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Porous-conductive chitosan scaffolds for tissue engineering II. *in vitro* and *in vivo* degradation

YING WAN[1, 5,*], AIXI YU[2], HUA WU[3], ZHAOXU WANG[4], DIJIANG WEN[5]
[1]*Department of Chemistry and Chemical Engineering, Royal Military College of Canada, PO Box 17000, Station Forces, Kingston, Ontario, Canada, K7K 7B4*
E-mail: ying.wan@rmc.ca
[2]*Department of Microsurgery, Zhongnan Hospital, Wuhan University, Wuhan, 430071, People's Republic of China*
[3]*Department of Nuclear Medicine, Xiamen First Hospital, Fujian Medical University, Xiamen, 301003, People's Republic of China*
[4]*Centre de Recherch, Hopital Saint-Francois d'Assise, CHUQ, Quebec, QC, Canada, G1L 3L5*
[5]*School of Materials Engineering, Suzhou University, Suzhou, 215021, People's Republic of China*

Porous-conductive chitosan scaffolds were fabricated by blending conductive polypyrrole (PPy) particles with chitosan solution and employing an improved phase separation method. *In vitro* and *in vivo* degradation behaviors of these scaffolds were investigated. In the case of *in vitro* degradation, an enzymatic degradation system was employed and lysozyme was used as a working enzyme. Meanwhile, the degradation products of scaffolds, glucosamine and *N*-acetyl-glucosamine, were also analyzed with a HPLC method. *In vivo* degradation of scaffolds was performed by subcutaneously implanting these scaffolds in rat for prescheduled time intervals. In the both cases, the weight-loss of scaffolds was monitored during the whole degradation process for evaluating the degradation of scaffolds. The changes in conductivity of scaffolds after*in vitro* or *in vivo* degradation were also measured using a four-point technique. It was observed that the pore parameters of scaffolds themselves could significantly influence the degradation behaviors of scaffolds but the PPy content in the scaffolds seemed not to impart its effect to the degradation of scaffolds. Degradation dynamics of scaffolds and conductivity measurements indicated that these scaffolds shown fairly different behaviors in their *in vitro* and *in vivo* degradation process. According to the results obtained from *in vitro* and *in vivo* degradation of scaffolds and based on some requirements of practical tissue engineering application, it was suggested that the PPy content in the scaffold should be slightly higher than 3 wt.% but lower than 6 wt.%.

1. Introduction

Tissue engineering approaches have been employed to restore and reconstruct the function of impaired tissues and organs for years [1]. In most cases, tissue engineering technique severs for many applications, such as culturing cell, tissue transplantation and tissue regeneration, by mainly using scaffolds that have various structures, properties and functions. Scaffolds therefore can play a very influential role in tissue engineering applications by providing the desirable three-dimension space for the transport of nutrients and metabolite as well as for the tissue organization [2]. The basic requirements on these scaffolds are their biocompatibility, biodegradability, absorbability, expectant mechanical strength, appropriate porous structure and easy processing for desired shape without inducing other unwanted effects [3–5]. Up to now, scaffolds have been widely developed by using either natural or synthetic biodegradable polymers. Most synthetic polymers involved this issue are polylactide, polyglycolide, poly (ε-caprolactone) or their copolymers [6]. However, these employed synthetic polymers have several obvious drawbacks, such as past biodegradation, hydrophobicity, acidic degradation product and absence of cell recognition sites [7, 8]. Hence, many natural polymers have been alternatively selected for fabricating scaffolds. Of the known natural polymers considered for biomedical applications, chitosan has received great attention because of its favorable biological properties, such as biocompatibility, biodegradability and bioactivity. Chitosan is a

*Author to whom all correspondence should be addressed.

functional and linear polysaccharide and structurally, is considered as a copolymer of glucosamine (GA) and *N*-acetyl-glucosamine (NAc-GA) units linked by β-1, 4 glycosidic bonds. It can be mainly obtained by *N*-deacetylation of chitin which is the principle component of crustacean shells and is the most abundant natural polysaccharide on the earth after cellulose [9]. Chitosan has been extensively investigated for many biomedical applications. In addition to the drug delivery, polyelectrolyte complex, sorption agent, enzyme immobilization and gene delivery [10–12] chitosan has also been used for the porous tissue engineering scaffolds [13].

In recent years, conductive scaffolds have aroused much interest in tissue engineering applications [14]. Several published reports have demonstrated that these conductive scaffolds have a capacity to support and modulate the growth of a few different types of cells, such as endothelial cells [15], nerve cells [16, 17], bone cells [18], and chromaffin cells [19]. So far, these conductive scaffolds have been mainly fabricated using biodegradable synthetic polymer and an intrinsically conductive polymer, polypyrrole (PPy). PPy has been chosen mainly due to its high conductivity and acceptable biocompatibility with mammalian cells [20, 21]. Noticing the weaknesses of synthetic polymers previously mentioned, it would be more attractive to build a conductive scaffold by using natural polymer, for instance, chitosan, as a matrix. However, a basic requirement should be kept in mind before such a conductive chitosan composite can be used for the tissue engineering (specially for the applications of implantation), that is, only a very low amount of PPy can be allowed to be incorporated into the chitosan matrix because PPy itself is nonbiodegradable. Although several studies have involved conductive hybrid materials composed of chitosan and PPy [22, 23], in those cases, those hybrid materials have been prepared through either *in situ* chemically synthesizing PPy in the presence of chitosan solutions or blending chemically synthesized PPy with chitosan solutions. It is generally accepted that PPy particles obtained from a common chemical synthesis method have a lower conductivity [24, 25]. As a consequence, a larger amount of PPy is required for this kind of scaffold to obtain an usable conductive property. Therefore, above hybrid materials are obviously unsuitable for the conductive scaffold fabrication. Recently, a microemulsion polymerization method has provided an effective technique to produce PPy particles with a higher conductivity [26]. This kind of PPy particle apparently becomes an attractive candidate for the conductive component in a scaffold. By using the microemulsion polymerization technique mentioned above and employing an improved phase separation method, we have successfully fabricated some porous-conductive chitosan scaffolds which have a conductivity close to 10^{-3} S. cm^{-1} with a low PPy loading of only around 2 wt% [27]. To date, there are no *in vitro* and *in vivo* degradable data available for these conductive scaffolds. The goals of this work were to investigate *in vitro* and *in vivo* biodegradable behaviors of these conductive scaffolds. In general, polysaccharides can be effectively degraded by enzymatic hydrolysis. It has been reported that numerous different enzyme systems can be employed for the biodegradation of chitosan [28, 29]. In the present study, a representative enzyme, lysozyme, was selected as the working enzyme because it had been most often used for the *in vitro* degradation of chitosan. Examinations on *in vivo* degradation behaviors of these scaffolds were performed by implanting them subcutaneously in rats for various prescheduled periods. The main attentions for *in vivo* degradation were focused on the changes in properties of scaffolds themselves, and relevant results for inflammatory reactions, histological and immunohistological observations will be given separately in follow-up reports.

2. Experimental

2.1. Materials

Chitosan was received in the form of flake from Fluka. All other chemicals were obtained from Aldrich and used as reagent grades, including pyrrole, dodecylbenzenesulfonic acid, butanol, hexane, ethanol, $FeCl_3 \cdot 6H_2O$, carbon tetrachloride, *n*-heptane, cyclohexane, acetic acid, *N*-acetyl-D-glucosamine, glucosamine, sodium hydroxide pellets, acetone and Triton X-100. The pyrrole monomer was purified by distillation and stored in a refrigerator at about 4 °C before use. Other chemicals were all used without further purification. Deionized water (resistivity $>7.5\times10^8$ Ω.cm) was used for all samples. Phosphate buffered saline (PBS) packets, lysozyme (40,000 U/mg) and collagenase (Type 1A) were purchased from Sigma and used as received.

