CHAPTER VII
GELATIN MICROSPHERES IMPREGNATED POROUS SCAFFOLDS OF 1,6-DIISOCYANATOHEXANE-EXTENDED POLY(1,4-BUTYLENE SUCCINATE) FOR CONTROLLED RELEASE OF PROTEIN

7.1 Abstract

Scaffolds nowadays can be proposed as a route of biological factors transportation besides being a supporting matrix for tissue regeneration. In this study, a protein delivery scaffold was fabricated from poly(1,4-butylene succinate) extended with 1,6-diisocyanatohexane (PBSu-DCH) by solvent casting and particulate leaching technique where the model protein (tetramethylrhodamine conjugated bovine serum albumin, BSA-Rhod) loaded gelatin microspheres were impregnated via matrix of hyaluronan-gelatin blends. Release of model protein was investigated in various releasing conditions and scaffold features i.e. pore size and proportion of hyaluronan and gelatin in matrix (HA:Gel). The release kinetics in media with various concentrations of ions (NaCl) suggests electrostatic interaction between positive charge gelatin microspheres and negative charge BSA-Rhod. The release of BSA-Rhod from scaffolds with 200-400 μm pores presented lower rates and amounts than from those with 400-500 μm pores for any HA:Gel proportion. The higher but favorable release was found in scaffolds impregnated with 1:1 HA:Gel matrix comparing to 1:3 or 3:1 particularly in minimum essential medium. Thus, controlled release of protein can be achieved by employing the microspheres and tailoring the proportion of HA-Gel matrix which is effective for PBSu-DCH scaffolds of either 200-400 or 400-500 μm pores.

(**Key-words**: scaffolds; gelatin microspheres; controlled release; poly(butylene succinate)

7.2 Introduction

Scaffold, an extracellular matrix simulated three-dimensional construct, serves as a supporting temporary matrix for cells to proliferate and maintain their differentiated functions during process of tissue engineering (Hutmacher, 2000; Mikos *et al.*, 2004). However, with a requirement of simultaneous control of complex cellular behaviors for the regeneration or replacement of lost cells and tissue, the concept of cellular guidance which is a new knowledge regarding the cell-material interaction in tissue regeneration (Causa *et al.*, 2007; Tessmar and Gopferich, 2007) has been concerned in designing scaffold to be a route to transport biological factors. The novel tissue engineering scaffold, therefore, can be considered as a special type of drug delivery apparatus (Tessmar and Gopferich, 2007) or a drug delivery scaffold.

The design of drug delivery scaffold has been demonstrated in several profiles. Drug or therapeutic agent may be either directly mixed in the scaffold's mass (Whang *et al.*, 2000; Hariraksapitak *et al.*, 2008) or carried in a separate delivery device which is further integrated into the scaffold (Holland *et al.*, 2005; Ungaro *et al.*, 2006). The separate drug delivery devices themselves have been designed in multiple forms and configurations. However, most systems base on encapsulation or entrapment of active substances in biocompatible polymeric devices (Baldwin and Saltzma, 1998) and their effectiveness in controlling drug release and enhancing tissue engineering have been widely studied (Urist, 1965; Syftestad and Caplan, 1984; Cho *et al.*, 1992; Hou *et al.*, 2000). Among multiple applicable drug delivery devices, Gelatin microspheres have been extensively studied for the accomplishment in controlled release of several therapeutic agents such as antihypertensive drug (Somerman *et al.*, 1983), signaling proteins like albumin (Lee *et al.*, 2007), chondroitin 6-sulfate (Freiberg and Zhu, 2004), bFGF (Kimura *et al.*, 2003), IGF and TGF-β (Holland *et al.*, 2005), or even the plasmid DNA (Kasper *et al.*, 2005). Gelatin microsphere evidently is a utilized drug delivery device.

Gelatin is biocompatibility and biodegradability thus commonly used in pharmaceutics and medicine. Gelatin is structurally a heterogeneous mixture of a single or multiple stranded polypeptides and their oligomers each of which contains

about 300-4,000 amino acids (Tabata and Ikada, 1998; Young *et al.*, 2005). Molecules of gelatin are polyelectrolyte, presenting diverse isoelectric point (IEP) which are about 3-5 and 7-9 for the alkaline and acidic treated gelatin, respectively. Gelatin, according to the different IEP, can be selectively designed to from complex with the oppositely charged molecule like proteins, to be the polyion complexation (Seyrek *et al.*, 2003; Dumetz *et al.*, 2007) which is quite stable and unlikely to dissociate simultaneously. Polyion complexes thus are durable than bonding between low molecular weight electrolytes (Young *et al.*, 2005).

This contribution proposes a novel drug delivery scaffold containing gelatin microspheres as separate drug delivery device. Porous scaffolds, in which gelatin microspheres were impregnated, were fabricated with 1,6-diisocyanatohexane-extended Poly(1,4-butylene succinate) (hereafter, PBSu-DCH) by solvent casting and particulate leaching technique. PBSu-DCH is a modification of the Poly(1,4-butylene succinate) (PBSu) that was developed by coupling PBSu with hexamethylene diisocyanate as a chain extender (Nikolic and Djonlagic, 2001). An introduction of such non-crystallizable units into the polymer chains, which causes both the melting temperature and the crystallinity to decrease, when compared with those of the pure polymer (Nikolic *et al.*, 2003) was anticipated to possess an improved biodegradability. The particle-leached, porous PBSu-DCH scaffold was proved in our previous study to be biocompatible with osteoblasts and suitable for scaffolding functions (Hariraksapitak *et al.*, 2008) particularly in the dental root socket.

In order to simulate environment of extracellular matrix (Alberts *et al.*, 2002), encapsulated gelatin microspheres were impregnated in the PBSu-DCH scaffold via a matrix produced by blending gelatin and hyaluronan (HA) (hereafter, HA-Gel matrix). Hyaluronan performs many functions in extracellular matrix like being a cells supporting structure, controls cell adhesion, proliferation, differentiation and cell mobility (Kikuchi *et al.*, 2001). It is also a medium in which the reaction between binding protein, proteglycan and other biomolecules like growth factor occurs (Alberts *et al.*, 2002). Blending of hyaluronan with other biomaterials was found to demonstrate better scaffolding properties than either material alone (Oerther *et al.*, 1998; Liu *et al.*, 1999; Oerther *et al.*, 1999; Hoffman, 2002; Chang *et al.*, 2003; Liu *et al.*, 2004). Additionally, the microspheres were expected to be securely

bound to the scaffold where release of drug, or the Tetramethylrhodamine conjugated albumin from bovine serum (BSA-Rhod); a model protein in this study, could be controlled.

With an aim to fabricate a new drug delivery scaffold of PBSu-DCH containing BSA-Rhod loaded gelatin microspheres impregnated via HA-Gel matrix. This study investigated the effect of the two factors of scaffold's pores sizes and proportion between HA and gelatin of the matrix on the release characteristics of BSA-Rhod.

7.3 Experimental section

7.3.1 Materials

Poly(1,4-butylene succinate) extended with 1,6-diisocyanatohexane (PBSu-DCH; pellet form; MW 06242; Batch #09717). Hyaluronan (MW $1.35x10^6$) was purchased from Coach Industries Inc (Japan). Gelatin from porcine skin (type A, Bloom no.170-180) was purchased form Fluka (Switzerland). Albumin from bovine serum, tetramethylrhodamine conjugate (MW 66,000 Da) was purchased from Molecular Probes Inc (USA). Saturated Glutaraldehyde aqueous solution (5.6 M) was purchased from Fluka (Switzerland). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased form Fluka (Switzerland). Acetone (AR grade) and Chloroform (AR grade) were purchased from Lab-Scan (Thailand). Sodium chloride (NaCl; powder form) was purchased from Ajax Finechem (Australia). All other chemical agents were of analytical grade and used without further purification.

7.3.2 Preparation of PBSu-DCH scaffolds

The PBSu-DCH scaffolds were fabricated by the solvent casting and particulate leaching technique. First, PBSu-DCH pellets were dissolved in chloroform at 50°C to obtain the PBSu-DCH solution at a fixed concentration of 15% w/v. The solution was left to cool down to room temperature. NaCl particles, a priori sieved to obtain particles with diameters in the range of 200-400 or 400-500 μm, were then added into the solutions at 35% weight ratio of NaCl to PBSu-DCH. The pasty suspensions were homogenized and a volume of the suspensions was filled

with slight pressing in Petri dishes to obtain molding samples. The moldings were placed in a fume hood overnight to allow evaporation of the solvent. NaCl particles were then leached out by immersing the moldings in deionized (DI) water for 48 h, with replacing of DI water every 8 h. The obtained scaffolds were dried in vacuo at room temperature for 24 h. The moldings cast in the Petri dishes were later cut into circular shape with 15 mm in diameter and 3 mm in height and kept in desiccators until use.

