Carbohydrate Polymers 84 (2011) 812–819

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Evaluation of chitosan/γ-poly(glutamic acid) polyelectrolyte complex for wound dressing materials

Ching Ting Tsao[b], Chih Hao Chang[a,c], Yu Yung Lin[b], Ming Fung Wu[d], Jaw Lin Wang[a], Tai Horng Young[a], Jin Lin Han[e], Kuo Haung Hsieh[b,*]

[a] Institute of Biomedical Engineering, College of Engineering and College of Medicine, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei City 10617, Taiwan
[b] Institute of Polymer Science and Engineering, College of Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei City 10617, Taiwan
[c] Department of Orthopedics, National Taiwan University Hospital and National Taiwan University College of Medicine, No. 1, Jen-Ai Road, Taipei City 10018, Taiwan
[d] Animal Medicine Center, College of Medicine, National Taiwan University, No. 1, Jen-Ai Road, Taipei City 10018, Taiwan
[e] Department of Chemical and Materials Engineering, College of Engineering, National Ilan University, No.1, Sec. 1, Shennong Rd., Ilan City, Ilan County 26047, Taiwan

ARTICLE INFO

Article history:
Received 1 October 2009
Received in revised form 11 March 2010
Accepted 14 April 2010
Available online 21 April 2010

Keywords:
Chitosan
γ-PGA
Polyelectrolyte complex (PEC)
Dressing
Regeneration

ABSTRACT

This study presents a novel design for an easily stripped polyelectrolyte complex (PEC), which consists of chitosan as a cationic polyelectrolyte and γ-poly (glutamic acid) (γ-PGA) as an anionic polyelectrolyte, as a wound dressing material. The physical and chemical properties of the chitosan/γ-PGA PECs are fully investigated. Experimental results show that the physical and chemical properties and the *in vitro* degradation of the chitosan/γ-PGA PECs directly reflect the degree of complex formation. In addition, chitosan/γ-PGA PECs provide suitable moisture content and exhibit good mechanical properties, both favorable for allowing dressing to be easily stripped off from the wound surface without damaging newly regenerated tissue. Furthermore, *in vivo* experiments are conducted on a mouse model to study wounds treated with the chitosan/γ-PGA PECs. Histological examinations reveal that, more than 50% of re-epithelialization and regeneration of the wound are achieved by dressing it with the chitosan/γ-PGA PECs. On the basis of wound-healing efficacy, chitosan/γ-PGA PECs can be potentially applied as a wound dressing material.

1. Introduction

In medical practice, dressing is usually required in the treatment of an open wound to achieving closure as soon as possible as infections delay healing and increase scar formation (Liu, Yao, & Fang, 2008). Generally, an effective wound dressing has the following properties: protecting the wound from foreign microbes; capable of absorbing any body fluids exuded from the wound area; permitting evaporation of moisture at a certain rate; and not adhering excessively to the wound in order to allow easy removal after healing. If the removal of the dressing is not correctly performed, additional damage to the wound prolongs the recovery period. A high water content material can prevent adhesion problems, which in turn promotes the tissue regeneration process.

Hydrogels are three-dimensional hydrophilic polymeric networks capable of absorbing and retaining a large amount of water (Dai, Ravi, & Tam, 2008). Their hydrophilic surface has low interfacial free energy when in contact with body fluids, rendering good biocompatibility. Thus, hydrogels have recently generated much interest as a drug carrier and an artificial tissue scaffold (Peppas, Bures, Leobandung, & Ichikawa, 2000). A hydrophilic polymeric backbone prevents hydrogels from dissolution in water by radical, chemical, or physical crosslinking (Hamidi, Azadi, & Rafiei, 2008). Radical crosslinking, though giving its high crosslinking qualities, also has the potential problem of residual radicals existing in the hydrogels (Hennink & van Nostrum, 2002). Chemical crosslinking, i.e. covalent bonds existing between different polymer chains, will induce unexpected bonding, which will lead to unfavorable results (Dai et al., 2008). Due to these safety concerns, the interest in physically crosslinked hydrogels is growing. These physical interactions are utilized to prevent dissolution between different polymer chains. Several physical modalities are exploited to design physically crosslinked hydrogels, including coiled–coil interactions (Yang, Xu, Kopeckova, & Kopecek, 2006; Zhang, Furst, & Kiick, 2006), hydrophobic interactions (Qu, Wirsen, & Albertsson, 2000), antigen–antibody interactions (Zhang, Bowyer, Eisenthal, & Hubble, 2007), stereo–complex interactions (Bos et al., 2005), and ionic interactions (Lin et al., 2005; Lin et al., 2007). Among the various ionic interactions, polyelectrolyte complex (PEC) hydrogel with two opposite charged agents is chosen for this study.

Chitosan, derived from chitin by alkaline deacetylation, is a type of polysaccharide constituted by N-glucosamine and N-acetyl-glucosamine units, in which the number of N-glucosamine units exceeds 50% (Hsieh, Tsai, Wang, Chang, & Hsieh, 2005). Chitosan

* Corresponding author. Tel.: +886 2 3366 3043; fax: +886 2 3366 3044.
E-mail address: khhsieh@ntu.edu.tw (K.H. Hsieh).

0144-8617/$ – see front matter.
doi:10.1016/j.carbpol.2010.04.034

is a slightly crystalline polysaccharide, which is insoluble when the pH value is around or above 7. However, the free amino groups of chitosan become protonated in an acidic environment, which makes the molecule soluble with a positive charge (Rinaudo, 2006; Sashiwa & Aiba, 2004). The high positive charge numbers of chitosan in a dilute acidic solution allows the formation of a polyelectrolyte complex hydrogel with polyanionic species (Rinaudo, 2008). Chitosan has been reported to have good hemostatic and antibacterial properties (Ishihara et al., 2001). Chitosan has also recently been used as a wound dressing in veterinary medicine owing to its ability to accelerate the healing process (Muzzarelli, 2009). Chitosan oligosaccharides have a stimulatory effect on macrophages, and both chitosan and chitin are chemo-attractants for neutrophils *in vitro* and *in vivo* (Chen, Wang, Liu, & Wang, 2008). Chitin oligomers and chitosan oligomers, generated by enzymatic degradation in a wound environment, exert significant biochemical effects, as studies have shown the migratory activity of mouse peritoneal macrophages are significantly enhanced (Moon et al., 2007; Mori et al., 2005; Okamoto et al., 2003).