2.2. Degree of deacetylation and molecular weight of chitosan

The first derivative UV spectrum of chitosan was recorded on a VARIAN Cary 5E UV-Visible Spectrophotometer and the collected data were used for calculating the degree of deacetylation (DDA) of chitosan [30]. A calibration curve was generated using the method of Tan *et al*. [31]. The DDA value of chitosan was thus determined as 77.4 (±2.3)%. The molecular weight of chitosan was estimated using 0.25 *M* CH_3COOH/0.25 *M* CH_3COONa as solvent system according to our previously reported method [32], and viscosity-average molecular weight of chitosan was measured to be 1.37 (±0.26) $\times10^6$.

2.3. Preparation of porous-conductive chitosan scaffold

PPy was first synthesized with a microemulsion polymerization technique [26]. The resulting PPy powder was successively washed with acetone and deionized water via a centrifugation method (IEC 21000 Centrifuge). This powder was then dispersed into a 0.5% (v/v) acetic acid aqueous solution to form a sort of suspension using an ultrasonic water bath and, this suspension served for further preparation of scaffold in the next step.

The details of how to prepare porous-conductive chitosan scaffolds could be found in our first report [27]. In a typical experiment, chitosan solution was first prepared by dissolving chitosan in 1.0% (v/v) acetic acid aqueous solution with a 1.5 wt% chitosan concentration. To this solution, the suspension of PPy was added with vigorous stirring at 60 °C for 5 h, and then the mixture was cast into a membrane on a plastic dish. After pre-evaporating, the obtained gelatinous membrane was immersed into NaOH solution with different concentrations for 24 h and then the membrane was exhaustively washed with deionized water until neutrality was achieved. This solid-like membrane was rapidly frozen using liquid nitrogen and lyophilized at −75 °C (EYELA FD-5N freeze-drier). The PPy content in the membrane was determined by the amount of pyrrole monomer used in the preparation of PPy. By changing the concentration of NaOH solution from 1.0 to 2.0 wt% with a step of 0.5 wt% and incorporating PPy with 3.0 or 6.0 wt%, two sets of scaffolds with various pore parameters were obtained. One set having 3.0 wt% of PPy content was code-named as PPy3/Chit-(1.0), PPy3/Chit-(1.5) and PPy3/Chit-(2.0), and another set loaded 6.0 wt% of PPy was designated as PPy6/Chit-(1.0), PPy6/Chit-(1.5) and PPy6/Chit-(2.0), respectively; where the number closely following PPy indicated the PPy content, and the number in the brackets denoted the concentration of NaOH solution employed.

Some non-porous PPy/Chitosan scaffolds with different PPy content were also prepared and used as controls.

2.4. *In vitro* degradation

In vitro degradation of scaffolds was studied by incubating scaffolds in an enzymatic degradation solution (EDS) and monitoring the weight-loss of scaffolds and the changes in their conductivity. The biodegraded products of scaffold were also analyzed.

0.1 *M* PBS solution with the pH 7.4 was first prepared by dissolving phosphate buffered saline powders in deionized water. To this PBS solution, lysozyme was added with a 2×10^4 U/ml lysozyme concentration to form EDS.

The dry scaffolds were cut into specimens of 1×1 cm^2 in size, and were precisely weighed. They were immersed in EDS in sealed tubes, and incubated at 37 °C in a reciprocal shaking incubator at 60 rpm for various periods up to 10 weeks without changing media. At predetermined time intervals, the scaffolds were taken out from the incubation medium and exhaustively washed with deionized water, and subsequently were frozen and lyophilized. The scaffolds were dried again in vacuum at ambient temperature to constant.

The degradation products were analyzed with a HPLC method. At the end of each predetermined incubation time interval, the medium solutions containing the degradation products of chitosan were filtered through a 0.5 μm filter and used for HPLC analysis. The chromatography was recorded on an Alltech HPLC system equipped with a UV-visible absorbance detector (Alltech Model 205) and a solvent pump (Alltech Model 325) at ambient temperature. An Adsorbosil C18 column (250 mm × 4.6 mm I.D.) was chosen, and the elution solvent was HPLC-grade water running at a flow-rate of 0.6 ml/min. HPLC analyses were carried out after 1, 3, 5, 7, 10, 15, 20, 25 and 30 days by measuring degradation product peaks area with glucosamine and *N*-acetyl-glucosamine as references [33].

2.5. *In vivo* degradation

In vivo degradation of scaffolds was conducted according to our previous method [34]. In brief, thirty-six male Wistar rats (350 ± 30 g, body weight) were randomly divided into six groups of 6 rats each for the implantation of scaffolds. A precisely weighed 1×1 cm^2 specimen was implanted subcutaneously in the back of the rat via the methods described in the literature [35, 36]. After awakening, the animal was sent back to the animal housing facility and fed with an unrestricted standard diet. *In vivo* degradation was carried out in such a manner that the time was altered at various intervals of 1, 2, 4, 6, 8 and 10 weeks, and each group was assigned to one of prescheduled implantation periods. At the end of the given implantation period, the animals were sacrificed and the samples were harvested along with the surrounding tissue. The tissue adhering onto the specimens was carefully cleaned with a published method [37]. Briefly, the freshly explanted samples were rinsed in deionized water, followed by being incubated in a collagenase solution (collagenase: 500 U/ml + buffer (0.05 *M* Tris-HCl, 0.36 m*M* $CaCl_2$, pH 7.5, with 0.02% w/v sodium azide)) at 37 °C for 24 h. After that, the samples were rinsed in 1% aqueous solution of Triton X-100, followed by a further intensive rinse in deionized water. Subsequently, these samples were frozen and lyophilized at −75 °C. They were dried again until their respective constant weight was reached.

The same cleaning protocol was applied to the non-implanted scaffolds to quantitatively evaluate the influence of collagenase on the chitosan matrix.

2.6. Measurement and morphology

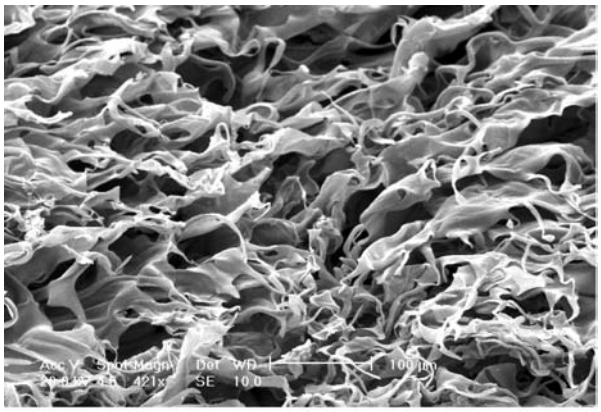
The conductivity of scaffold was measured using a four-point probe technique at a constant current of 0.5 mA at ambient temperature. The scaffolds were dried to constant weight before the measurements were made. Six replicate measurements were made for each kind of sample and average values were reported.