7.3.3 Preparation of BSA-Rhod loaded gelatin microspheres (BSA-Micros)

Gelatin microspheres were firstly prepared by a thermal gelation technique (Esposito *et al.*, 1996) with modification. In detail, 10 ml of 15% w/v gelatin aqueous solution was prepared at 40°C. Then 0.1%, on the weight of dry gelatin, of the tetramethylrhodamine conjugated Albumin from bovine serum (BSA-Rhod) was added and homogeneously mixed. The solution was drip into 200 ml of Soya oil preheated at 40°C under continuously stirring at 1,000 rpm with a homogenizer to form water-in-oil emulsion. After 10 min, temperature of the emulsion was reduced to be 4°C with an ice bath while stirring was continued for an additional 30 min to allow for physically thermal gelation of the gelatin. Afterward, 200 ml of pre-cooled (4°C) acetone was added and stirred for the next 60 min in order to dehydrate and flocculate the coaceravate droplets. The microspheres were collected by filtering the mixture through a 1 μm-pored sintered glass filter under vacuum, washed three times with 100 ml of cool acetone, and dried in air at room temperature over 24 h.

To crosslink the gelatin microspheres, 250 mg of the dry microspheres was suspended in 10 ml of acetone-water (2:1, v/v) containing 1% (w/v,~100mM) Glutaraldehyde solution and stirred at 4°C, 500 rpm for 1 h. The crosslinked microspheres were collected with a sintered glass filter and washed with precooled acetone. The crosslinked microspheres were then suspended in 20 ml of 10 mM aqueous glycine solution containing 0.1 wt% of Tween 80, shaken at 37 °C, 50 rpm for 1 h to block the residual aldehyde groups of the unreacted glutaraldehyde. The crosslinked microspheres were then washed twice with 60 ml of the cool deionized water (4°C), with cool acetone, filtered, and eventually air-dried at room temperature

for over 24 h. The as-prepared microspheres were stored at 4°C and protected from light until ready to use.

7.3.4 Preparation of impregnated PBSu-DCH scaffolds

PBSu-DCH scaffolds were impregnated with the neat HA-Gel blend or the HA-Gel blend suspending with BSA-Micros by a simple dispersion method. In detail, the 2% (w/w) aqueous solution of a HA and gelatin mixture (HA:Gel were 1:1, 1:3 and 3:1 by wt.) was firstly prepared at 50°C and left to cool down to room temperature. The 1 mmole of NaCl was then added and being mixed for 30 min with an aim to facilitate blending between HA and gelatin. The resulting mixture would become clearer and more translucent. Then the calculated amount of EDC (1 mmole) was added and reacted under the 200 rpm stirring at room temperature for 2 h to crosslink the polymers. For the suspension of BSA-Micros in HA-Gel blend, it was further prepared by adding the crosslinked BSA-Micros in the HA-Gel mixture at the concentration of 2.5 mg% (w/v). The suspension was continuously stirred until the microspheres were well dispersed as observed.

For impregnation, 200 μl of the neat HA-Gel mixture or the mixed suspension which contained 5 mg of BSA-Micros (equal to 5 μl of the BSA-Rhod) was evenly dropped on top of a circular shape PBSu-DCH scaffold with a micropipette, followed by immersing the piece of scaffold in ethanol for 30 min, air-dried at room temperature for over 24 h and kept in desiccators until use.

7.3.5 Characterization

7.3.5.1 Morphological observation

A BSA-Micros impregnated PBSu-DCH scaffold was randomly selected from each group of samples and cut into 2 pieces along the center. The cut pieces were mounted on copper stab by pointing up the top and the cut edges. The BSA-Micros were mounted directly on a separated copper stab. All specimens were coated with gold using a JEOL JFC-1100 sputtering device, and observed for their microscopic structure using JEOL JSM-5200 scanning electron microscopy (SEM).

7.3.5.2 Confocal Laser Scanning Microscopy Imaging

Distribution of the BSA-Rhod within gelatin microspheres was investigated using a confocal laser scanning microscopy (CLSM) (Olympus FluoView™ FV1000, Japan). The fluorescent dye was excited by Helium-Neon (green) laser at 543 nm. The images were scanned by 8 bit plane mode with 3 channel dimensions and the image size was 512×512 pixels. The neat gelatin microspheres were imaged as the control.

7.3.5.3 Water absorption capability

The test of water absorption capability was carried out on the neat impregnated PBSu-DCH scaffold. Briefly, the scaffold specimens were first dried, weighed, and individually immersed in 2 mL of 10 mM phosphate buffer saline solution (PBS; pH 7.4) at room temperature. At a given time point, the specimens were taken out, blotted on a glass plate which was set at ~30° from a horizontal baseline for 5 s to remove excess water, and immediately weighed. The amount of water absorbed in the specimens was determined according to the following equation:

$$\text{Water absorption (\%)} = [(W_w - W_d)/W_w] \times 100,$$

where W_d and W_w are the weight of the specimens before and after submersion in the medium, respectively. The experiment was carried out in pentuplicate and the measurements were carried out at different time intervals within a period of 72 h.

7.3.5.4 Actual loading of BSA-Rhod in microspheres

Actual loading of the BSA-Rhod in gelatin microspheres were detected in 37% HCl by centrifugal method. 5 mg of BSA-Micros were suspended in 100 µL of 37% HCl in a 1.5 ml microcentrifuge tube. The tube was then placed in a 37°C shaking water bath at 70 rpm for 1 h. The suspension was diluted with 10 mM PBS with 0.15 M NaCl to the volume of 1 mL, slightly vertex and centrifuged at 5,000 rpm for 10 min. The supernatant was collected and quantified for BSA-Rhod using spectrofluorometer (Cary Eclipse™) at 541 and 572 nm of the excitation and emission wavelength respectively. The amount of BSA-Rhod was determined by correlating to a BSA-Rhod (Molecular Probes™) standard curve over the concentration range 1-50 µg/ml ($r2=0.99^6$). The experiment was carried out in triplicate and the results were presented in terms of Encapsulating efficiency of BSA-

Rhod (EE) and Loading capacity of gelatin microspheres (LC), which were determined according to the following equation (Freiberg and Zhu, 2004):

$$\text{Encapsulating efficiency (\%)} = \frac{\text{total } \mu g \text{ BSA-Rhod encapsulated}}{\text{initial } \mu g \text{ BSA-Rhod loaded}} \times 100$$

$$\text{Loading capacity (\%)} = \frac{\text{total mg BSA-Rhod encapsulated}}{\text{total mg microspheres}} \times 100$$

7.3.5.5 In vitro BSA-Rhod release

In vitro release of BSA-Rhod from gelatin microspheres and the microspheres impregnated PBSu-DCH scaffold were investigated by a standard sampling-separation method. The 5 mg of BSA-Micros were separately immersed in 1 mL of 10 mM PBS with 0.15, 0.5 or 1.0 M NaCl, 1 mL of the prepared Simulated Body Fluid (SBF) (Oyane *et al.*, 2003; Ohtsuki, 2008) or 1 mL of the minimum essential medium (MEM; Gibco™, Invitrogen). All samples were incubated in a shaking water bath (70 rpm) at 37°C. At a given time point, 500 µl of the releasing medium (hereafter, the sample solution) was withdrawn and an equal amount of fresh medium was added to maintain a constant volume of the medium. The sample solution was centrifuged at 5000 rpm for 5 min at room temperature and the amount of CBP-Rhod in the sample solution was determined by spectrofluorometry at 541 and 572 nm for the excitation and emission respectively, as previously described. An average value was calculated at each time point. The experiment was done in triplicate.

The release of BSA-Rhod form the microspheres impregnated PBSu-DCH scaffold was investigated in 1 mL of 10 mM PBS with 0.15 M NaCl and in MEM. Scaffolds were separately immersed in 2 mL of releasing medium and tested with the same procedure as mentioned above. The experiments were run on the scaffold specimens prepared with all conditions in triplicate and the mean ± sd. values were reported.

7.3.6 Statistical analysis

Data were analyzed using the SPSS software version 14.0 for window. Initially, the normal distribution was assessed by the Shapiro-Wilk test. The normal distribution data, representing the homogeneity of the variances, shown by the

Levene's test, were then investigated by the one-way analysis of variance (ANOVA) with the Tukey HSD post hoc multiple comparisons. Otherwise, the Dunnett T3 would be applied if the data did not exhibit the homogeneity of the variances.

7.4 Results and Discussion

7.4.1 Morphological observation

Pictures of the as-prepared gelatin microspheres and scaffolds are shown in Figure 7.1 a-b. The microspheres apparently are fine powder with different color according to BSA-Rhod content. The selected SEM images demonstrate spherical geometry with a smooth surface, on which the macroscopic pores were not detected for both the neat and BSA-Rhod loaded microspheres (Figure 7.2). Aggregation of various-sized microspheres into small clusters can be detected in both cases. In our opinion, such aggregation was caused by the direct contact between the adjacent particles once the solvent was expelled during microspheres preparation. The electrical charge on the surface of particles might be diminished in dry environment so that the electrostatic repulsive force was also weakened; as a consequence, repulsion among particles was unlikely illustrated. The microscopic morphology of the neat and BSA-Rhod gelatin microspheres were not different when detected by SEM, thus.