γ-Poly(glutamic acid) (γ-PGA) is an unusual anionic, natural polypeptide made of D- and L-glutamic acid units, connected by amide linkages between the α-amino and γ-carboxylic acid groups. With microbial fermentation, γ-PGA is currently produced on an industrial scale in high yield (Richard & Margaritis, 2001). γ-PGA is water soluble, biodegradable, and edible, and with its good tissue affinity, gains interest for biological applications (Ashiuchi, Kamei, & Misono, 2003; Prodhomme, Tutt, Glennie, & Bugg, 2003; Shih & Van, 2001). Its high anionic charge number allows γ-PGA to form PEC hydrogel with chitosan in an appropriate pH range.

In this study, chitosan/γ-PGA PECs with different compositions were prepared via a simple complex formation by varying the repeated unit ratios of chitosan to γ-PGA. The physicochemical properties of the PECs were examined by Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). Also, the water uptake, the compressive modulus, the water vapor transmission rates, and the *in vitro* degradation of the chitosan/γ-PGA PECs were fully investigated. *In vivo* evaluation of the chitosan/γ-PGA PECs as wound dressing materials was conducted using a mouse model.

2. Experimental

2.1. Reagents

Chitosan (average molecular weight: 300 kDa; degree of deacetylation: 97%, PDI = 1.47) was purchased from the G-HT Co., Hsinchu, Taiwan. γ-PGA calcium salt (average molecular weight: 1250 kDa) was purchased from the VEDAN Co., Taichung, Taiwan. Acetic acid (CH_3COOH), sodium hydroxide (NaOH), phosphate buffered saline (PBS), ethyl ether ($C_2H_5OC_2H_5$), and all other chemicals were purchased from the Sigma–Aldrich Corp., St. Louis, MO, USA.

2.2. Preparation of chitosan/γ-PGA polyelectrolyte complexes (PECs)

The chitosan/γ-PGA PECs containing varied molar ratios of amine groups of chitosan to carboxylic acid groups of γ-PGA ([$-NH_2$]: [–COOH] = 75/25, 50/50, 25/75) were prepared for this study. First, chitosan powder was well dispersed in a previously prepared γ-PGA aqueous solution. The weight percentage of chitosan and γ-PGA in the prepared solution was 4%, and then 1% acetic acid was added, the chitosan powder dissolved immediately due to the protonated amine groups of chitosan. The homogeneous PEC is subsequently formed through a complex formation between $-NH_2$ of chitosan and –COOH of γ-PGA. These PECs was then immersed in a 1N NaOH aqueous solution and washed with deionized water to a pH value around 7. The neutral PECs were further freeze-dried to a porous structure of the PECs; the freeze-dried PEC was found to shrink to 75–80% of its original size. The PECs were categorized into five groups, according to the molar ratios of amine groups ($-NH_2$) of chitosan (named C) to carboxylic acid groups (–COOH) of γ-PGA (named P), and thus, the degree of complex formation was defined as complex formed in an ionic solution:

$$\text{Degree of complex formation} = \frac{-NH_3{}^+ {}^-OOC-}{[-NH_2]_C + [-COOH]_P} \times 100\%$$

The description of the chitosan/γ-PGA PECs is listed in Table 1. As an example of the nomenclature used herein, C50P50 indicates that the molar ratio of amine groups ($-NH_2$) of chitosan to carboxylic acid groups (–COOH) of γ-PGA in this specimen was 50/50; the degree of complex formation = 50, which indicates that a complex formation between $-NH_2$ of chitosan and –COOH of γ-PGA was theoretically completed without free $-NH_2$ or –COOH groups in the PECs.

The neat chitosan (C100) were prepared by the immersion-precipitation method (Denkbas & Ottenbrite, 2006). In brief, the C100 was formed with solification by immersing the chitosan solution (4 wt% in 1% acetic acid) into 1N NaOH. The C100 was then neutralized with deionized water, and freeze-dried to remove any excess water. The neat γ-PGA (P100) cannot be formed as hydrogel, as it is water soluble.

2.3. Characterization of the PECs

2.3.1. Chemical structure

Fourier transform infrared spectroscopy (FTIR, Spectrum 100, PerkinElmer, Fremont, CA, USA) was used to examine the peak variation of the amine and carboxylic acid groups of the chitosan/γ-PGA PECs. Powdered PEC was mixed with dry KBr and pressed into a transparent disc, with a ratio of 1/10.

2.3.2. Morphology

To investigate the morphology, the cross-sections of the chitosan/γ-PGA PECs and the neat chitosan were observed using scanning electron microscopy (SEM, JSM-6700, JOEL Ltd., Tokyo, Japan), operated at an accelerating voltage of 20 kV. The PECs were first coated with an ultrathin layer of gold–palladium in an ion sputter and then examined by SEM.

2.3.3. Water uptake

To calculate the water uptake of the chitosan/γ-PGA PECs and the neat chitosan, the dry PECs were weighed and then immersed in 0.05 M PBS at room temperature. After a specific time interval, the excess water was removed with filter paper and the swollen PECs were weighed again. The water uptake was calculated through the following equation:

$$\text{Water uptake} = \frac{W_s - W_d}{W_d} \times 100\% \quad (1)$$

where the water uptake is expressed in %, and W_d and W_s are the weights of the PECs, before (dry) and after (swollen) being placed in PBS, respectively. The results are presented as a mean value with a standard deviation ($n = 6$).

2.3.4. Compressive modulus

A universal testing machine was used to determine the compressive modulus of the chitosan/γ-PGA PECs and the neat chitosan, in both dry and swollen states, by compressing the sample discs (13 mm diameter and 3 mm thickness) at a constant rate of 5 mm/min (Tangsadthakun et al., 2007). The swollen counterpart

Table 1
Description of the chitosan/γ-PGA PECs.