After experiencing different periods of *in vitro* or *in vivo* degradation, the weight-loss (W_{loss}) of samples was calculated via following formula:

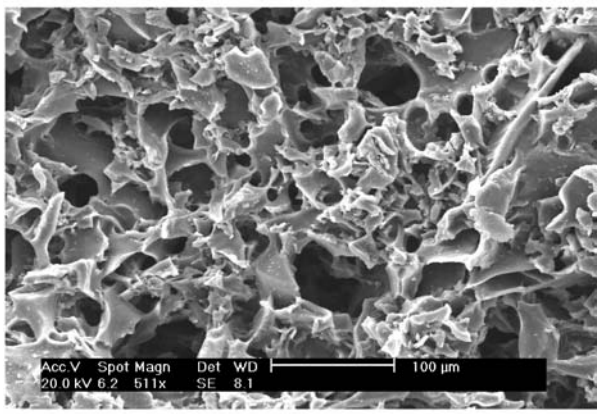
$$W_{loss} = (W_{init} - W_{deg})/W_{init} \quad (1)$$

where W_{init} is the initial weight of scaffold before degradation and, W_{deg}, the surplus weight of scaffold after degradation.

The dry scaffolds were examined under a Philips XL-30 scanning electron microscope (SEM) and the pore



(a)



(b)

Figure 1 SEM micrographs of scaffolds (a) PPy3/Chit-(1.5) (porous surface), (b) PPy3/Chit-(1.5)(cross-section area).

size of scaffold was estimated via the analysis of SEM images from cross-section areas.

3. Results and discussion

3.1. Pore parameters, conductivity and morphologies of scaffolds

The details for determining the pore parameters of scaffolds can be found in our previous reports [27, 38]. It is obviously necessary to present their basic information before any comparison and discussion can be made. Fig. 1 shows the surface and cross-section morphologies of PPy3/chit-(1.5) scaffold. It reveals that pores inside the scaffold are interconnected and the pore size ranges from 5 to 60 μm (in cross-section area). We found that the pore volume, porosity and pore size of all scaffolds mainly varied with the concentration of NaOH solution employed for the preparation of scaffolds, and the PPy content in the scaffolds nearly did not influence the pore parameters of scaffolds. In addition, it was also observed that the conductivity of scaffold was mainly governed by the PPy content and also comparably varied with pore parameters at different rates. Some basic information about the pore parameters and conductivity for two series of scaffolds was summarized in Table I. Based on the data listed in Table I, it can be concluded that there are no substantial differences in the pore parameters for the mutually corresponding scaffold in each set.

3.2. *In vitro* degradation

In vitro degradation behaviors of chitosan itself in lysozyme degradation system have been intensively investigated. In the case of porous chitosan scaffolds, for the different application purposes, the concentration of lysozyme in degradation media had varied from 10^3 to 2×10^5 U/ml, leading to quite different degradation rates [39–41]. Unfortunately, since the concentration of lysozyme altered within such a big span, many published results were nearly incomparable. In addition, there is no standard method available for *in vitro* biodegradation investigation of chitosan scaffold. At a compromise level, in the present study, the concentration of lysozyme was selected as 2×10^4 U/ml, which was almost a medium-strong concentration compared to the data reported in the literature.

The conventional assays for examining degradation behaviors of scaffolds fall into two categories: those that measure the variance in scaffold itself (for instance, the changes in appearance, in weight or in some other physcochemical properties) and those that measure the degradation products of scaffolds. Monitoring the weight-loss of scaffold is a suitable and reliable method and it has been widely employed for the investigation of degradation behaviors of scaffolds [42, 43].

TABLE I Pore parameters and conductivity of PPy/chitosan scaffold[a].

Scaffolds	Pore volume (ml/g)	Porosity (%)	Pore size (μm)[b]	Concentration of NaOH (wt%)	Conductivity (S. cm^{-1})
Non-porous PPy/chitosan (PPy content: 3 wt.%)					0.086
PPy3/Chit-(1.0)	1.14	47.1	2–40	1.0	2.82×10^{-2}
PPy3/Chit-(1.5)	1.57	55.7	5–50	1.5	1.17×10^{-2}
PPy3/Chit-(2.0)	1.92	61.4	10–70	2.0	0.74×10^{-2}
Non-porous PPy/chitosan (PPy content: 6 wt.%)					0.297
PPy6/Chit-(1.0)	1.26	48.9	5–40	1.0	8.96×10^{-2}
PPy6/Chit-(1.5)	1.63	54.3	5–60	1.5	7.12×10^{-2}
PPy6/Chit-(2.0)	2.08	62.8	10–70	2.0	5.03×10^{-2}

[a]The values for pore volume, porosity and conductivity were the average values from five specimens for each sample.
[b]The pore size of scaffold was estimated from SEM images in the cross-section area.

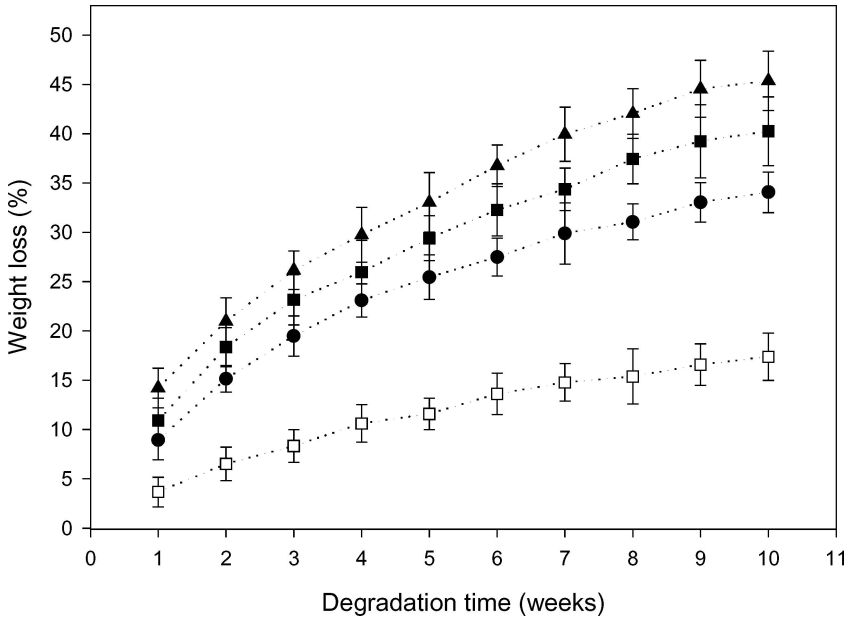


Figure 2 Weight-loss of scaffolds after incubation in EDS. (▲) PPy3/Chit-(2.0), (■) PPy3/Chit-(1.5), (●)PPy3/Chit-(1.0), (□) non-porous PPy/Chitosan (PPy content: 3 wt.%).

3.2.1. Effect of pore parameters on the degradation of scaffolds

Fig. 2 illustrates the weight-loss of scaffolds after incubation in EDS for various time intervals. Several characteristics can be drawn: (1) compared to non-porous PPy/Chitosan scaffold, porous scaffolds present a significant higher weight-loss rate and a much larger weight-loss within the same interval of degradation time; (2) the weight-loss changes with pore parameters of scaffolds, i.e., the scaffold with a bigger pore size and a higher porosity has a greater weight-loss; (3) the weight-loss does not alter linearly with the degradation time and the scaffolds are degraded relatively fast in the first a few weeks. Although chitosan possesses a higher hydrophilicity, the lysozyme can only progressively cleave chitosan molecules on the surface or the superficial layer of scaffolds. Porous scaffolds, especially for those ones having a bigger pore size and a higher porosity, obviously provide a larger surface area and more active sites for lysozyme to react upon, and thus more chitosan molecules in the scaffolds can be cleaved and a larger weight-loss of scaffold is inevitably resulted in. A non-porous scaffold only exposes its two surfaces to the degradation medium, and as expected, a corresponding and much lower weight-loss of scaffold is concomitantly observed. Since the activity of lysozyme should be relatively high in the first a few weeks during the overall degradation process and *in vitro* degradation was performed without changing degradation media, the weight-loss rate should be greater in the first a few weeks than other following weeks.