The impregnated scaffolds exhibit color of gelatin microspheres throughout the bulk defining that proper distribution of the impregnated microspheres was achieved by the procedure we decided (Figure 7.1 c-e). Selected SEM images illustrating microstructure of the scaffolds when being viewed on the top and longitudinal cutting surface are shown in Figure 7.3-7.4. For any given type of the scaffolds, there was no significant difference in the microstructure observed along both directions. A well-defined cubical porous structure, with the polymer mass conforming to the cubic particles of NaCl used in the fabrication process, was evident in scaffolds prepared with any given condition. In addition, the inter-pore connectivity containing clusters of gelatin microspheres was generally observed on both the top and longitudinal cut specimen. As observed closely on every sample of HA-Gel proportion, gelatin microspheres were extensively attached on scaffold's wall with HA-Gel matrix without deterioration of their geometry (Figure 7.5).

However, exceptional for the quite small particles, almost microspheres did not thoroughly submerge in the thin sheet of HA-Gel matrix. They were partially covered. This manifestation may be responsible for controlled release of the absorbed protein since the releasing medium could transport through the exposed microspheres differently from the one covered under HA-Gel matrix.

7.4.2 Water absorption capability

The test of water absorption capability within 72 h at room temperature on both the neat impregnated and non-impregnated PBSu-DCH scaffold is graphically shown in Figure 7.6. The non-impregnated scaffolds, for both 200-400 and 400-500 μm pores sizes, demonstrated an indifferent water absorption profile, i.e., an abrupt increase in the level of water absorption during the first 24 h at ~94%. The result, even of the scaffolds with smaller pores sizes of 200-400 μm used in this study, was similar to that of our previous study in 2008 on the same material (Hariraksapitak *et al.*, 2008) and the study of Park et al. in 2002 on a collagen-hyaluronan sponge (Park *et al.*, 2002). The non-impregnated PBSu-DCH scaffolds themselves exhibited relatively high water absorption capability.

For the impregnated scaffolds, HA-Gel matrix seems to accelerate scaffolds' water absorption no matter what proportion it was. Absorption reached the highest level at ~ 93-94% within the first 1-3 h of submersion time. Nonetheless, water absorption of the impregnated scaffold was evidently lesser than that of the non-impregnated one. Data analysis with the Univariate analysis of variance; test of between-subjects effects, demonstrated significant different of water absorption capability at 24 h by the influence of interaction between scaffold's pores sizes and HA:Gel matrix proportion. The result was reasonable since HA and gelatin in the matrix were crosslinked with EDC in which both amide and ester bond were expected to be generated (Prestwich, 2001) respectively, between the carboxyl group of the gelatin and/or of the glucuronic acid in hyaluronan and amino group of the gelatin, and between such carboxyl group and the hydroxyl group of gelatin and/or of hyaluronan (Tomihata and Ikada, 1997; Sannino *et al.*, 2005). The HA-Gel matrix was afterward dehydrated and precipitated with ethanol and became unpredictably random network inside the scaffolds. The data were thus reorganized into six discrete groups and analyzed further with one-way ANOVA. The result exhibited influence

of scaffold pores sizes which was significantly observed only on the 1:1 HA:Gel matrix. Impregnating with HA:Gel matrix seemed to insignificantly decrease water absorption except for the 1:1 and 1:3 matrix in 200-400 and 400-500 μm pores scaffolds, respectively (Figure 7.6 a-b).

The rapid and high water absorption capability of the as-prepared scaffolds resembles the characteristic of hydrogel material which are very hydrophilic (Drury and Mooney, 2003). From scaffolding standpoint, the as-prepared scaffolds were obviously good hydrated environment in which cells including their products could be safely protected and effective transportation of nutrition was promoted (Hoffman, 2002).

7.4.3 Loading of BSA-Rhod in microspheres

The concept of polyion complexation, of about electrostatic interactions between positively or negatively charged, high molecular weight electrolytes and their oppositely charged partners, had been used for the coupling of controlled release carrier and therapeutic agent in this study. With isoelectric point *(pI)* about 9 similar to that of collagen (Vandervoort and Ludwig, 2004; Young *et al.*, 2005), Gelatin A (acidic gelatin) exhibiting a net positive charge at neutral pH was chosen to react with BSA which is a globular protein containing a net negative charged at physiological conditions (Menon and Zydney, 1998). Considering the studied condition, pH of the as-prepared gelatin solution was 5.2. BSA still rendered a net negative charge in such pH condition since BSA *pI* was about 4.7. It was expected that direct mixing of the BSA in gelatin aqueous solution provoked electrostatic interactions between those two opposite charged molecules.

Loading of BSA-Rhod in the prepared microspheres was qualitatively verified by their CLSM images as shown in Figure 7.7. As observed, the color image of BSA-Micros illustrates much higher intensity of luminosity than that of the neat microspheres which were hardly detectable devoid of the correspondent grayscale images. The consistent red color inside BSA-Micros indicates well the good internal distribution of BSA-Rhod (Figure 7.7 c).

Quantitatively, the obtained encapsulation efficiency of BSA-Rhod ranges between 72-78% with an average of 75% (Table 7.1). It was roughly at the lower value of the encapsulation of the crude bone protein (70-90%) which was

directly absorbed into the post-crosslinked, dry gelatin microspheres as demonstrated in our previous study, and was also lower than that of the BSA loaded gelatin microspheres prepared in the mixture of toluene and chloroform by Mladenovska et al. in 2002 (~80-95%) (Mladenovska *et al.*, 2002). Since BSA-Rhod is readily soluble in water (Zhang *et al.*, 2007), crosslinking of gelatin-BSA-Rhod microspheres with glutaraldehyde in the mixture of acetone and water, and being washed afterward with cool water during filtrating collection was capable to extract a substantial amount of BSA-Rhod from the microspheres (Ugwoke *et al.*, 1997). However, the 75% of the encapsulation efficiency seems to be comparable with that found in certain literatures (Esposito *et al.*, 1996; Ugwoke *et al.*, 1997; Vandervoort and Ludwig, 2004).

Loading capacity determines the capability of a carrier to contain a drug at utmost quantity. Loading capacity of the as prepared gelatin microspheres was ~75 μg BSA-Rhod per 100 mg microspheres. It was about 75% of the theoretical loading which was initially decided at 0.1% (0.10 mg of BSA-Rhod per 100 mg of microspheres). The obtained loading capacity is in the range of growth factor therapeutic dose that promotes bone healing (30-80 μg of IGF-1) (Luginbuehl *et al.*, 2004) and much more than the physiologic concentration of BMP-2 in normal bone which is ~2 ng/g and sufficient for bone healing (Wang *et al.*, 1990). The study of Brown et al. in 1998 demonstrated a direct relationship between loading capacity of albumin and concentration of the loaded protein in gelatin/chondroitin 6-sulphate microspheres, which ranged from 2.0 mg/ml to 12.5 mg/ml (Brown *et al.*, 1998). In this study, concentration of the loading BSA-Rhod was only 1.25 mg/ml due to limited amount of the substance. Thus, the increasing loading capacity of the prepared microspheres might be achieved by increase the concentration of the loaded BSA-Rhod.

7.4.4 In vitro BSA-Rhod release

7.4.4.1 Effect of ionic strength on BSA-Rhod release form gelatin microspheres

With an aim to study the electrostatic interaction, release of the BSA-Rhod from microspheres was initially conducted in PBS with various ionic strengths of 0.15, 0.5 and 1.0 M, being models of the low, medium and high ionic

strength, respectively (Zhang *et al.*, 2007). The release profiles are shown in Figure 7.8 and the data were curve-fitted to the semi-empirical equation based on a power-law expression as (Ritger and Peppas, 1987a; Arifin *et al.*, 2006)

$$Mt/M\infty = kt^n$$

where $Mt/M\infty$ is the fractional release of the BSA-Rhod (%), k is a constant concerning the structure and geometry of the releasing device, and n is the releasing exponent indicating the mechanism of drug release. Results are presented in Table 7.2.

As observed, release of BSA-Rhod in low ionic strength medium was consecutively much higher than in high ionic strength medium (Figure 7.8), corresponding with the study of Lee et al. in 2007 for BSA release from collagen gels (Lee *et al.*, 2007). Release kinetic demonstrates comparable n values but remarkably different k values among conditions of different ionic strengths. The interaction between protein-protein and protein-salt has to be taken into account in order to describe the result.