Nomenclature	Composition		Degree of complex formation (%)
	Weight ratio chitosan/γ-PGA	Molar ratio [$-NH_2$] of chitosan/[–COOH] of γ-PGA	
C100	4/0	100/0	0
C75P25	3.06/0.94	75/25	25
C50P50	2.09/1.91	50/50	50
C25P75	1.07/2.93	25/75	25
P100	Water soluble		0

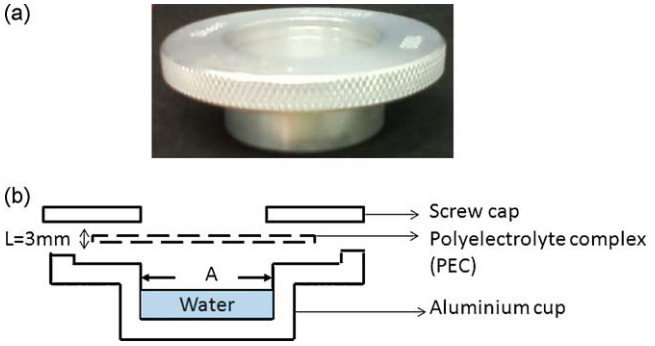

Fig. 1. (a) Photograph of permeability cup. (b) Schematic representation of the water vapor transmission rate test.

was prepared by immersion in 0.05 M PBS at room temperature for 5 h. The slopes of the compressive stress–strain curves from 5% to 35% deformation were used to calculate the compressive modulus. The results are presented as a mean value with a standard deviation ($n = 6$).

2.3.5. Water vapor transmission rate

The water vapor transmission rate (WVTR) across the chitosan/γ-PGA PECs and the neat chitosan was determined according to ASTM (F-1249-06), and a permeability cup was applied (Sheen Instruments Ltd. Surrey, England). The chitosan/γ-PGA PECs and the neat chitosan were cut into 20 mm × 20 mm with a thickness of 3 mm, and mounted on the mouth of cylindrical aluminium cups (38 mm diameter) containing 10 ml water, and then placed in an incubator at 37 °C, as schematically shown in Fig. 1. The WVTR was calculated using the following equation:

$$\text{WVTR} = \frac{W_o - W_f}{A \times 10^6} (\text{g/m}^2/\text{day}) \quad (2)$$

where, WVTR is expressed in g/m^2/day, A (mm^2) is the bottle mouth area, W_o (gram) and W_f (gram) are the weights of the device before and after being placed in an incubator for 24 h, respectively. The results are presented as a mean value with a standard deviation ($n = 6$).

2.3.6. In vitro degradation

The *in vitro* degradation of the chitosan/γ-PGA PECs and the neat chitosan was performed according to ASTM (F-1635-95), by measuring weight changes throughout the degradation time under simulated physiological conditions. The dry PECs were weighed, then immersed in 0.1 M PBS containing 58,1 00 units/ml lysozyme at 37 °C. After a specific time interval, the PECs were removed from the simulated medium and thoroughly rinsed with distilled water before being placed in a freezer. The PECs were freeze-dried for 24 h to remove excess water, and weighed again. The weight remaining was calculated using the following equation:

$$\text{Weight remaining}(\%) = 100 - \left(\frac{W_o - W_d}{W_o \times 100}\right) \quad (3)$$

where, weight remaining is expressed in %, and W_o and W_d are the weights of the PECs before and after degradation for a specific time interval, respectively. The results are presented as a mean value with a standard deviation ($n = 6$).

2.4. In vivo study for wound dressing application

Animal use and care was performed in compliance with the "Guide for the Care and Use of Laboratory Animals," as prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. ICR mice (male, 6 weeks-old) were anesthetized with ethyl ether. A 1-cm-wide wound was cut with scissors on the back of each shaved mouse down to the panniculus carnosus layer. Mice were dressed with chitosan/γ-PGA PECs or neat chitosan and wrapped with surgical tape. Wound closure observation was assessed by digital camera. The wound closure rate was calculated using the following equation:

$$\text{Wound closure rate} = \frac{L_o - L_f}{L_o} \times 100\% \quad (4)$$

where, the wound closure rate is expressed as a percentage %, and L_o and L_f are the length of the originally created wound, and the length of the wound postoperative for a specific time interval, respectively. The results are presented as a mean value with a standard deviation ($n = 6$).

In addition, the wounds and the surrounding skin (1.5 cm × 1.5 cm) were fixed with 4% formaldehyde solution and stained with hematoxylin–eosin (H&E) reagent for histological examinations.

3. Results and discussion

3.1. Characterization of the PECs

3.1.1. Chemical structure

Fig. 2 shows the FTIR spectra of neat chitosan, the various ratios of chitosan/γ-PGA PECs and neat γ-PGA. The characteristic peaks of neat chitosan (C100) appeared at 3450, 3300, and 1560 cm^{-1}, corresponding to its hydroxyl (–OH), free amine ($-NH_2$), and N–H bending vibrations coupled with C–N stretching vibrations, respectively (Brugnerotto et al., 2001). Those of the neat γ-PGA (P100) appeared at 3400, 1620, and 1560 cm^{-1}, corresponding to the N–H bending, carbonyl (C=O) bond vibrations, and amide II bands (Lin et al., 2005). The FTIR spectra of the chitosan/γ-PGA PECs for C75P25, C50P50, and C25P75 featured characteristics similar to those of their parent polymers. However, the intensity of the free amine peak at 3300 cm^{-1} of the chitosan/γ-PGA PECs decreased, as compared with those of neat chitosan. Furthermore, a peak, which arose in each chitosan/γ-PGA PECs but not observed in neat chitosan or γ-PGA, was observed at 1400 cm^{-1}. It was attributed to the $-NH_3^+$ of chitosan complexing with $-COO^-$ of γ-PGA (Simsek-Ege, Bond, & Stringer, 2003). As the degree of complex formation increased, the peak intensity of the complex formation at 1400 cm^{-1} in chitosan/γ-PGA PECs also increased. These results suggest that a

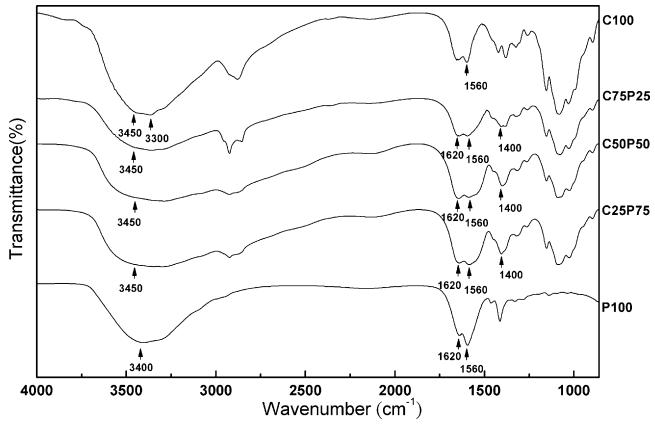

Fig. 2. FTIR spectra of neat chitosan, different chitosan/γ-PGA PECs and neat γ-PGA.

