Biological Inc. Beijing, China); poly(lactic-co-glycolic) acid copolymer with mean MW 10000, PLGA 75/25, and terminal COOH (Jinan Daigang Biotechnology Co. Ltd, Shandong Province, China); rhBMP-2 ELISA kit (Sino Biological Inc. Beijing, China); Poloxamer (BASF, Germany); chitosan thioglycolic acid hydrogel (supplied by Tianjin University, China). All solvents were analytical grade.

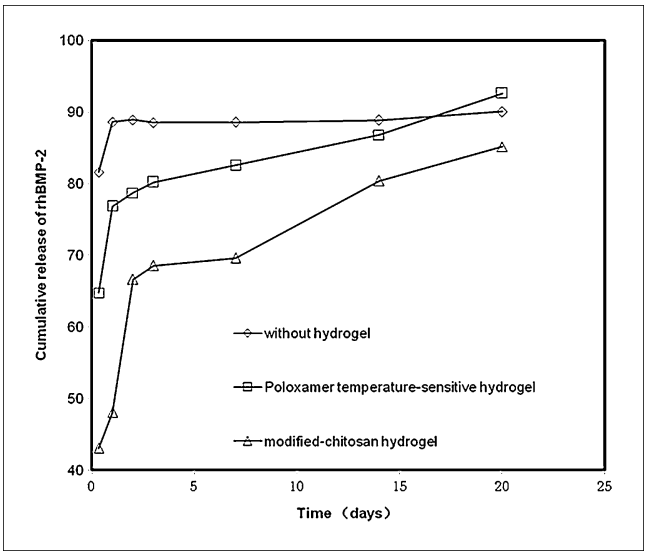


Fig. 5: Release profiles of rhBMP-2 systems combined with CS-TA and P407 hydrogel. Error bars indicate the standard deviation (n = 3)

3.2. Fabrication of microspheres

The microspheres were fabricated using a previously described water-in-oil-in water (W/O/W) double-emulsion-solvent-extraction technique (Oldham et al. 2000). First, 50 μg of solid state rhBMP-2 was homogenised with 150 μl PBS (pH 7.4), which serves as the inner water phase. The concentration of rhBMP-2 was 0.33 μg/μl. For the oil phase, 400 mg PLGA was dissolved in 1 ml of dichloromethane. An emulsion of the inner water and oil phases was formed by homogenisation for 30 s with an ultrasound in an ice bath. The resulting emulsion was immediately dropped into 65 ml of ice-cooled 1% polyvinyl alcohol (PVA, w/v) using a vortex at 5600 rpm to create the double emulsion and, subsequently, the microspheres. The dichloromethane was evaporated for 3 h with magnetic stirring at low speed. The microspheres were collected by centrifugation, washed three times with distilled deionised water, and vacuum-dried to obtain a free-flowing powder. The rhBMP-2 microspheres were stored at 4 °C.

3.3. Characteristics of rhBMP-2 microspheres

3.3.1. Particle size and morphology

Dynamic light diffraction measurements were performed to determine particle size. A small quantity of rhBMP-2 microspheres was dispersed in 5 ml H_2O. Subsequently, 20 μl of the dispersion was analysed in quintuplicate. The particle surface was observed with light microscope and the Environmental Scanning Electron Microscopy (SEM).

3.3.2. Encapsulation efficiency

Both the outer phase of the W/O/W emulsion and the washing water, which was regarded as loss during fabrication, were assessed for encapsulation efficiency. An rhBMP-2 ELISA kit (Sino Biological Inc. Beijing) was used to determine the amount of rhBMP-2 in the washing water. The amount of rhBMP-2 in the microspheres was calculated by subtracting the rhBMP-2 in the washing water from the total amount of rhBMP-2 used during fabrication. The formula for encapsulation efficiency is

$$\text{Encapsulation efficiency} = \frac{\text{Total amount-not encapsulated amount}}{\text{Total amount}} \times 100\%$$

3.3.3. Characterisation of rhBMP-2 microsphere-hydrogel composites

3.3.3.1. Hydrogel morphology. The two hydrogels described above were painted on a strip of double-sided tape and observed with Environmental Scanning Electron Microscopy.

3.3.3.2. Evaluation of thermosensitivity and viscosity of hydrogels. The temperature sensitivity of each hydrogel was evaluated by placing the hydrogels in a 37 °C water bath. The time required for the hydrogel consistency to change from a fluid to a gel was recorded. The viscosity of different concentrations of each hydrogel was measured with an NDJ-9S digital display viscosity instrument (Tianping Lab Instrument Co., Ltd, Shanghai, China).

3.3.3.3. Safety evaluation of hydrogels - haemolytic test. Fresh rabbit red blood cells were used to evaluate the ability of CS-TA and poloxamer hydrogels to induce haemolysis. Different concentrations of the thermosensitive hydrogels were prepared (25%, 50% and 100% of the hydrogels) for the haemolytic test: 2 ml 25% CS-TA gel, 4 ml 50% CS-TA gel, 8 ml 100% CS-TA gel (with or without PEGDA), 25% poloxamer gel, 50% poloxamer gel, and 100% poloxamer gel. Anti-coagulated rabbit blood (0.2 ml) was added to (i) different concentrations of the hydrogels, which were incubated in cell nutritional fluid at 37 °C for 72 h in advance of the assay; (ii) physiological saline solution (negative control); and (iii) distilled water (positive control).

The contents of the tubes were gently mixed and incubated in a 37 °C water bath for 1 h. The suspension was then centrifuged at 2000 rpm for 5 min and absorbance of the supernatant of each tube was measured by ultraviolet spectroscopy (DU-640 nucleic Acid and Protein Analyzer, BECKMAN COULTER, USA) at 545 nm. Samples were run in triplicate. Results were analysed using SPSS statistical software.

3.4. In vitro release of rhBMP-2 microspheres and their composites

Eighty milligrams of rhBMP-2 microspheres was added with 2.4 ml phosphate buffered saline (PBS pH 7.4), and 2.4 ml poloxamer hydrogel, 2.4 ml CS-TA hydrogel or 2.4 ml vehicle. The three rhBMP-2 sustained-release systems were homogenised and divided into 24 0.1 ml Eppendorf tubes, which were then diluted with PBS buffer to a final volume of 1 ml.
Each sample was placed in a constant temperature shaker (maintained at 37 °C, 100 rpm) and analysed at each time point (8 h, 1 d, 2 d, 3 d, 7 d, 14 d, 21 d). A sandwich ELISA kit (Sino Biological Inc. Beijing) was used to determine the concentration of rhBMP-2. All batches were assessed in triplicate.

Acknowledgements: The authors would like to thank Yongling Huang and Juan He at Baimtec Material Co. Ltd. for their assistance with the Scanning Electron micrograph for rhBMP-2 microspheres and hydrogels. This work was made possible in part by funding from the National High Technology Research and Development Program ("863" Program) of China (2007AA021903).

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