BSA-Rhod, in this study, was mixed in gelatin solution of pH 5.2 at 40 °C. In such pH and temperature, BSA was in compact (N) form and chain unfolding did not occur since chain conformation alters at pH below 4.5, or at temperature over 56°C in general (Michnik, 2003; Michnik *et al.*, 2005). BSA thus probably reacted with gelatin as a non-gelling, soluble filler-liked component which interspersed throughout gelatin network. Interaction between those two proteins might proceed with several contributions i.e. electrostatic and van der Waals forces, hydrogen bonding, hydration effects, salt bridging, and ion binding (Dumetz *et al.*, 2007) which was difficult to be identified. Chemical crosslinking with bifunctional glutaraldehyde induced intra-and intermolecular covalent bond by reacting predominantly with free amino groups particularly that of lysine as (Molin *et al.*, 1978):

$$\text{Protein-}NH_3^+ + \text{OHC-}(CH_2)_3\text{-CHO} \rightarrow \text{Protien-N=CH-}(CH_2)_3\text{-CHO}$$

The resulting gelatin microspheres, therefore, did not undergo appreciable degradation in aqueous solution. Cumulative release of BSA-Rhod within the first 24 h which were less than 10% in every test groups reveals that

release was predominantly determine by gelatin degradation (Young *et al.*, 2005). However, the different influence of ionic strengths on BSA-Rhod release kinetic indicates a considerable contribution of electrostatic interaction between BSA-Rhod and gelatin carrier as well (Young *et al.*, 2005). Many studies have proved that increasing the ionic strength induces the protein interaction potential changes smoothly from a repulsive to an attractive potential (Seyrek *et al.*, 2003; Dumetz *et al.*, 2007; Zhang *et al.*, 2007). At low ionic strength (0.15 M NaCl), the net surface charge is screened leading to dominant neutralization of charges on the protein so that the total interaction exhibits the repulsive interaction (Seyrek *et al.*, 2003; Zhang *et al.*, 2007). BSA-Rhod consequently released out from gelatin microspheres at the highest rate and amount. At a moderate ionic strength (0.5 M NaCl), the surface charges are so sufficiently screened that the strength of proteins interactions are rather weak but still stronger than in low ionic strength. Upon the increase of ionic strength (1.0 M NaCl), the surface charges are highly screened, and protein-salt interaction is dominated by the unfavorable interaction between the salt ions and the hydrophobic residues of the protein, producing a "salting out" effect. The overall interaction potential was dominated by an additional attractive potential leading to lowest release of BSA-Rhod (Dumetz *et al.*, 2007; Zhang *et al.*, 2007). An increase of ionic strength, therefore, decreases the repulsive force and weakens the correlation between protein molecules in solution.

7.4.4.2 Effect of releasing medium on BSA-Rhod release form gelatin microspheres

Release of the BSA-Rhod from microspheres was further studied in c-SBF (conventional SBF) and MEM to investigate release kinetics in the simulated condition of human body and in-vitro culturing environment. The c-SBF was selected because the ion concentration (and pH value) can be maintained over a period of up to 8 weeks to be approximately equal to those of human blood plasma (Oyane *et al.*, 2003). The release profiles were evaluated together with that in 0.15 M NaCl-PBS as shown in Figure 7.9 and data analysis was done as mention above (Table 2).

The released profiles in c-SBF and 0.15 M NaCl-PBS were identical as expected though releasing parameters (k and n values) of the 0.15 M

NaCl-PBS is somewhat higher. The c-SBF contains Na^+ and Cl^- ions at the concentration of 0.142 and 0.147 M respectively (Oyane *et al.*, 2003). Thus, ionic strength of the c-SBF is slightly lesser but quite closed to that of 0.15 M NaCl-PBS. Electrostatic interaction between BSA-Rhod and gelatin should be slightly more repulsive in c-SBF than in 0.15 M NaCl-PBS, bringing about the comparable range of higher BSA-Rhod release. The studies of BSA-Rhod release from the impregnated scaffold, therefore, were further conducted in 0.15 M NaCl-PBS and MEM only.

On the contrary, release of BSA-Rhod in MEM is significantly higher. Releasing parameter shows a similar n, but much larger k value than that of in PBS. The MEM used in this study contained plenty of much high molarity inorganic salts comparing with those in PBS or c-SBF, i.e. NaCl 117.24 M, KCl 5.33 M, $CaCl_2$ 1.8 M, NaH_2PO_4-H_2O 1.01 M (Invitrogen, 2009). According to electrostatic interaction, such high ionic strength should promote aggregation of protein and retard release of BSA-Rhod but the results were not as expected. It is plausible that degradation of gelatin microspheres might concurrently occurred and concealed the effect of electrostatic interaction due to such high concentration of inorganic salts assisted in the attack of water on the peptide bonds and cross-links, and led to degradation of gelatin matrix (Bosch and Gielens, 2003). BSA-Rhod release in MEM, therefore, was mainly controlled by degradation or dissolution process.

Considering the kinetic parameters of BSA-Rhod release from the prepared microspheres in any releasing medium (Table 7.2), n values range between 0.32 and 0.40. As the theoretical n values for the Fickian diffusion and the Case-II transport are 0.43 and 0.85 respectively for monodispersed spheres, and are 0.30 and 0.45 respectively for multiple-sized microspheres (Ritger and Peppas, 1987a, b), mechanism of BSA-Rhod release from gelatin microspheres should be the combination of diffusion and dissolution.

7.4.4.3 BSA-Rhod release form impregnated PBSu-DCH scaffolds

The study of BSA-Rhod release from impregnated PBSu-DCH scaffolds was conducted on two variables; scaffold's pore size and HA:Gel proportion. According to Figure 7.10, the release profiles of 200-400 μm scaffolds in PBS present lower rates and amounts than those of 400-500 μm groups for any

HA:Gel proportion. Astoundingly, release profiles within group of 400-500 μm scaffolds are indistinguishable suggesting that all releases of BSA-Rhod are comparable, and also to that of the gelatin microspheres. Kinetic parameters of these samples are approximately alike for both *k* and *n* and indifferent much from values of the gelatin microspheres (Table 7.3). Such phenomenon indicates incompetence of the mentioned scaffold on the aspect of being a control released device. Though scaffold with 400-500 μm pores demonstrates insignificantly higher water absorption determined by weight change (Figure 7.6), the consequences on BSA-Rhod release is noticeable. The larger pore size of scaffolds, therefore, may conceal effect of HA:Gel matrix of which influence is more prominent in the 200-400 μm groups.

As observed on the 200-400 μm scaffolds, sample of 1:1 exhibits indifferently lower release profile comparing to gelatin microspheres while the 1:3 and 3:1 are significantly lower. Apparently, release profiles seem not to be compatible with water absorption capability of the correspondently neat, impregnated scaffold (Figure 7.10) as the 1:1 samples presents the lowest water absorption capability but the highest BSA-Rhod release. Kinetic parameter of these samples also shows variety of *k* and *n* values. All evident suggests that BSA-Rhod release in this group was controlled by multiple factors, especially by the HA:Gel matrix.

According to SEM (Figure 7.5), the randomized, partial coverage of the incorporated gelatin microspheres by network of HA-Gel matrix has been generally observed. The function of HA-Gel matrix, therefore, was not only in affixing microspheres onto scaffolds' walls, but also in restricting the transportation of releasing medium, as well as BSA-Rhod, through the gelatin microspheres and scaffold. The unequal ratio of HA to gelatin (1:3, 3:1) was found to obviously retard BSA-Rhod release more than the equal one, even in MEM where the degradation of gelatin microspheres was expected to occur (Figure 7.11).

In MEM, impregnation of gelatin microspheres in the PBSu-DCH by HA-Gel matrix apparently decelerates the release of BSA-Rhod. The influence of pores size seems to be negligible since release profiles of an individual HA:Gel proportion are quite comparable between those two scaffolds with different pores sizes, though that of the 1:1 is slightly higher for the 400-500 μm sample. In

addition, kinetic parameters of the samples are much varied similar to that of the 200-400 μm samples in PBS release. Therefore, it was plausible that BSA-Rhod release was mainly determined by the resistance of HA-Gel matrix to be degraded by MEM while diffusion from gelatin microspheres took minor counterpart. The study of Liu et al. in 2004 demonstrated the better resistance to lysozyme degradation of the Chitosan-Gelatin-Hyaluronan composite membrane at the higher amount of HA composition (Liu *et al.*, 2004). On the contrary, the higher amount of gelatin in HA-Gelatin hydrogel films was also found to increase density of disulfide-crosslink in the study of Shu et al. in 2003 (Shu *et al.*, 2003). At a constant total concentration, the equal amount of HA:Gel, thus, provide HA-Gel matrix that resist to MEM degradation at minimum. From the practical standpoint, however, an optimal amount of initial protein release is necessary for the early process of tissue regeneration (Baldwin and Saltzma, 1998). The as-prepared scaffold of either 200-400 or 400-500 μm provided favorable BSA-Rhod release when being impregnated with 1:1 HA:Gel matrix, since up to 11.43% (493 ng) and 14.07% (666 ng) of BSA-Rhod was detected in both scaffold within the first 10 h, respectively. The obtained result is consistent with the study of Holland et al. in 2005, on the insulin-like growth factor-1 release form the Oligo(poly(ethylene glycol)fumarate)–gelatin microspheres composite in which burst release was ~14% (Holland *et al.*, 2005).