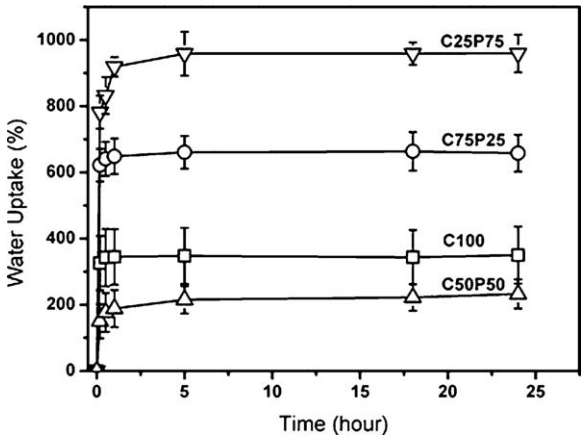

Fig. 3. Water uptake of neat chitosan and different chitosan/γ-PGA PECs as a function of incubation time.

complex formation existed between chitosan and γ-PGA, which results in the chitosan/γ-PGA PECs.

3.1.2. Water uptake

The water uptakes for the various ratios of chitosan/γ-PGA PECs and the neat chitosan are presented in Fig. 3. All chitosan/γ-PGA PECs became saturated after being immersed for 5 h. The water uptake decreased as the degree of complex formation of the chitosan/γ-PGA PECs increased. The water uptake of C50P50, with the highest degree of complex formation, had the lowest water uptake ($232 \pm 44\%$). With the same degree of complex formation, the water uptake of C25P75 ($959 \pm 97\%$) was larger than that of C75P25 ($675 \pm 55\%$) which is due to the higher hydrophilicity and greater water absorption capabilities of the free –COOH groups in γ-PGA than that of the free $-NH_2$ groups in chitosan existing in the chitosan/γ-PGA PECs.

3.1.3. Morphology

In tissue engineering, biomaterials must have a porous structure to ensure cell attachment and proliferation as well as tissue growth, in addition to providing a passage for nutrient flow. The morphologies of the various ratios of chitosan/γ-PGA PECs and the neat chitosan, as revealed by SEM photographs in Fig. 4, indicate that there were interconnected porous structures throughout the PECs. The three-dimensional porous structure was achieved through the processes of complexing interactions, which squeezed out water and subsequently induced solidification, as described in Section 2. Therefore, the pore size of the chitosan/γ-PGA PECs decreased as the degree of complex formation of the chitosan/γ-PGA PECs increased. The neat chitosan (C100), having no complex formation, had the largest pore size, estimated at 175 ± 15 μm. C50P50, with the highest degree of complex formation, had the smallest pore size of 50 ± 10 μm. As previously mentioned, C25P75 and C75P25

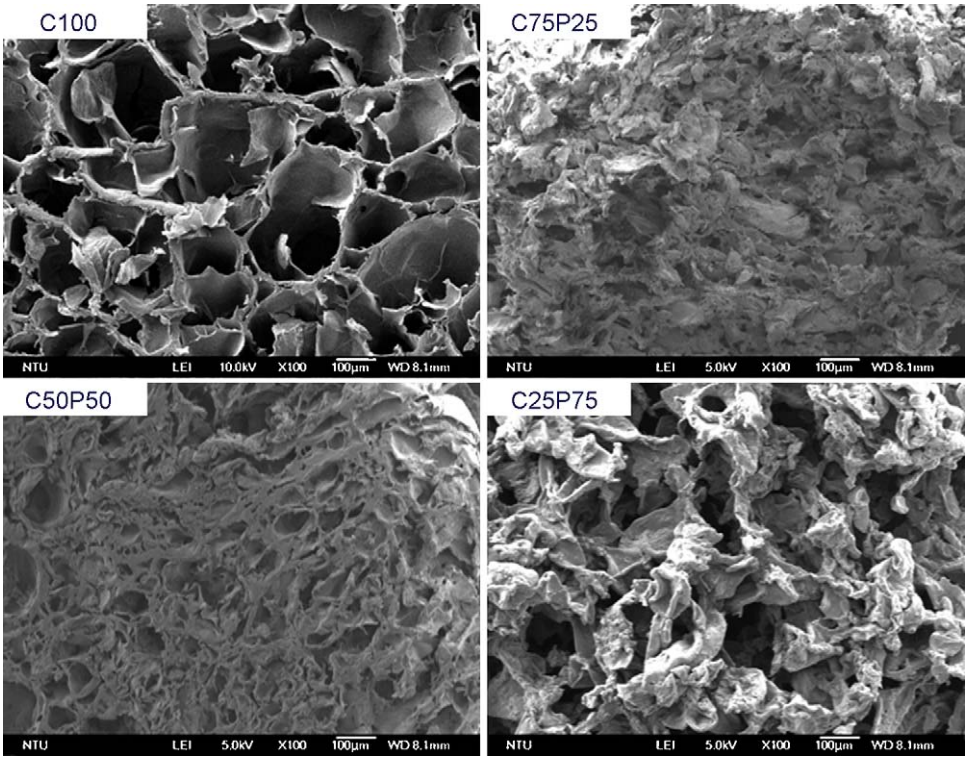

Fig. 4. SEM photographs of cross-sections of neat chitosan and different chitosan/γ-PGA PECs.

Table 2
Compressive modulus of neat chitosan and different chitosan/γ-PGA PECs.