3.2.2. Analysis of degradation products

To find out more details occurred during degradation process, the degradation products of chitosan, i.e., GA and NAc-GA, were also analyzed. Two representative HPLC chromatographs of degradation products are depicted in Fig. 3. It is noted that after 5 days incubation in EDS a much larger amount of GA product is detected than that of NAc-GA product for each kind of scaffold by comparing respective peak area. Moreover, it is again seen that the non-porous PPy/Chitosan is more slowly degraded since corresponding peak area of its degradation products is much smaller than that of PPy3/Chit-(1.5). By continuously measuring the peak area of two kinds of degradation products, the variance in peak area for each kind of scaffold with degradation time is illustrated in Fig. 4. The curves show that, in both cases, the GA product increases comparatively fast until the degradation time approaches around 10 days and after that the curves related to GA products tend to extend in a slowly increasing manner. On the other hand, NAc-GA product is released apparently fast after about 7 days incubation in EDS. In addition, in both cases, the amount of NAc-GA product is significantly less than that of GA before degradation time reaches 20 days, and thereafter, it increases relatively fast and finally approaches almost the same amount of GA product when the degradation time is extended to 30 days. Mi *et al*. [44] has suggested a mechanism for the degradation of chitosan in lysozyme system, in which GA oligomers and NAc-GA oligomers are randomly cleaved by lysozyme, implying GA and NAc-GA should be released with an almost same probability. Based on this degradation mechanism, the much lesser NAc-GA product in the first 20 days of degradation may be ascribed to the lesser average amount of NAc-GA on the chitosan backbones because as previously stated in experimental section, the DDA of chitosan was around 77% (the corresponding content of NAc-GA in chitosan backbone is around 23%). However, the tendency that the amount of NAc-GA product is less than that of GA has not been maintained during the whole degradation process. The fact that the amount of NAc-GA product approaches almost the same amount of GA product at the end of degradation

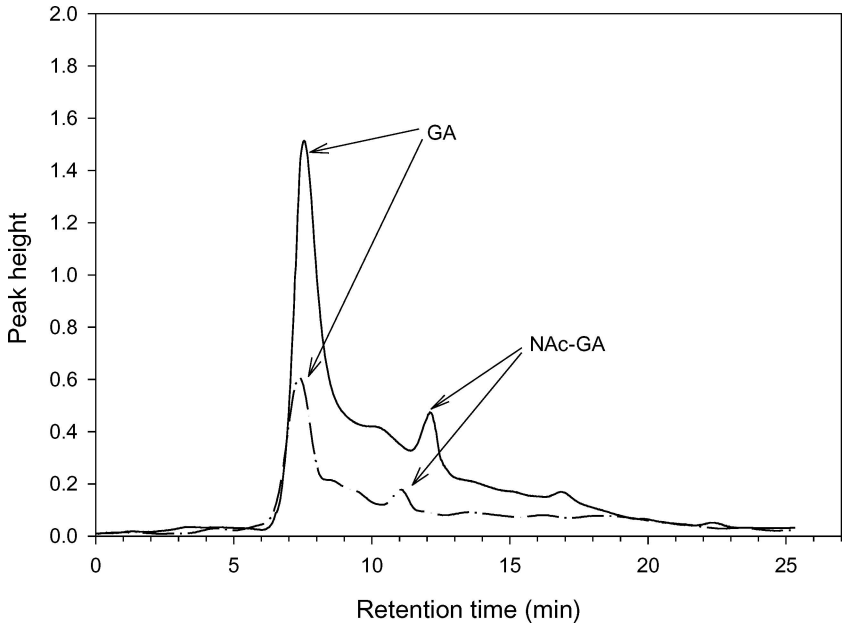


Figure 3 HPLC chromatographs of degradation products of scaffolds after 5 days incubation in EDS. (—) PPy3/chit-(1.5), (– · –) non-porous PPy/Chitosan (PPy content: 3 wt.%).

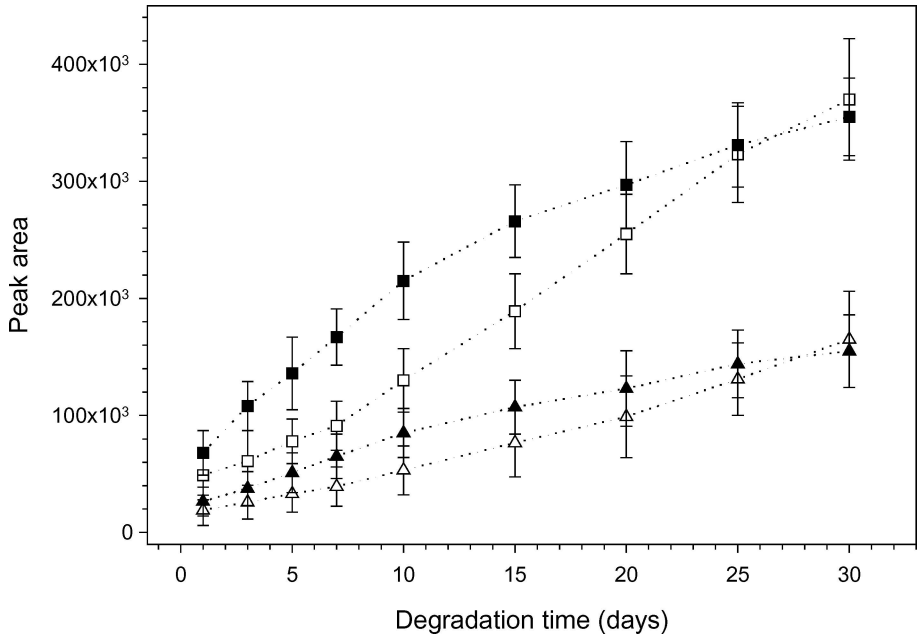


Figure 4 GA and NAc-GA products of PPy3/Chit-15 and non-porous PPy/chitosan scaffolds as a function of degradation time. (■) GA (PPy3/Chit-(1.5)), (□) NAc-GA (PPy3/Chit-(1.5)); (▲) GA (non-porous PPy/Chitosan, PPy content: 3 wt.%), (△) NAc-GA (non-porous PPy/Chitosan, PPy content: 3 wt.%).

process may reveal the lysozyme somewhat preferentially cleaves NAc-GA oligomers at the later stage of degradation process. The results obtained above may suggest that an integrated degradation mechanism for chitosan being degraded in lysozyme system still need exploring.

3.2.3. Effect of PPy content on the degradation scaffolds

Fig. 5 presents the weight-loss of scaffolds with different PPy content after incubation in EDS for various intervals. It shows that the graphs of weight-loss versus degradation time are approximately kept in almost the same shape for two kinds of scaffolds, and the curve corresponding to the weight-loss of PPy3/Chit-(1.5) is nearly integrally shifted upward to a certain amount, indicating two kinds of scaffolds behave almost in the same way and the PPy content in the scaffold does not significantly affect the degradation behavior of scaffold. These results are acceptably reasonable because PPy itself is nonbiodegradable and the PPy3/Chit-(1.5) and PPy6/Chit-(1.5) have almost the same pore parameters (see Table I) except for the difference in respective PPy content. Accordingly, during the whole degradation process, the greater weight-loss of PPy3/Chit-(1.5) can be attributed to its lower PPy content.