Apparently, the release of BSA-Rhod can be successfully controlled for a slower rate and lesser amount by impregnating the protein-loaded gelatin microspheres in PBSu-DCH with HA-Gel matrix. Scaffolds with smaller pores sizes of 200-400 μm provide better control of the release, as required, by mean of tailoring proportion of HA:Gel mixture which was unachievable with the larger 400-500 μm scaffolds in PBS. A temporal and spatial control of protein release may be obtained by formulations of scaffold preparation.

7.5 Conclusion

A porous scaffold of Poly(1,4-butylene succinate) extended 1,6-diiocyanatohexane (PBSu-DCH) fabricated by solvent cast and particulate leaching technique was successfully modified to be a protein delivery scaffold by

impregnating protein-loaded gelatin microspheres with HA-Gel matrix. Mechanism of BSA-Rhod release was believed to be the combination of diffusion and dissolution or degradation of the carrier as observed from the releasing kinetic parameters. Many factors play roles and synergistically control on the release of BSA-Rhod from the as-prepared scaffold which were electrostatic interaction between protein and gelatin, scaffold's pores sizes, proportion of HA and gelatin in the HA:Gel matrix and type of releasing medium. In an environment where degradation of the device is enhanced by enzymatic digestion such as in the human body, controlled release of protein can be achieved by tailoring the proportion of HA-Gel matrix which is effective in scaffold with either small or large pores sizes.

7.6 Acknowledgements

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Table 7.1 Encapsulating efficiency of the BSA-Rhod and Loading capacity of the as-prepared gelatin microspheres

Sample No.	Encapsulating Efficiency (%) ($\mu g_{\text{BSA-Rhod encap}}/\mu g_{\text{loaded BSA-Rhod}}$)	Loading Capacity (%) ($mg_{\text{detected BSA-Rhod}}/mg_{\text{spheres}}$)
1	76.392	0.0764
2	72.334	0.0723
3	77.853	0.0779
Average	75.526 ± 2.859	0.0755 ± 0.003

Table 7.2 Constant (k), kinetic exponent (n) and correlation coefficient (r2) of the BSA-Rhod release for 24 h from gelatin microspheres.

	Gel Microspheres		
Release media	k	n	r^2
PBS			
0.15 M NaCl	0.3825	0.3826	0.9933
0.50 M NaCl	0.1090	0.4041	0.9653
1.00 M NaCl	0.0693	0.3265	0.9355
SBF	0.2946	0.4414	0.9751
MEM	2.5314	0.2867	0.9667

Table 7.3 Constant (k), kinetic exponent (n) and correlation coefficient (r2) of the BSA-Rhod release for 10 h from gelatin microspheres and BSA-Micros impregnated PBSu-DCH scaffolds

Delivery Material	Release media					
	0.15 M NaCl PBS			MEM		
	k	n	r^2	k	n	r^2
Gel Microspheres	0.3604	0.3943	0.9895	1.7867	0.3548	0.9955
PBSu-DCH 200-400 μm						
1:1 HA:Gel	0.1562	0.5115	0.9824	0.2944	0.5650	0.9764
1:3 HA:Gel	0.0602	0.5654	0.9723	0.0632	0.5483	0.8742
3:1 HA:Gel	0.2251	0.4018	0.9926	1.2496e-5	2.0148	0.8721
PBSu-DCH 400-500 μm						
1:1 HA:Gel	0.2611	0.4645	0.9961	1.3898	0.3461	0.9632
1:3 HA:Gel	0.4573	0.3683	0.9821	0.2024	0.4447	0.9759
3:1 HA:Gel	0.3857	0.4070	0.9714	0.0699	0.5345	0.9547

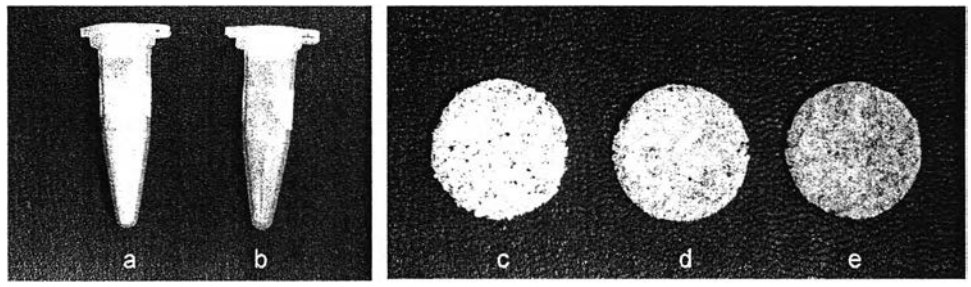


Figure 7.1 Picture of the as-prepared neat gelatin microspheres (a), BSA-Rhod loaded gelatin microspheres (b), HA-Gel blends impregnated PBSu-DCH scaffold (c), neat Gel-Micros impregnated PBSu-DCH scaffold (d) and BSA-Micros impregnated PBSu-DCH scaffold (e).

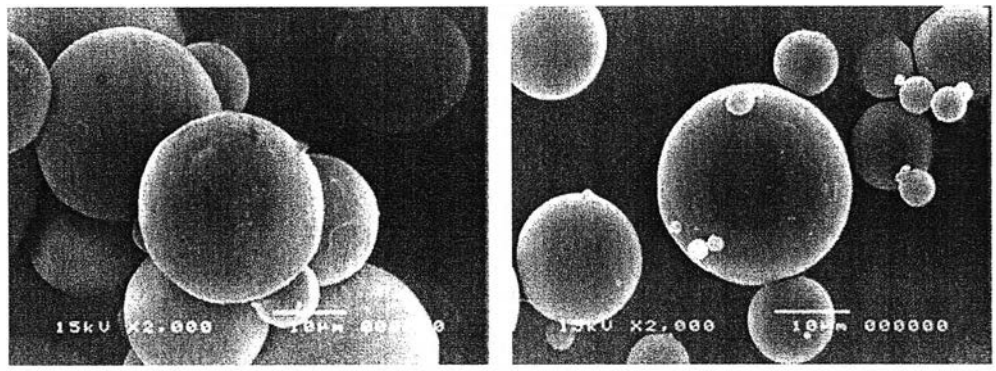


Figure 7.2 SEM images illustrating morphology of the as-prepared neat gelatin microspheres (a), and BSA-Rhod loaded gelatin microspheres (b)