	Compressive modulous (MPa)	
	Dry	Swollen
C100	0.45 ± 0.085	0.084 ± 0.012
C75P25	0.59 ± 0.079	0.116 ± 0.017
C5OP50	1.91 ± 0.176	0.182 ± 0.026
C25P7S	0.33 ± 0.125	0.039 ± 0.009

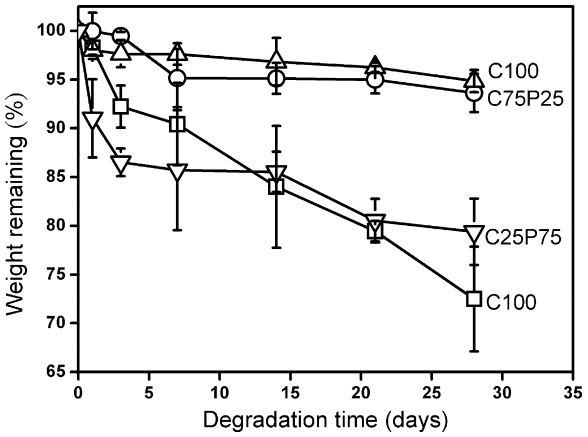

Fig. 5. Weight remaining of different chitosan/γ-PGA PECs and neat chitosan as a function of degradation time.

had the same degree of complex formation; however, C25P75 (110 ± 10 μm) had a larger pore size than C75P25 (80 ± 10 μm), as shown in Fig. 4. The different pore sizes of C25P75 and C75P25 can be reasonably explained by the different water absorption capabilities between the free –COOH groups in C25P75 and the free $–NH_2$ groups in C75P25. Due to the greater water absorption characteristic of the free –COOH groups than that of the free $–NH_2$ groups, C25P75 absorbed much more water than C75P25 in the preparation process. Thus, C25P75 resulting in a larger pore size than C75P25, upon being freeze-dried.

3.1.4. Compressive modulus

The mechanical properties of the various ratios of chitosan/γ-PGA PECs and neat chitosan, in both dry and swollen states, were illustrated as compressive modulus, as depicted in Table 2. The compressive modulus of dry chitosan/γ-PGA PECs was about one order larger than that of their swollen counterparts. The compressive modulus increased as degree of complex formation of the chitosan/γ-PGA PECs increased. The results suggest that complex formations between polyelectrolyte polymers influenced the mechanical properties of the chitosan/γ-PGA PECs. At the same degree of complex formation, the compressive modulus increased as the chitosan content increased, as seen in C75P25, which is attributed to the higher modulus of chitosan through the chemical structure of a 6-member ring in the chitosan main chain.

Table 3
Water vapor transmission rate of neat chitosan and different chitosan/γ-PGA PECs.

	Water vapor transmission rate
C100	2110 ± 115
C75P25	1952 ± 85
C50P50	1775 ± 78
C2SP7S	2034 ± 48

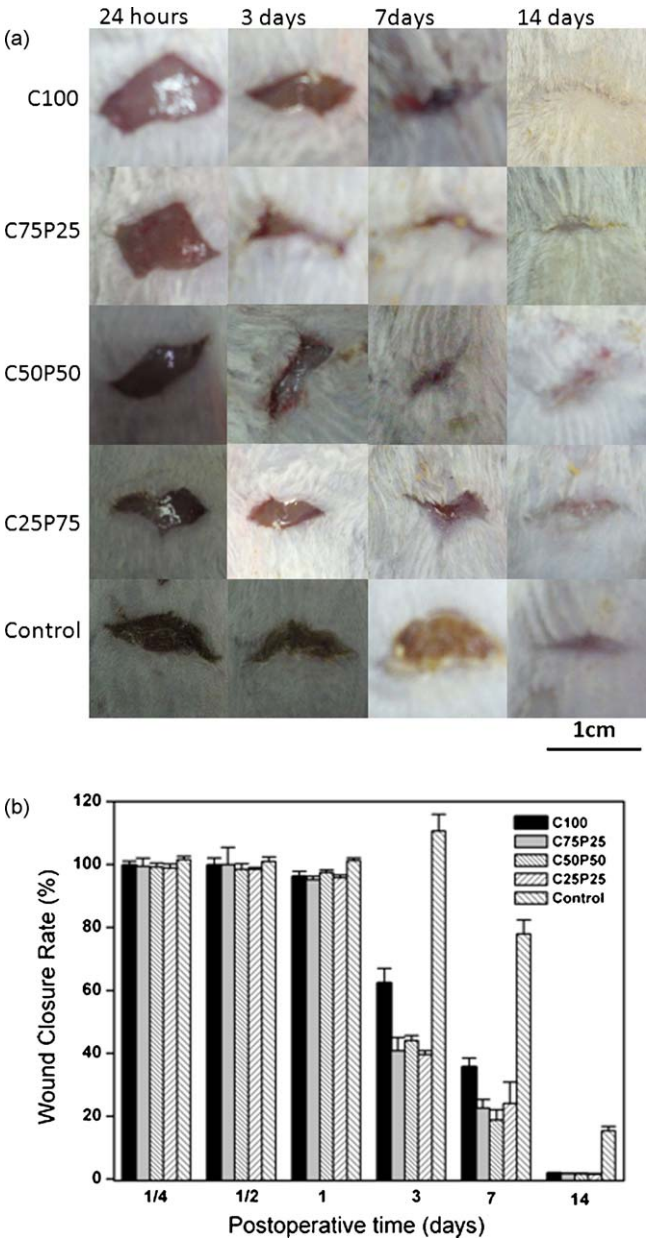

Fig. 6. (a) Macroscopic photographs of the wounds treated with neat chitosan, different chitosan/γ-PGA PECs and the control. (b) Wound closure rate of the wounds treated with neat chitosan, different chitosan/γ-PGA PECs and the control.

3.1.5. Water vapor transmission rate

Table 3 presents the water vapor transmission rate (WVTR) of the various ratios of chitosan/γ-PGA PECs and neat chitosan. As indicated, the WVTR for the neat chitosan (C100) was about 2110 g/m^2/day; whereas, the chitosan/γ-PGA PECs was between 1775 and 2034 g/m^2/day. This is attributed to the largest pore size existing in the neat chitosan. C50P50 with the highest degree of complex formation had the lowest WVTR of around 1775 g/m^2/day. At the same degree of complex formation, the transmission of water vapor through the PECs increased as the γ-PGA content increased. Therefore, the WVTR of C25P75 was larger than that of C75P25, which is resulted from the free –COOH groups high content of γ-PGA in C25P75. An ideal dressing should maintain evaporative water loss from the skin at an optimal rate. The evaporative water loss rate for normal skin is 204 ± 12 g/m^2/day, while that for injured skin can range from 279 ± 26 g/m^2/day for a first-degree burn to 5138 ± 202 g/m^2/day for a granulating wound. The water vapor