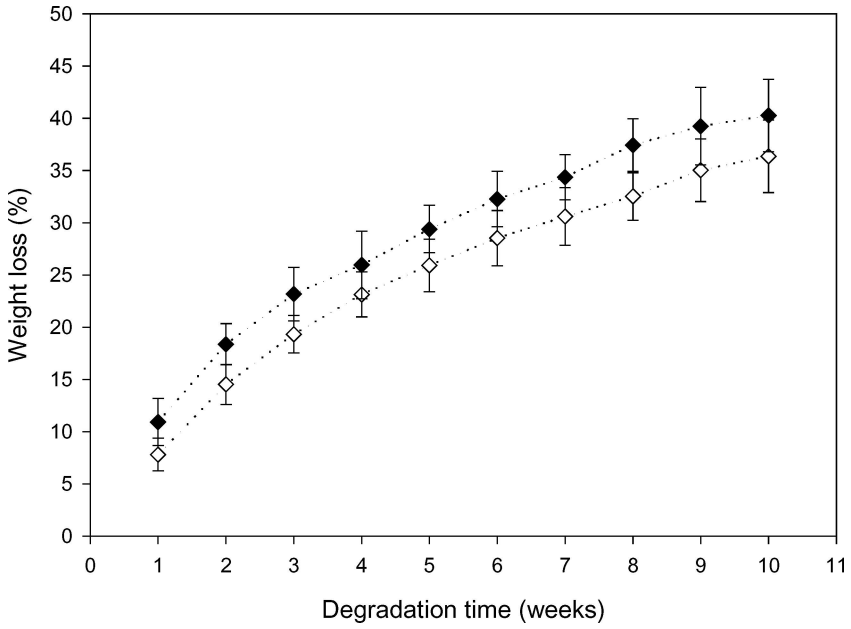


Figure 5 Weight-loss of scaffolds with different PPy contents after incubation in EDS. (♦) PPy3/Chit-(1.5), (◇) PPy6/Chit-(1.5).

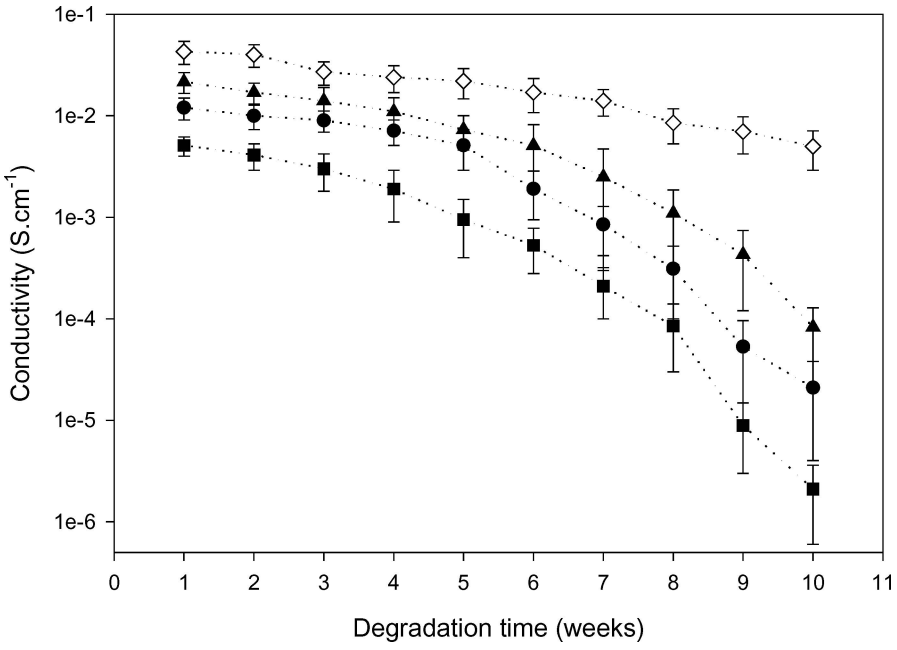


Figure 6 Variation in conductivity of scaffolds (PPy3/Chit set) after incubation in EDS. (◇) non-porous PPy/Chitosan (PPy content: 3 wt.%), (▲) PPy3/Chit-(1.0), (●) PPy3/Chit-(1.5) and (■) PPy3/Chit-(2.0).

3.2.4. Variation of conductivity

The variations of conductivity of two series of scaffolds with the degradation time are displayed in Figs. 6 and 7, respectively. Two graphs for non-porous PPy/Chitosan scaffolds are also given in both figures and used as controls. Only a slight decrease in conductivity for non-porous PPy/Chitosan scaffolds is observed in both figures no matter whether the PPy content in the scaffold is 3 or 6 wt%. As shown in Fig. 6, the scaffolds in PPy3/Chit series exhibit a faster decrease in conductivity along with degradation time. On the other hand, a relatively slow-moving decline in conductivity for the scaffolds in PPy6/Chit series is noticed in Fig. 7. In respect to the same PPy content, it can be seen in Figs. 6 and 7 that the scaffold having a higher porosity and a larger pore size remains a lower conductivity (see Table I). In addition, comparing Fig. 6 with Fig. 7, as far as the similar pore parameters (see Table I) are concerned, the results indicate that, within a same time interval of degradation, the scaffold containing lesser PPy possesses a distinctly lower conductivity compared to the one containing more PPy.

The conductive property of scaffold stems from the contribution of PPy particles which could build a conductive network inside the scaffold. Since PPy is non-biodegradable, the decrease in conductivity of scaffold after incubation could be attributed to the damage to the matrix of scaffold did by degradation media since some newly created flow and pores inside the scaffold will inevitably break many conductive paths on

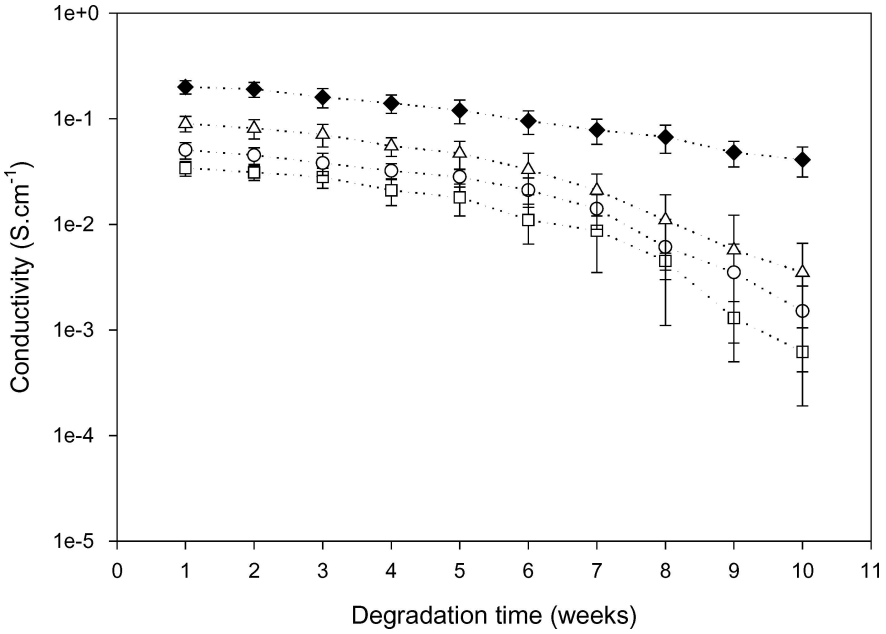


Figure 7 Variation in conductivity of scaffolds (PPy6/Chit set) after incubation in EDS. (♦) non-porous PPy/Chitosan (PPy content: 6 wt.%), (△) PPy6/Chit-(1.0), (○) PPy6/Chit-(1.5) and (□) PPy6/Chit-(2.0).

the conductive network. Several SEM micrographs in Fig. 8 exhibit the changes in morphologies of scaffolds before and after incubation. After 3 weeks incubation in EDS, the original thin solid bottom skin of scaffold has been seriously denuded and some pits and pores can be easily viewed (Fig. 8(b)). At the same time, the enlarged pore size on top surface (Fig. 8(c)) and in the cross-section (Fig. 8(d)) is distinctly evidenced (compared to the SEM images in Fig. 1).