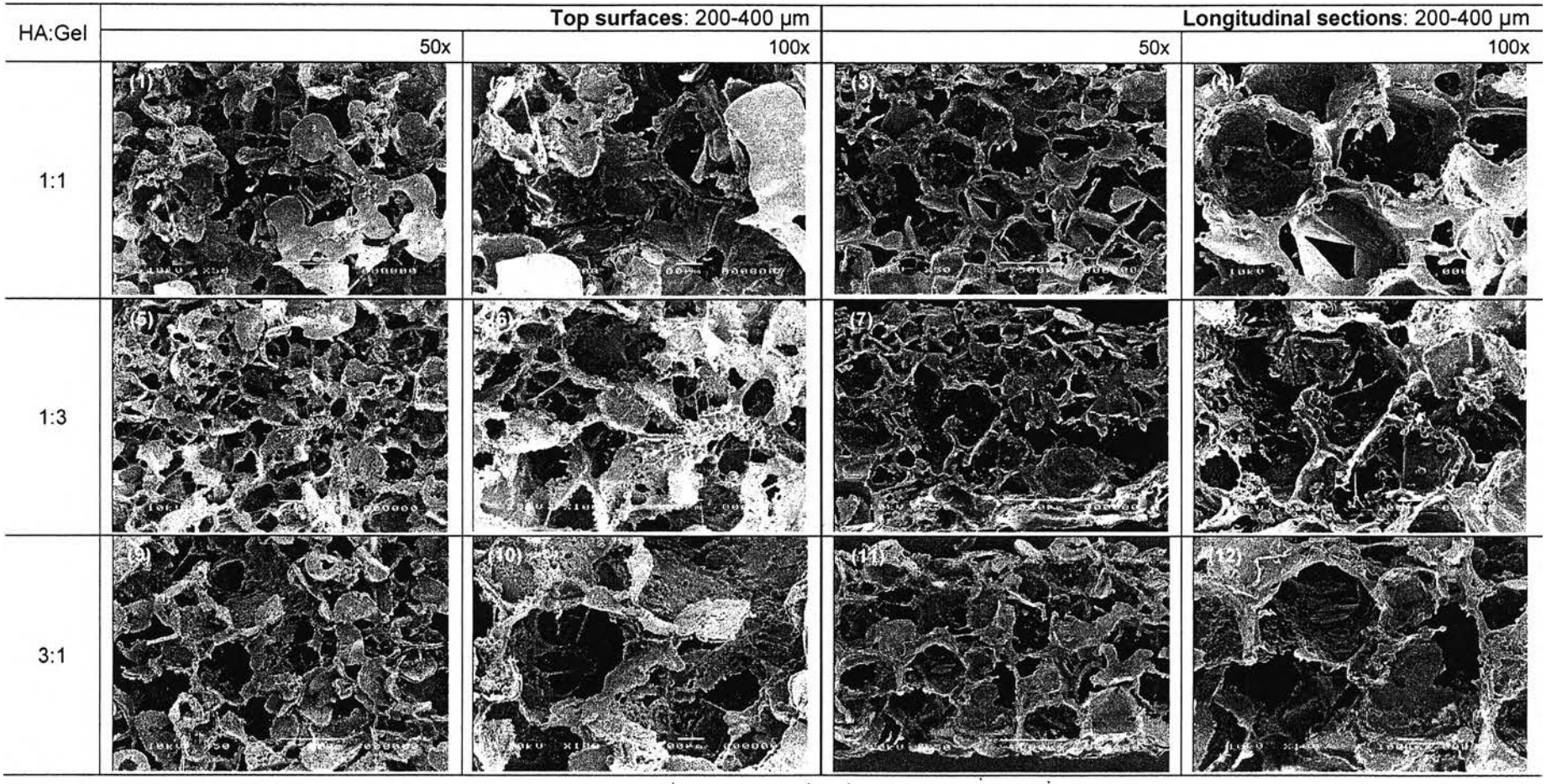


Figure 7.3 SEM images illustrating structure of the BSA-Micros impregnated PBSu-DCH scaffolds with pores of 200-400 μm pores, being viewed on the top and the longitudinally cutting surface: (1-4) 1:1 HA:Gel, (5-9) 1:3 HA:Gel, (9-12) 3:1 HA:Gel.

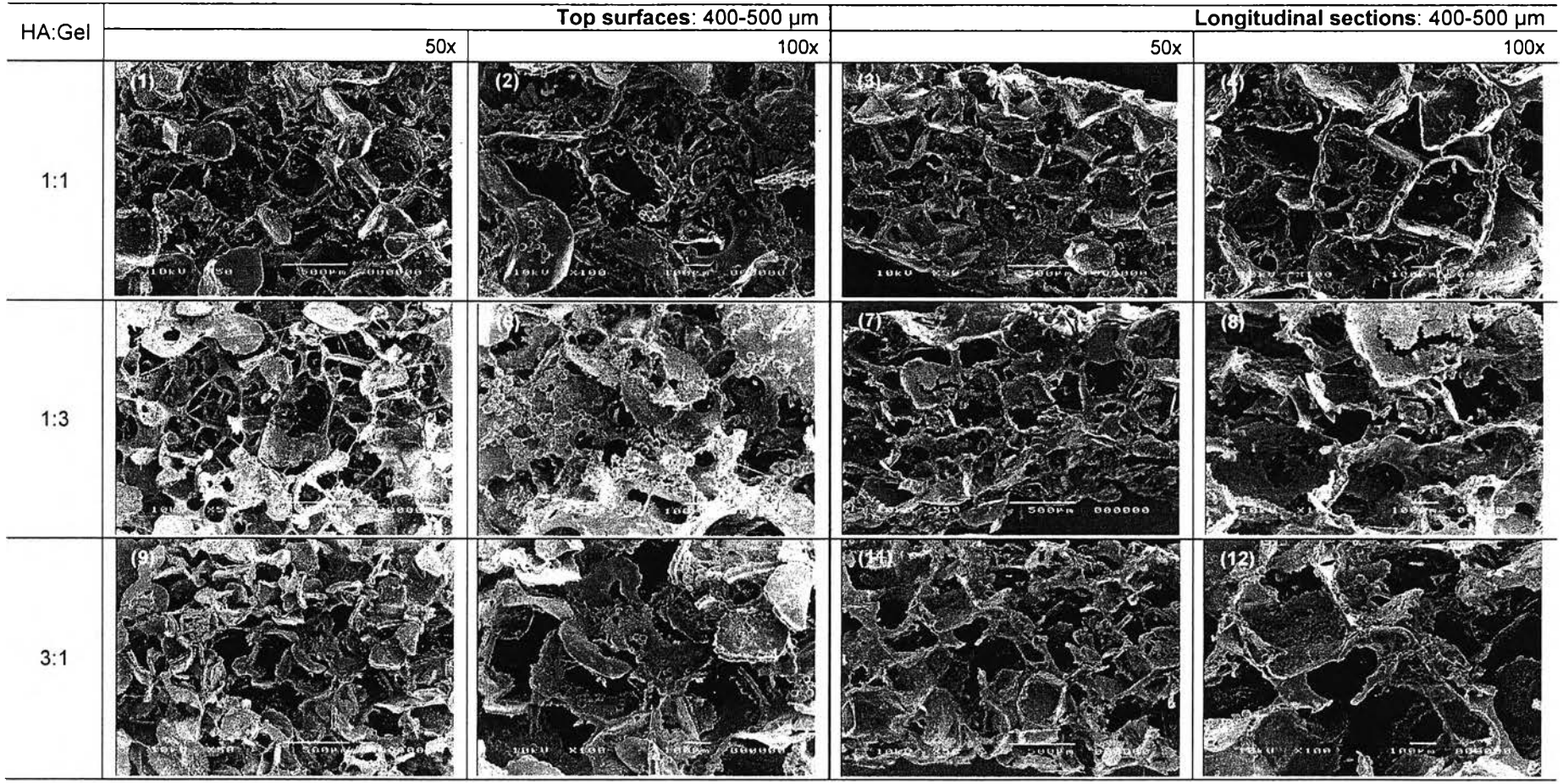


Figure 7.4 SEM images illustrating structure of the BSA-Micros impregnated PBSu-DCH scaffolds with pores of 400-500 μm, being viewed on the top and the longitudinally cutting surface: (1-4) 1:1 HA:Gel, (5-9) 1:3 HA:Gel, (9-12) 3:1 HA:Gel.