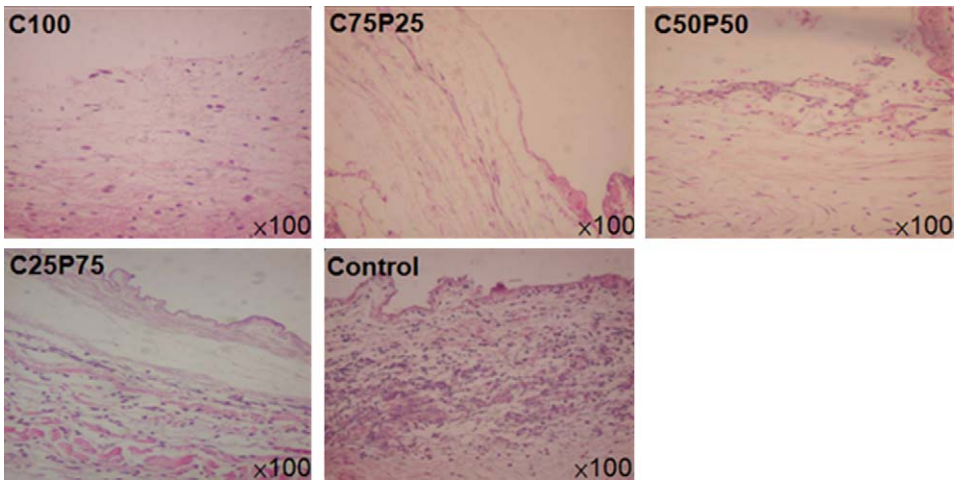

Fig. 7. Histological examinations of mice skin showing healing in the wounds treated with neat chitosan, different chitosan/γ-PGA PECs and the control, at hour 6 postoperatively.

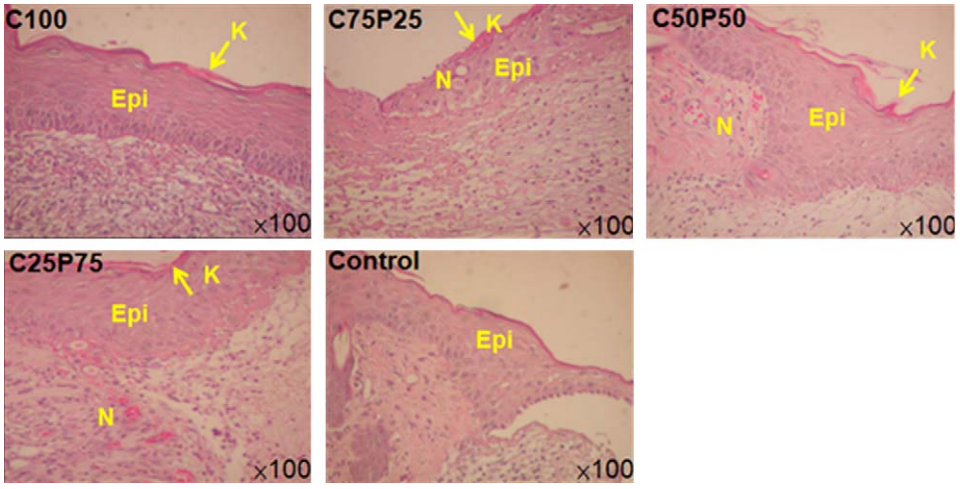

Fig. 8. Histological examinations of mice skin showing healing in wounds treated with neat chitosan, different chitosan/γ-PGA PECs and the control, on day 3 postoperatively (K: keratin, Epi: epidermis, N: neovascularization. Arrows refer to keratin).

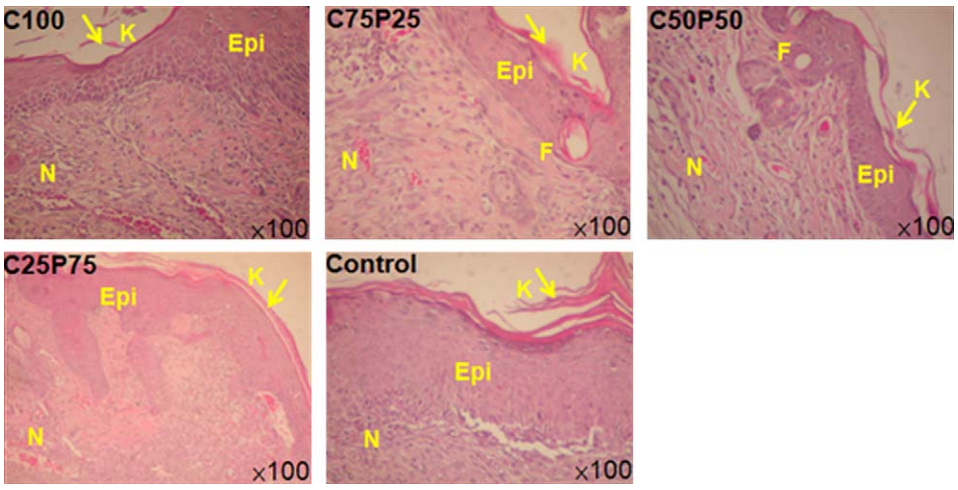

Fig. 9. Histological examinations of mice skin showing healing in the wounds treated with neat chitosan, different chitosan/γ-PGA PECs and the control, on day 7 postoperatively (K: keratin, Epi: epidermis, N: neovascularization, F: hair follicle. Arrows refer to keratin).

permeability of a wound dressing should prevent excess dehydration and the buildup of exudates. It is recommended that a rate of 2000–2500 g/m^2/day, which is the mid-range of the evaporative water loss rate from injured skin, would provide an adequate level of moisture without the risk of wound dehydration (Kim et al., 2007). In this study, the WVTR of the chitosan/γ-PGA PECs was about 1700–2100 g/m^2/day, which falls in the mid-range of the recommended evaporative water loss rate of injured skin. Therefore, the chitosan/γ-PGA PECs could control evaporative water loss at a suitable rate to yield a moist environment.

3.1.6. *In vitro* degradation

The *in vitro* degradation of the various ratios of chitosan/γ-PGA PECs and neat chitosan, shown as weight remaining after degradation, is depicted in Fig. 5. Overall, all the chitosan/γ-PGA PECs (C25P75, C50P50, C75P25) showed milder degradation than the neat chitosan (C100) after 28 days of incubation. This suggests that, unlike neat chitosan, the complex formation between chitosan and γ-PGA had a physical crosslinking effect that prevents the cleavage of PECs. As the degree of complex formation increased, the degradation rate reduced. C50P50, with the highest degree of complex formation, lost very little weight throughout the entire process, as compared with C25P75 and C75P25. Under identical degrees of complex formation, C25P75 and C75P25 showed different degradation trends. C25P75 had the faster degradation rate of 21% weight loss after 28 days incubation, while C75P25 had a slower degradation rate of only 7% weight loss after 28 days. This may be attributed to the local excess of –COOH of γ-PGA in C25P75, which catalyzed the degradation reaction of the chitosan (Liu et al., 2006). This may also be attributed to the larger pore size of PEC C25P75, which allows the low network structures to be exposed to lysozyme, resulting in increased degradation rates.