3.3. *In vivo* degradation

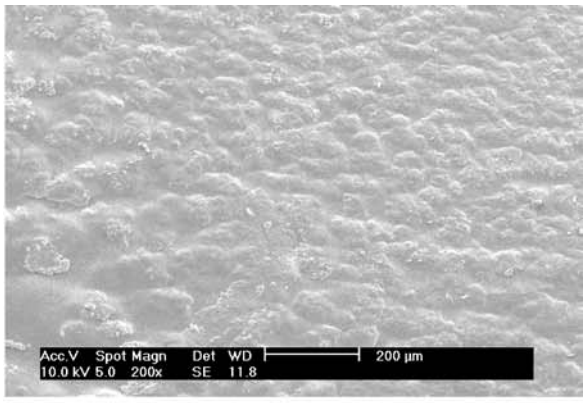
Since the PPy content in scaffold did not substantially influence the degradation behavior of scaffolds based on the results obtained above, at a simple level, only those scaffolds in PPy3/Chit series were selected for further *in vivo* degradation investigations.

3.3.1. Cleaning method and its effect

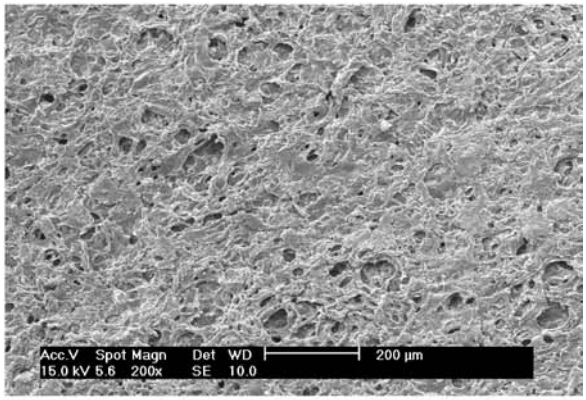
The freshly explanted scaffolds were found to be partially or completely anchored by the surrounding tissue (mainly collagenous tissue with or without a small amount of fatty tissue) to a different extent depending on the various degradation time intervals. In order to quantitatively analyze the weight-loss of explanted scaffolds and the changes in their conductivity after a period of *in vivo* degradation, these attached tissues have to be carefully removed. Several common methods by using chemicals, such as sodium bicarbonate and/or bleaching agents, may not be feasible because in many cases the chemical cleaning either was insufficient and left residual impurities, or was too harsh and damaged the scaffold itself [45, 46]. The enzymatic cleaning method employed in the present study is proven to be effective. Fig. 9 shows SEM images of one explanted scaffold cleaned by a collagenase solution described in the experimental section. It is observed that before the cleaning the scaffold is almost totally covered by anchored tissues both on the surface (Fig. 9(a)) and inside the pores (Fig. 9(b)) and after an enzymatic cleaning the micro-porous structure is clearly visible again both on the surface area (Fig. 9(c)) and cross-section area (Fig. 9(d)). Although enzymatic treatment is an effective method, however, it has been stated that the collagenase can also degrade chitosan matrix itself [47]. To estimate the extent of how seriously the collagenase can affect the chitosan matrix, several non-implanted specimens from PPy3/chit-(1.5) series were treated using exact the same method applied to the explanted scaffolds. The variation in weight-loss and conductivity of resulting non-implanted scaffolds is illustrated in Fig. 10. After experiencing a period of 24 h incubation, it is observed in Fig. 10 that the total weight-loss is less than 3% even considering experimental errors, and the corresponding decrease in conductivity is so small that it can be thought as a negligible quantity. These results indicate that the collagenase only slightly degrades chitosan matrix in this short period of incubation time and it does not markedly influence the conductivity of non-implanted samples. On the basis of above results and in considering the fact that surfaces of the freshly explanted scaffolds were partially or totally covered by collagenous tissue and fatty tissue after a few weeks of implantation, the effect of collagenase on the chitosan matrix will be further remarkably weakened, and it is reasonable to believe that the property of explanted scaffolds will not exhibit any substantial change although they are cleaned with the collagenase solution. Therefore, the effect of collagenase will not be taken into account for the evaluation of scaffolds after *in vivo* degradation in the following sections.

3.3.2. Weight-loss of scaffold after in vivo degradation

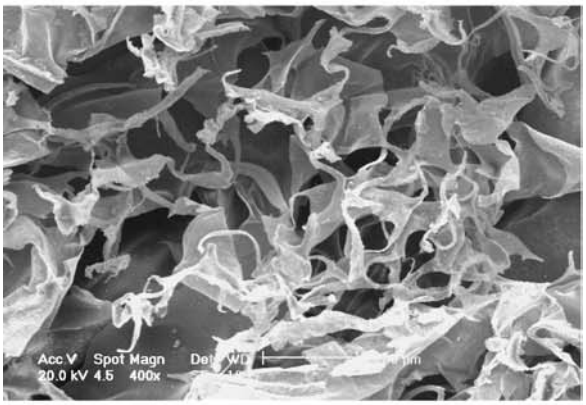
After implanting in rats for various prescheduled time intervals, the scaffolds (PPy3/Chit series) were



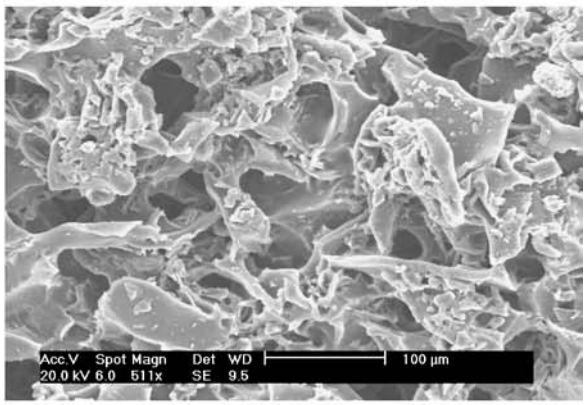
(a)



(b)



(c)



(d)

Figure 8 SEM micrographs of PPy3/Chit-(1.5) scaffolds after incubation in EDS. (a) Bottom solid skin (before incubation), (b) Bottom solid skin (after 3 weeks incubation), (c) Top surface (after 3 weeks incubation) and (d) Cross-section area (after 3 weeks incubation).

explanted and cleaned with the same methods described in the experimental section. They were dried to their respective constant weight prior to weight-loss measurements. The corresponding results are given in Fig. 11. The weight-loss of scaffolds after implantation presents a noticeable different behavior in comparison with those non-implanted scaffolds degraded in enzymatic medium (see Fig. 2). In the overall period of implantation, the weight-loss gradually increases with increasing degradation time but it varies at relatively low rate in the first a few weeks and becomes slightly fast in the later period. In addition, the magnitude of weight-loss of scaffolds after implantation is also fairly smaller than those non-implanted ones in the whole range of degradation time. The progressive increase in the weigh-loss of implanted scaffolds can be ascribed to the *in vivo* degradation environment. Unlike *in vitro* enzymatic degradation system, the concentration and activity of lysozyme and other enzymes in rat should be maintained at an almost constant level, which keeps degrading the chitosan matrix at a constant degradation rate and leads to a successive increase in the weight-loss. However, the concentration of lysozyme in rat may be lower than that used for *in vitro* enzymatic degradation system and thus, resulting in a lesser amount of weight-loss during the different degradation time intervals.