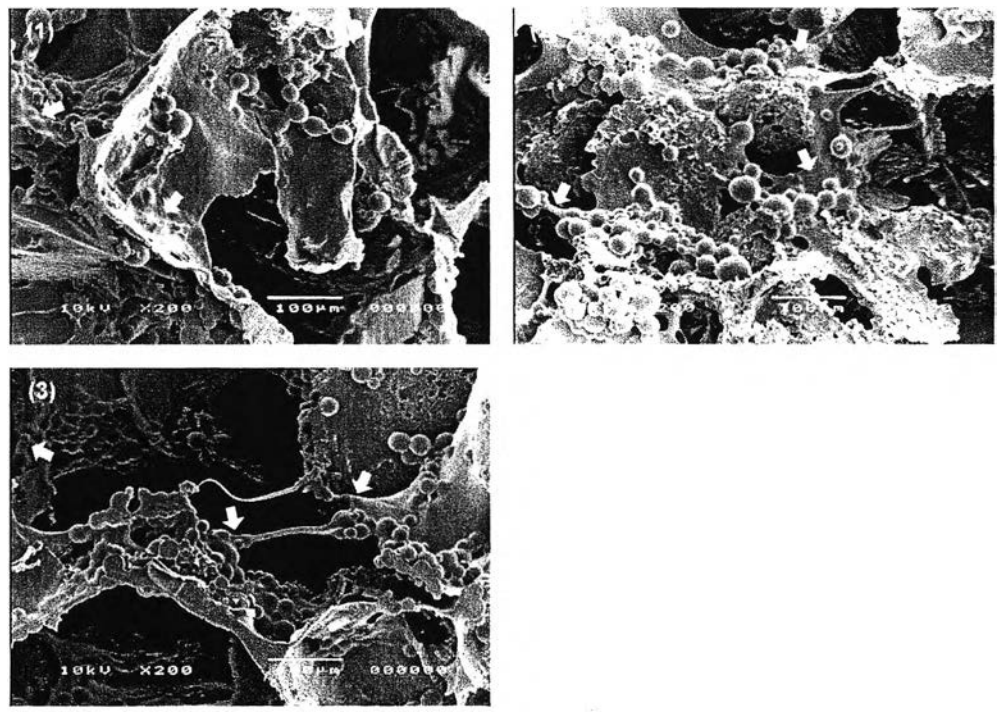
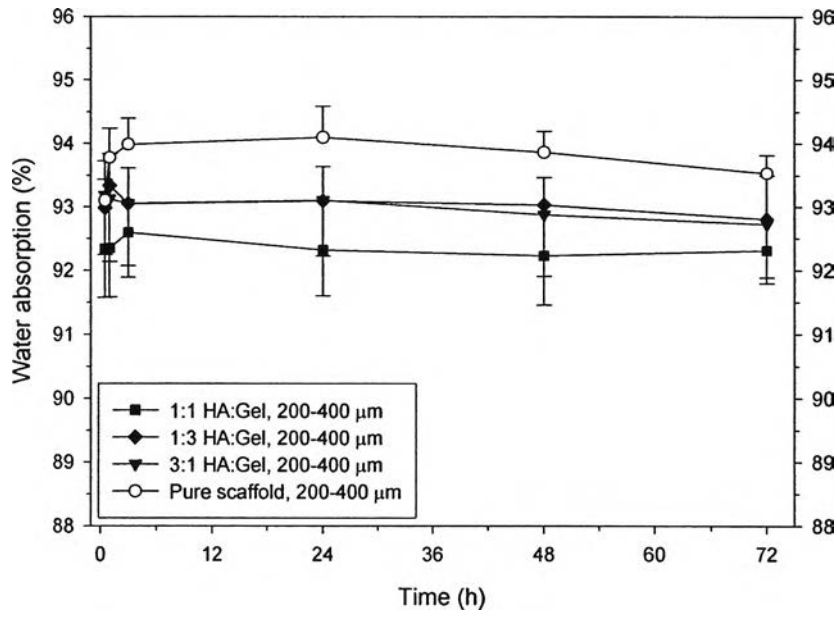
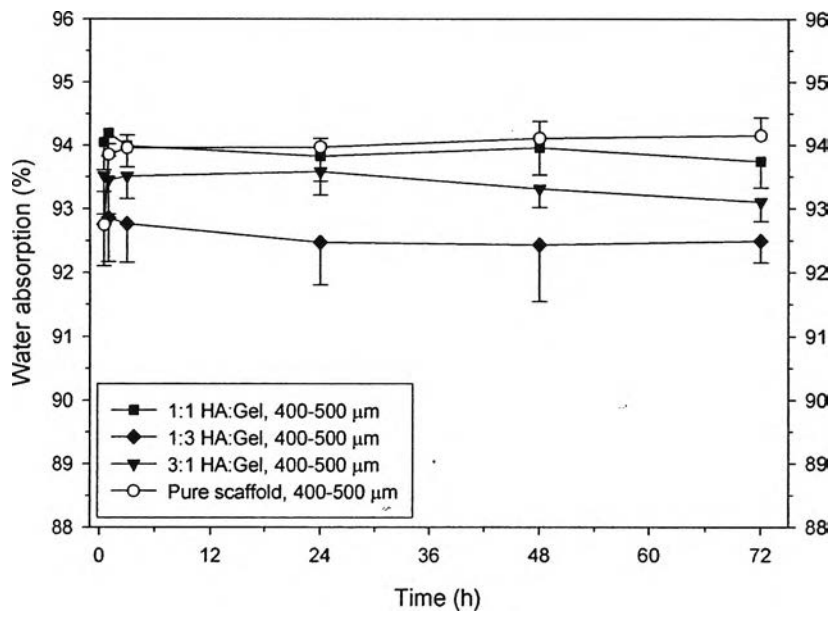


Figure 7.5 Selected SEM images illustrating attachment of the BSA-Micros onto the surfaces of PBSu-DCH scaffold with pores of 200-400 µm through HA-Gel matrix (white arrow): (1) 1:1 HA:Gel, (2) 1:3 HA:Gel, (3) 3:1 HA:Gel.



(a)



(b)

Figure 7.6 Water absorption capability of the neat and HA-Gel blend impregnated PBSu-DCH scaffold with pores of 200-400 μm (a) and 400-500 μm (b) at various HA:Gel ratios.

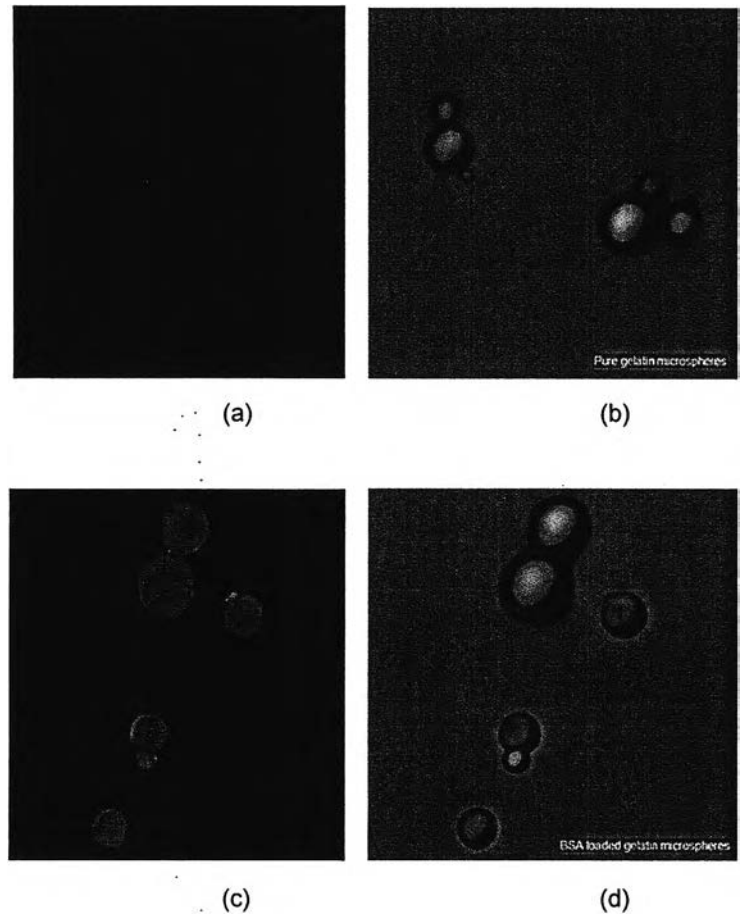


Figure 7.7 CLSM color and correspondent grayscale images of the as-prepared neat gelatin microspheres (a,b), and BSA-Rhod loaded gelatin microspheres (c,d).

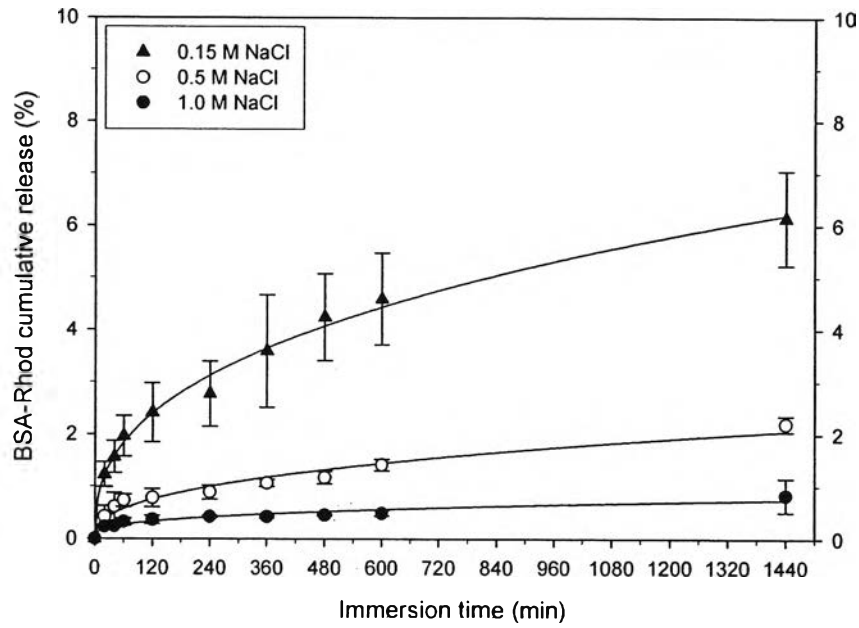


Figure 7.8 BSA-Rhod cumulative release (%) from gelatin microspheres in PBS containing various malarity of NaCl.