3.2. Wound closure observation

Fig. 6a presents the macroscopic photographs of wounds treated with neat chitosan, the various ratios of chitosan/γ-PGA PECs, and the control. The wounds treated with chitosan/γ-PGA PECs or neat chitosan healed faster than those of the control. The chitosan/γ-PGA PECs were found to adhere more uniformly to the wound surface than neat chitosan. The uniformly adhered chitosan/γ-PGA PECs absorbed the wound exudates, thus, reduced the risk of dehydration. The phenomenon of inflammation was found in the control (Kondo & Ohshima, 1996). When the chitosan/γ-PGA PECs were removed, they were easily stripped off without damage to the regenerated wound. The wound closure rates of wounds treated with neat chitosan, chitosan/γ-PGA PECs, and the control are illustrated in Fig. 6b. The wounds treated with the chitosan/γ-PGA PECs had healed 60% by day 3, postoperatively. On day 14, the wounds treated with the chitosan/γ-PGA PECs had healed almost completely, whereas, this phenomenon was not observed in the control.

3.3. Histological observation

Histological examinations of wounds treated with neat chitosan, the various ratios of chitosan/γ-PGA PECs, and the control, recorded at hour 6, on day 3, and on day 7, postoperatively, are illustrated in Figs. 7–9, respectively. All chitosan/γ-PGA PECs performed well to block inflammatory cell infiltration, as compared with the control at hour 6, postoperatively, as shown in Fig. 7. As the chitosan content increased in the chitosan/γ-PGA PECs, the suppression of inflammatory cells increased. Neat chitosan (C100) did not show excellent suppression, which indicates that γ-PGA plays an important role in the reduction of inflammatory reaction.

As shown in Fig. 8, the re-epithelialization was activated early when wounds were treated with chitosan/γ-PGA PECs, as compared to the control on day 3, postoperatively. The epidermis of wounds treated with neat chitosan (C100) seemed unable to achieve a steady state. Furthermore, the keratin formation was more integrated in those wounds treated with chitosan/γ-PGA PECs than with the neat chitosan or the control. It is reported that wounds re-epithelialize more rapidly under moist conditions (Chiu, Lee, Chu, Chang, & Wang, 2008). Chitosan has been used in veterinary medicine owing to its ability to accelerate the healing process. With the strong hydrophilic properties and good tissue affinity of γ-PGA, the chitosan/γ-PGA PECs create a favorable environment for epithelial cells to proliferate. The extent of epithelialization of a wound is increased with an increase of γ-PGA content in the chitosan/γ-PGA PECs. Furthermore, neovascularization occurred in wounds treated with C25P75 and C50P50. Moist-state material covering a wound absorbs the exudates and maintains the wound naturally with suitable moisture. These results indicated that a chitosan/γ-PGA PEC wound dressing can provide the proper environment for wounds to heal, through maintaining sufficient moisture, thus, reducing the risk of dehydration.

As shown in Fig. 9, on day 7, postoperatively, the histological examinations of wounds treated with chitosan/γ-PGA PECs showed that the tissue is regenerated very well, as compared with the control. Furthermore, new hair follicle formation could be seen. These histological observations under *in vivo* assessment reveal that chitosan/γ-PGA PECs can meet efficacy requirements, and may be a potential material for wound dressing.

4. Conclusion

In this research, chitosan and γ-PGA formed structurally stable polyelectrolyte complexes through simple complex formations. The results showed that, by increasing the degree of the complex formation lowered the water uptake, reduced the pore size of the porous structure, decreased the *in vitro* degradation, but increased the compressive modulus of the chitosan/γ-PGA PECs. Furthermore, the chitosan/γ-PGA PECs provided adequate moisture, and thus, reduced the risk of dehydration in the presence of γ-PGA. Finally, animal studies revealed that wounds treated with the chitosan/γ-PGA PECs healed significantly faster than wounds given no treatment. Inflammatory phenomena were suppressed and reduced, and re-epithelialization was activated in wounds treated with the chitosan/γ-PGA PECs. After healing, the chitosan/γ-PGA PECs were easily stripped off from the wound surface without damaging the newly regenerated tissue. The results of this study indicated that chitosan/γ-PGA PECs are potential materials for wound dressings.

References

Ashiuchi, M., Kamei, T., & Misono, H. (2003). Poly-gamma-glutamate synthetase of *Bacillus subtilis*. *Journal of Molecular Catalysis B-Enzymatic*, *23*(2–6), 101–106.
Bos, G. W., Hennink, W. E., Brouwer, L. A., den Otter, W., Veldhuis, T. F. J., van Nostrum, C. F., et al. (2005). Tissue reactions of in situ formed dextran hydrogels crosslinked by stereocomplex formation after subcutaneous implantation in rats. *Biomaterials*, *26*(18), 3901–3909.
Brugnerotto, J., Lizardi, J., Goycoolea, F. M., Arguelles-Monal, W., Desbrieres, J., & Rinaudo, M. (2001). An infrared investigation in relation with chitin and chitosan characterization. *Polymer*, *42*(8), 3569–3580.
Chen, C. L., Wang, Y. M., Liu, C. F., & Wang, J. Y. (2008). The effect of water-soluble chitosan on macrophage activation and the attenuation of mite allergen-induced airway inflammation. *Biomaterials*, *29*(14), 2173–2182.
Chiu, C. T., Lee, J. S., Chu, C. S., Chang, Y. P., & Wang, Y. J. (2008). Development of two alginate-based wound dressings. *Journal of Materials Science-Materials in Medicine*, *19*(6), 2503–2513.
Dai, S., Ravi, P., & Tam, K. C. (2008). pH-Responsive polymers: Synthesis, properties and applications. *Soft Matter*, *4*(3), 435–449.