3.3.3. Variation in conductivity of scaffold after In vivo degradation

Fig. 12 illustrates the variation of conductivity of scaffolds after *in vivo* degradation. Since some conductive pathways in the conducting network could be damaged during the degradation process of scaffold, a progressive decrease in conductivity of scaffolds should be concomitantly observed starting at the early stage of degradation process. It is seen in Fig. 11 that these scaffolds after implantation show a lesser weight-loss compared to those non-implanted scaffolds degraded in enzymatic degradation system, as expected, a relatively mediate change in conductivity of scaffolds after implantation is also observed in Fig. 12 (compare with Fig. 6) because a lesser weight-loss of scaffold implies a lesser extent of damage to the conducting network inside the scaffold.

In general, an electrical potential for the study of electro-physiological behavior of biological tissue or organs is selected lower than 150 mV. A 100 mV electrical potential is a common value applied to the nerve conduits [14, 17]. Meanwhile, the electrical current in the range between 0.6 and 400 μA has been demonstrated to be biologically effective in both *in vitro* and *in vivo* investigations [48, 49]. Thus, an approximate estimation can be made to select a practical conductivity for these scaffolds when they are used for nerve conduits. Usually, a nerve conduit has a size with an inner diameter of 4 mm, a length of 10 mm and a wall-thickness of 2 mm. Accordingly, if some scaffolds with a conductivity between 10^{-3} and 10^{-4} S.cm^{-1} are selected, their resistance will lie in the range between 10^4 and $10^5\Omega$. When a 100 mV electrical potential is

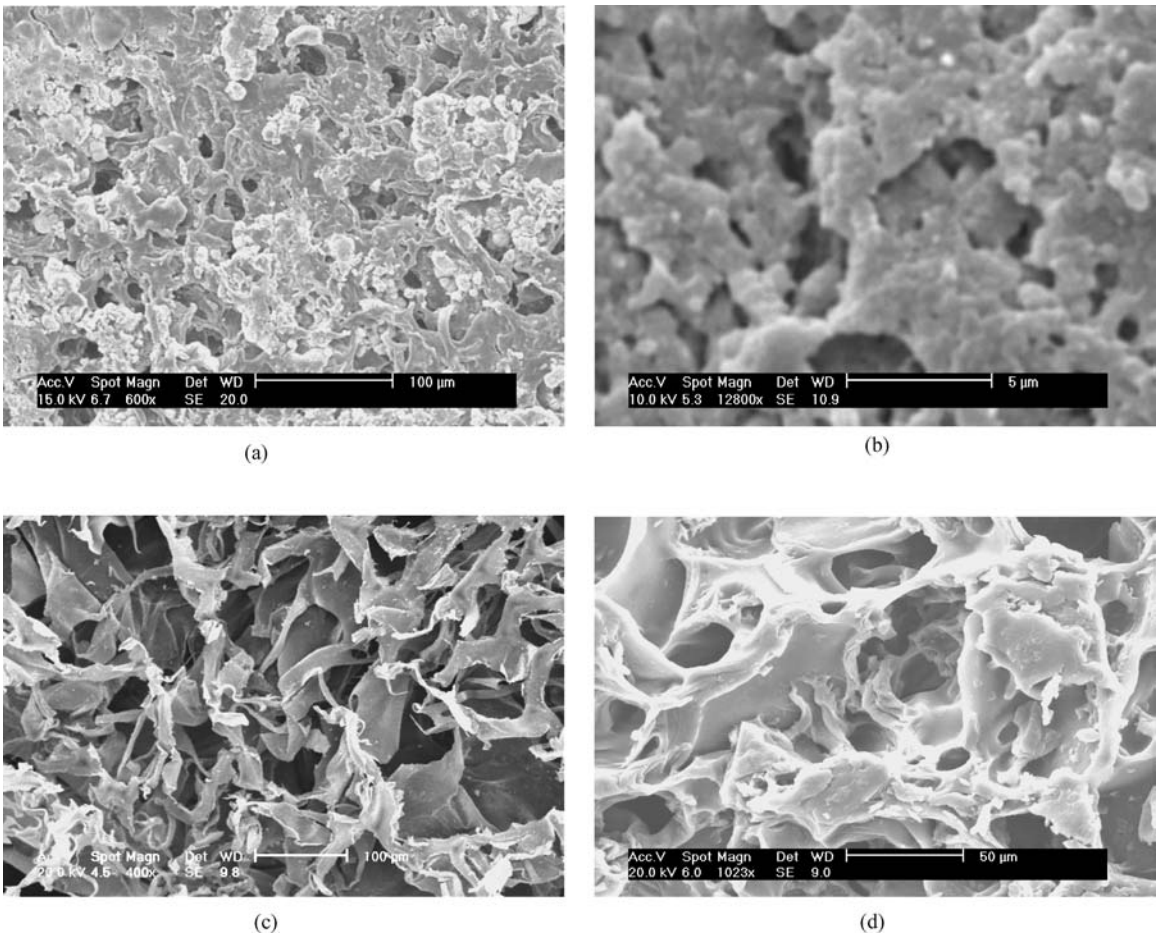


Figure 9 SEM micrographs of explanted PPy3/Chit-(1.5) scaffolds before and after the cleaning in collagenase solutions. (a) Porous surface of explanted scaffold before the cleaning, (b) Cross-section area of explanted scaffold before the cleaning, (c) Porous surface of explanted scaffold after the cleaning and (d) Cross-section area of explanted scaffold after the cleaning.

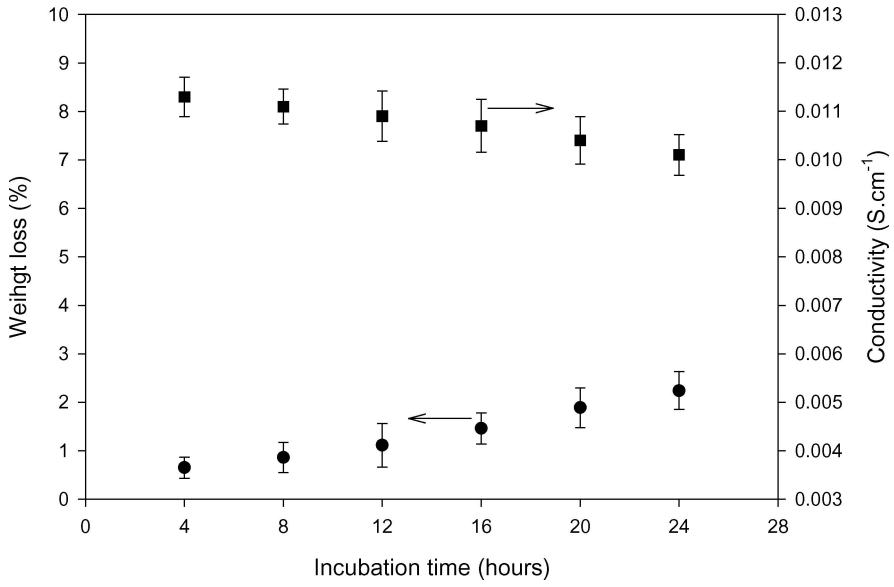


Figure 10 Variation in conductivity and weight-loss of non-implanted scaffolds (PPy3/Chit-(1.5)) cleaned in collagenase solutions.