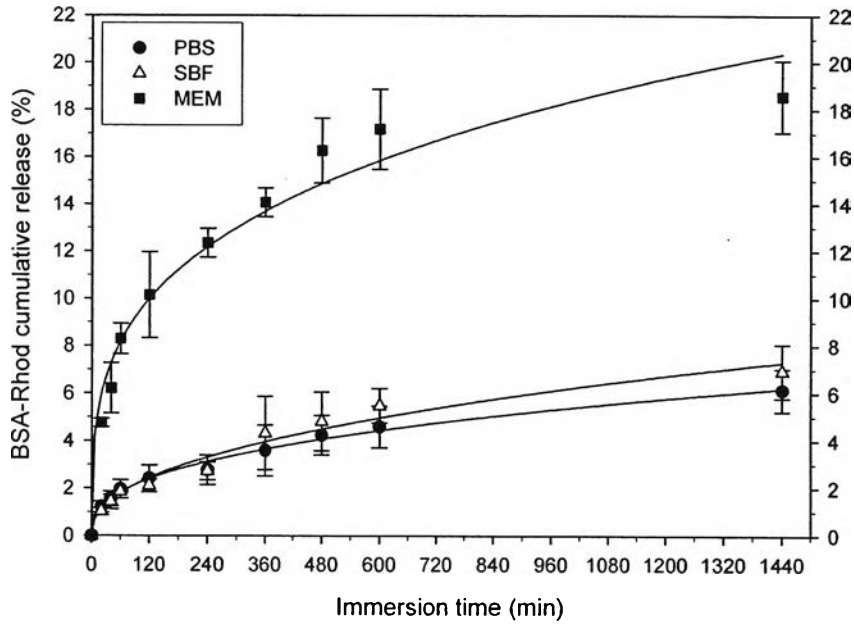
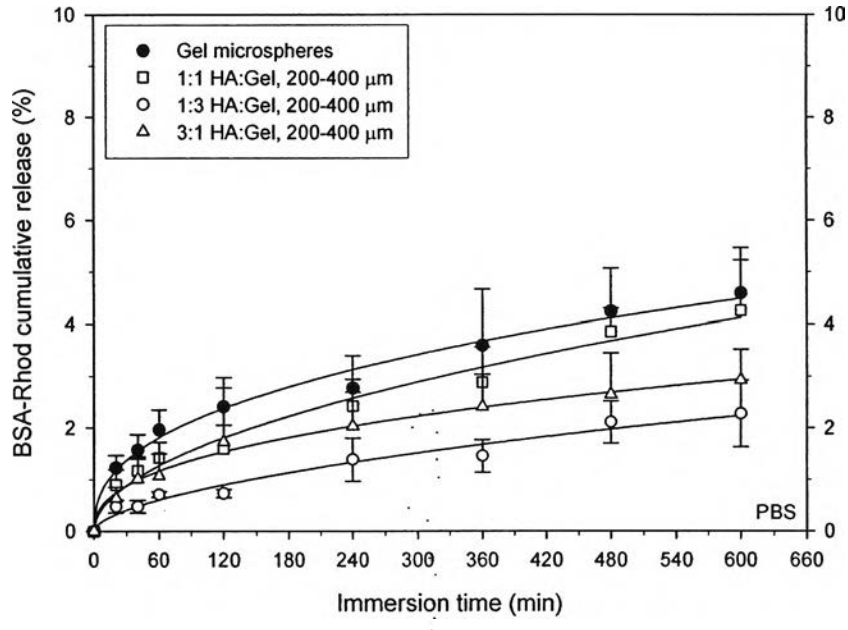
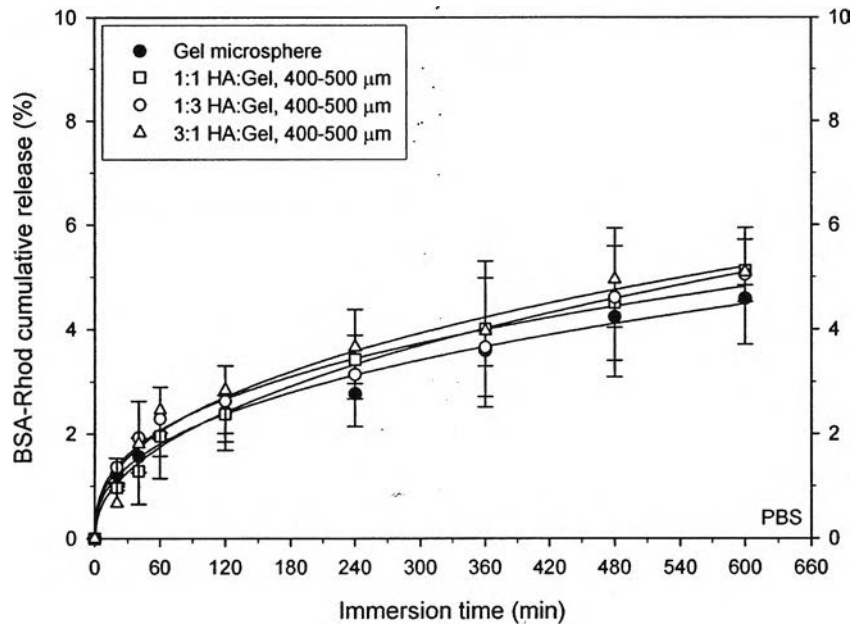


Figure 7.9 BSA-Rhod cumulative release (%) from gelatin microspheres in PBS (0.15 M NaCl), SBF and MEM.

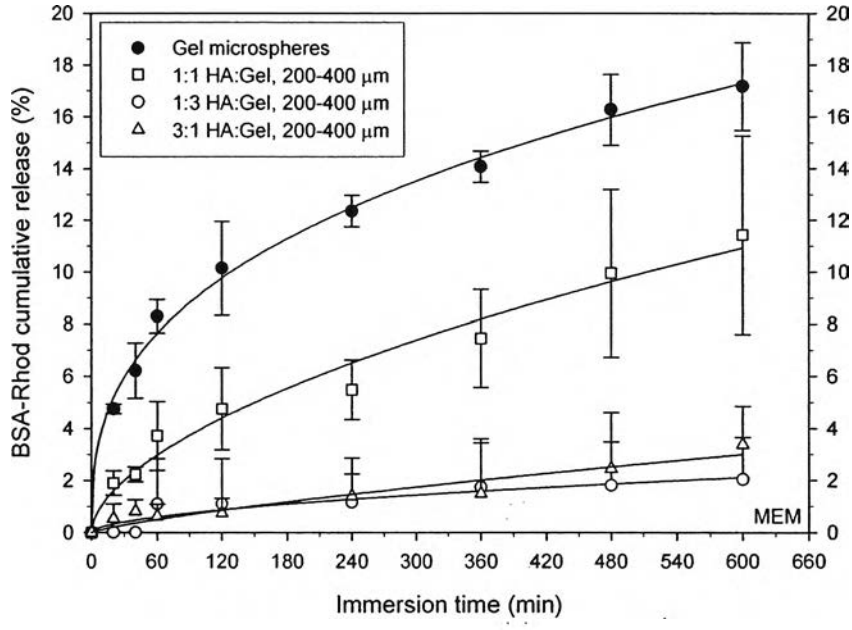


(a)

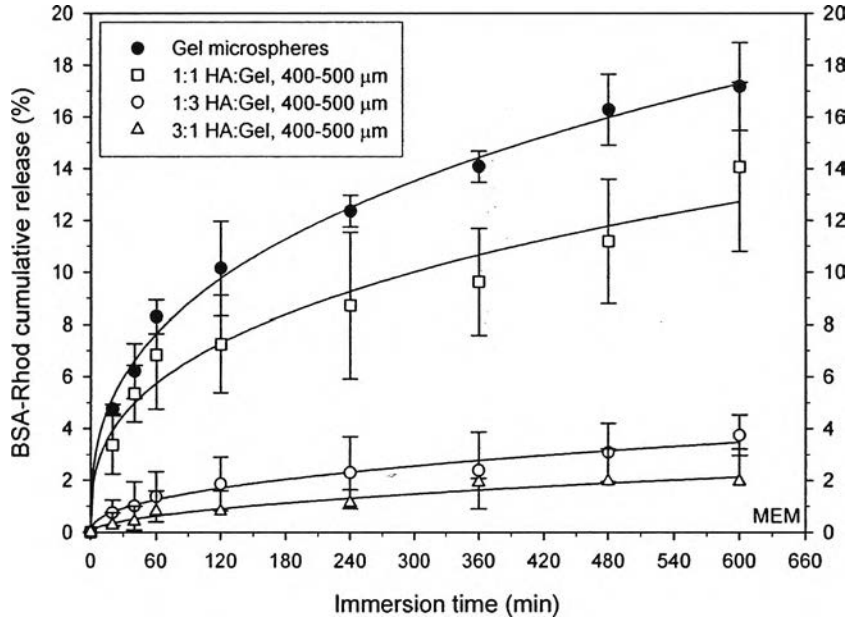


(b)

Figure 7.10 BSA-Rhod cumulative release (%) from gelatin microspheres and BSA-Micros impregnated PBSu-DCH scaffold with pores of 200-400 (a) and 400-500 μm (b) at various HA:Gel ratios in PBS.



(a)



(b)

Figure 7.11 BSA-Rhod cumulative release (%) from gelatin microspheres and BSA-Micros impregnated PBSu-DCH scaffold with pores of 200-400 (a) and 400-500 μm (b) at various HA:Gel ratios in MEM.