Denkbas, E. B., & Ottenbrite, R. M. (2006). Perspectives on: Chitosan drug delivery systems based on their geometries. *Jounal of Bioactive and Compatible Polymers*, *21*(4), 351–368.

Hamidi, M., Azadi, A., & Rafiei, P. (2008). Hydrogel nanoparticles in drug delivery. *Advanced Drug Delivery Reviews*, *60*(15), 1638–1649.

Hennink, W. E., & van Nostrum, C. F. (2002). Novel crosslinking methods to design hydrogels. *Advanced Drug Delivery Reviews*, *54*(1), 13–36.

Hsieh, C. Y., Tsai, S. P., Wang, D. M., Chang, Y. N., & Hsieh, H. J. (2005). Preparation of gamma-PGA/chitosan composite tissue engineering matrices. *Biomaterials*, *26*(28), 5617–5623.

Ishihara, M., Ono, K., Sato, M., Nakanishi, K., Saito, Y., Yura, H., et al. (2001). Acceleration of wound contraction and healing with a photocrosslinkable chitosan hydrogel. *Wound Repair and Regeneration*, *9*(6), 513–521.

Kim, I. Y., Yoo, M. K., Seo, J. H., Park, S. S., Na, H. S., Lee, H. C., et al. (2007). Evaluation of semi-interpenetrating polymer networks composed of chitosan and poloxamer for wound dressing application. *International Journal of Pharmaceutics*, *341*(1–2), 35–43.

Kondo, T., & Ohshima, T. (1996). The dynamics of inflammatory cytokines in the healing process of mouse skin wound: A preliminary study for possible wound age determination. *International Journal of Legal Medicine*, *108*(5), 231–236.

Lin, Y. H., Chung, C. K., Chen, C. T., Liang, H. F., Chen, S. C., & Sung, H. W. (2005). Preparation of nanoparticles composed of chitosan/poly-gamma-glutamic acid and evaluation of their permeability through Caco-2 cells. *Biomacromolecules*, *6*(2), 1104–1112.

Lin, Y. H., Mi, F. L., Chen, C. T., Chang, W. C., Peng, S. F., Liang, H. F., et al. (2007). Preparation and characterization of nanoparticles shelled with chitosan for oral insulin delivery. *Biomacromolecules*, *8*(1), 146–152.

Liu, B. S., Yao, C. H., & Fang, S. S. (2008). Evaluation of a non-woven fabric coated with a chitosan Bi-layer composite for wound dressing. *Macromolecular Bioscience*, *8*(5), 432–440.

Liu, N., Chen, X. G., Park, H. J., Liu, C. G., Liu, C. S., Meng, X. H., et al. (2006). Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli*. *Carbohydrate Polymers*, *64*(1), 60–65.

Moon, J. S., Kim, H. K., Koo, H. C., Joo, Y. S., Nam, H. M., Park, Y. H., et al. (2007). The antibacterial and immunostimulative effect of chitosan-oligosaccharides against infection by Staphylococcus aureus isolated from bovine mastitis. *Applied Microbiology and Biotechnology*, *75*(5), 989–998.

Mori, T., Murakami, M., Okumura, M., Kadosawa, T., Uede, T., & Fujinaga, T. (2005). Mechanism of macrophage activation by chitin derivatives. *Journal of Veterinary Medical Science*, *67*(1), 51–56.

Muzzarelli, R. A. A. (2009). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*, *76*(2), 167–182.

Okamoto, Y., Inoue, A., Miyatake, K., Ogihara, K., Shigemasa, Y., & Minami, S. (2003). Effects of chitin/chitosan and their oligomers/monomers on migrations of macrophages. *Macromolecular Bioscience*, *3*(10), 587–590.

Peppas, N. A., Bures, P., Leobandung, W., & Ichikawa, H. (2000). Hydrogels in pharmaceutical formulations. *European Journal of Pharmaceutics and Biopharmaceutics*, *50*(1), 27–46.

Prodhomme, E. J. F., Tutt, A. L., Glennie, M. J., & Bugg, T. D. H. (2003). Multivalent conjugates of poly-gamma-D-glutamic acid from *Bacillus licheniformis* with antibody F(ab') and glycopeptide Ligands. *Bioconjugate Chemistry*, *14*(6), 1148–1155.

Qu, X., Wirsen, A., & Albertsson, A. C. (2000). Novel pH-sensitive chitosan hydrogels: Swelling behavior and states of water. *Polymer*, *41*(12), 4589–4598.

Richard, A., & Margaritis, A. (2001). Poly(glutamic acid) for biomedical applications. *Critical Reviews in Biotechnology*, *21*(4), 219–232.

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science*, *31*(7), 603–632.

Rinaudo, M. (2008). Main properties and current applications of some polysaccharides as biomaterials. *Polymer International*, *57*(3), 397–430.

Sashiwa, H., & Aiba, S. I. (2004). Chemically modified chitin and chitosan as biomaterials. *Progress in Polymer Science*, *29*(9), 887–908.

Shih, I. L., & Van, Y. T. (2001). The production of poly-(gamma-glutamic acid) from microorganisms and its various applications. *Bioresource Technology*, *79*(3), 207–225.

Simsek-Ege, F. A., Bond, G. M., & Stringer, J. (2003). Polyelectrolye complex formation between alginate and chitosan as a function of pH. *Journal of Applied Polymer Science*, *88*(2), 346–351.

Tangsadthakun, C., Kanokpanont, S., Sanchavanakit, N., Pichyangkura, R., Banaprasert, T., Tabata, Y., et al. (2007). The influence of molecular weight of chitosan on the physical and biological properties of collagen/chitosan scaffolds. *Journal of Biomaterials Science-Polymer Edition*, *18*(2), 147–163.

Yang, J. Y., Xu, C. Y., Kopeckova, P., & Kopecek, J. (2006). Hybrid hydrogels self-assembled from HPMA copolymers containing peptide grafts. *Macromolecular Bioscience*, *6*(3), 201–209.

Zhang, L., Furst, E. M., & Kiick, K. L. (2006). Manipulation of hydrogel assembly and growth factor delivery via the use of peptide-polysaccharide interactions. *Journal of Controlled Release*, *114*(2), 130–142.

Zhang, R., Bowyer, A., Eisenthal, R., & Hubble, J. (2007). A smart membrane based on an antigen-responsive hyrogel. *Biotechnology and Bioengineering*, *97*(4), 976–984.