applied to these scaffolds, an electrical current between 100 and 10 μA can be expected, which is a biologically effective current. Therefore, only some scaffolds with a conductivity between 10^{-3} and 10^{-4} S.cm^{-1} can be considered as candidates for the practical application. Many applications concerning the regeneration of tissues, such as the regeneration of sciatic nerve and peripheral nerve in rat, may require the scaffolds serving for a period of 1–2 months or even longer [50], hence, a basic requirement that needs to be met is to maintain the conductivity of scaffolds between 10^{-3} and 10^{-4} S.cm^{-1} even after these scaffolds have been *in vivo* degraded more than 8 weeks. In addition, another factor that should be considered for this type of scaffold

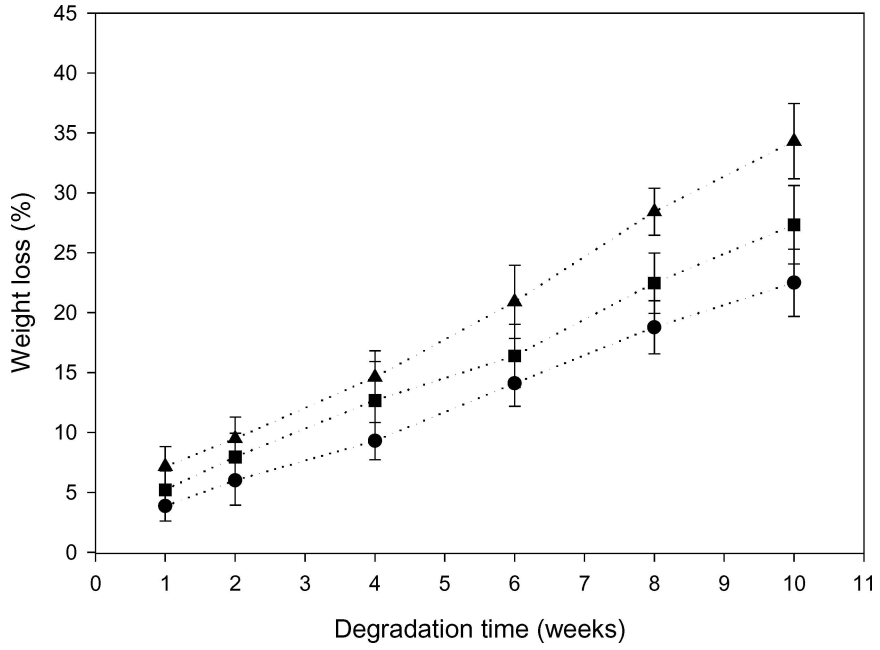


Figure 11 Weight-loss of scaffolds after implantation in rat. (▲) PPy3/Chit-(2.0), (■) PPy3/Chit-(1.5), (●)PPy3/Chit-(1.0).

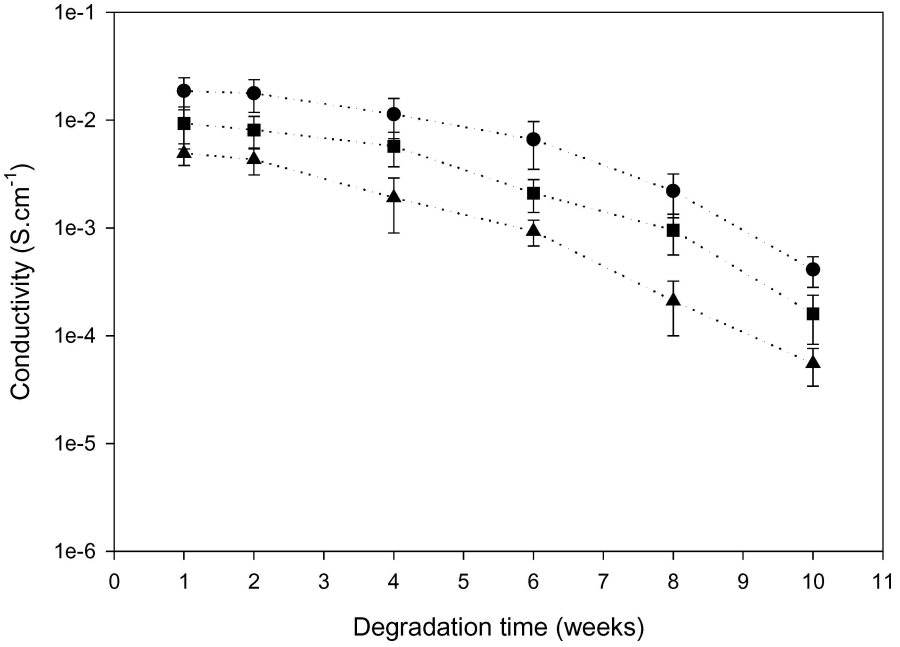


Figure 12 Variation in conductivity of scaffolds after implantation in rat. (●) PPy3/Chit-(1.0), (■) PPy3/Chit-(1.5), (▲) PPy3/Chit-(2.0).

is that the PPy content has to be controlled as low as possible because of nonbiodegradable property of PPy. Combining the observations of *in vivo* degradation with the results of *in vitro* degradation and, considering for a relatively long tissue engineering application, consequently, the PPy content in these scaffolds should be slightly higher than 3 wt% but lower than 6 wt%.

4. Conclusion

In vitro degradation and *in vivo* degradation of porous-conductive chitosan scaffolds presented fairly different behaviors. In the case of *in vitro* degradation in a lysozyme system, it was found that (1) the pore parameters of scaffolds themselves could significantly influence the degradation behaviors of scaffolds but the PPy content in the scaffolds seemed not to impart its effect to the scaffolds; (2) the weight-loss of scaffolds did not alter linearly with the degradation time and the scaffolds were degraded relatively fast in the first a few weeks; (3) degradation products, GA and NAc-GA, were released at relatively different rates and the degradation behavior for NAc-GA component indicated that chitosan was degraded in a way which was not strictly in agreement with proposed mechanism for the degradation of chitosan in lysozyme degradation system. The cleaning method employed collagenase in the present study was proven to be quite effective and only a slight its effect on chitosan matrix was observed. After being subcutaneously implanted in rat for various implantation time intervals, the explanted scaffolds cleaned with the collagenase solution exhibited a different behavior

in their degradation dynamics compared with those *in vitro* degraded in lysozyme degradation system, i.e., the weight-loss of scaffolds after implantation gradually increased with increasing degradation time in the overall period of implantation and varied at relatively low rate in the first a few weeks and became slightly fast in the later period. Furthermore, implanted scaffolds shown a lesser weight-loss and a relatively small change in conductivity compared to non-implanted scaffolds. Combining the observations of *in vivo* degradation with the results of *in vitro* degradation, and considering that these scaffolds would serve for a relatively long tissue engineering application, the PPy content in these scaffolds should be slightly higher than 3 wt% but lower than 6 wt%.

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