Effect of Experimental Parameters on the Formation of Chitosan–Poly(acrylic acid) Nanofibrous Scaffolds and Evaluation of their Potential Application as DNA Carrier

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ABSTRACT: Gene delivery from tissue engineering scaffolds has demonstrated the ability to promote gene transfer and stimulate new tissue formation. In this article we report a novel nanofibrous scaffold based on polyelectrolyte complexes as a vehicle for delivery of DNA. When polycation chitosan (CS) was dropped into polyanion poly(acrylic acid) (PAA) suspension and freeze-dried, CS-PAA nanofibrous scaffold with diverse microstructure would be formed under different experimental conditions. The nanofiber size was affected by the CS molecular weight, the concentration of CS, the volume ratio of CS to PAA, the reaction temperature, the incubation time, and the final pH of the suspension as well. By using adipic acid as branch promoter, adjusting the pH value of the CS solution to 3, and then dropping CS into the PAA solution at a ratio of 3 : 1, a nanofibrous structure with average diameter 140 nm is obtained after the suspension is freeze-dried. These nanofibrous scaffolds were nontoxic and can encapsulate plasmid DNA very well. Transgenic expression in human dermal fibroblasts seeded on the nanofibrous scaffolds was significant after 14 days compared to lipofectamine controls. This result indicates that CS-PAA nanofibrous scaffold have favorable characteristics for nonviral gene delivery to mammalian cell, and have the potential to enhance gene transfer in tissue engineering. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1769–1780, 2010

Key words: self-assembly; swelling; nanofiber; chitosan; gene delivery

INTRODUCTION

Gene therapy is a rapidly progressing technology devised for the treatment of a variety of diseases. This approach is based on the principles of correcting the basis of a disease at their origin by delivery and subsequent expression of exogenous DNA, which encodes for a missing or defective gene product. Gene therapy strategies were originally designed using viral particles for DNA delivery. However, they develop a high immunogenicity after repeat administration as the mammalian immune system has developed strategies to eliminate viral invaders as well.[1] These limitations have motivated the development of nonviral vectors capable of delivering DNA while providing some of the advantages of viral vectors.

One approach to control the duration and location of gene expression with nonviral vectors is to immobilize DNA constructs in localized depots with polymeric scaffolds that provide a platform for localized DNA delivery.[2] Gene expression is only typically desired at the site, at which tissue formation is desired. In fact, localizing gene expression can alleviate unwanted side events at other sites in the body.[3] Thus, ideal scaffolds for gene delivery should incorporate methods of fabrication that allow for control of the structure and morphology of the biomaterial from the nanoscopic to macroscopic and molecular to macromolecular levels. Scaffolds should be able to offer site-specific delivery of DNA in a controllable and sustainable manner. Additionally, the scaffold should be able to protect the DNA from the biological system until it is released.

Polymer scaffold fabricated from polyesters, such as poly(lactideco-glycolide) and poly(l-lactic acid), and natural polymers, such as, collagen and hyaluronan can be incorporating DNA either in the naked or condensed state. They also have been formulated into nano- and micro-spheres, as well as three-dimensional scaffolds capable of DNA delivery and supporting cell growth.[4] These materials are

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typically biocompatible and easily processed, making them useful scaffolds for tissue engineering. The natural scaffold for most tissues is the extracellular matrix (ECM), which is composed of ground substances and collagens. The collagen fibers maintain structure and mechanically stability. The collagen fibrous structure is organized in a fiber network composed of collagen fibers that are formed hierachically by nanometer-scale fibrils. Due to large surface area to volume ratio, flexibility in surface functionalities, and superior mechanical performance compared with any other known form of the material, the interaction between polymer nanofibers and cell has been widely discussed. Therefore, ideally the dimensions of the building fibers of the scaffold should be on the same scale with those of natural ECM. In efforts to mimic this natural scaffold, many groups are exploring the development of biomaterial composites incorporating biological agents, such as growth factors and other key cell regulatory molecules.[5] Additionally, a wide variety of polymers, polymer processing techniques, and fabrication techniques are being explored for the incorporation of plasmid DNA into delivery vehicles of various geometries and tissue engineering scaffolds.

Self-assembly is a process, whereby atoms, molecules, and molecular aggregates organize and arrange themselves through weak and noncovalent forces such as hydrogen bonding, electrostatic interactions, and hydrophobic forces into stable and structurally well-defined functional entities at the meso- and nano-scale dimensions. Self-assembled materials through purposeful manipulation potentially offering novel property and functionality that cannot be achieved by conventional organic synthesis. Although such ordered and reproducible structures are very common in biology, they are a tremendous challenge for the material scientist. Therefore, much effort has been focused on investigating the use of biological molecules for nanotechnology applications.

Polyelectrolyte complex (PEC) formation is mainly caused by the strong coulomb interaction between the oppositely charged polyelectrolytes. Under special conditions, polyelectrolytes with weak ionic groups and significantly different molecular weights at nonstoichiometric mixing ratios, water soluble PEC on a molecular level can be prepared, which were comprehensively studied by the groups of Kabanov.[6] These PEC consist of a long host molecule and shorter sequentially attached guest polyions of opposite charge, i.e., they contain single stranded hydrophilic and double stranded hydrophobic sections.

Chitosan (CS) is a second abundant nature polymer in the world. Its nontoxic, biodegradable, and biocompatible has attracted significant interests in various novel applications, such as pharmaceutical and biomedical engineering.[7] CS has free amino groups on its backbone, leading CS to be a cationic polyelectrolyte. Poly(acrylic acid) (PAA) is considered a pH and electrically sensitive material due to the ionic repulsion between its anionic groups. PAA contains carboxylic groups that become ionized in solutions with a pH that support a p*K*a value of 4.7. The amino group of CS can interact with the carboxylic acid groups of PAA through electrostatic attraction resulting in the formation of **PEC**.[8] The interaction between CS and PAA has been exploited to produce different biomaterials in the form of micro/nano particles, films, hydrogels, and foams.[9] Our group has reported the preparation of nanofibrous structure of CS-PAA in adipic acid aqueous solution by a modified dropping method.[10] However, little work was focused on finely tuning the microstructure of CS-PAA nanofibrous scaffold.

In this article, we focused on the investigation of microstructure and nanostructure formation of CS-PAA nanofibrous structure by the complexation between CS and PAA. The influence of various experimental parameters on the formation of nanofibers, including the volume ratio of CS to PAA, final suspension pH, concentration and molecular weight of CS, incubation time and reaction temperature has been investigated. The mechanism governing the formation of the CS-PAA nanofibers is also discussed. Furthermore, the plasmid DNA encapsulation ability and cell transfection of these CS-PAA nanofibrous scaffolds was also evaluated.

EXPERIMENTAL

Materials

Chitosan (CS, $\overline{M}_w$ = 400,000 and 600,000) was purchased from Fluka biochemical (USA). The degree of deacetylation was about 88% determined by IR method. PAA (viscosity 8000–12000cp) was obtained by Showa (Japan). Adipic acid was purchased from Sigma (USA). All other reagents were of analytical grade and used without further purification. Dulbecco's minimum Eagle's medium (DMEM) for cell culture was purchased from Gibco (USA), supplemented with 10% fetal bovine serum (FBS), 100 mg/mL penicillin, and 100 mg/mL streptomycin. The fluorescent protein reporter gene pEGFP-N_2, a 4.7 kb pcDNA encoding GFP, driven by a cytomegalovirus immediate early promoter was purchased from Clontech (USA). E. Z. N. A. Plasmid Maxiprep Kit was purchased from Omega Bio-tek (USA). The plasmid was propagated in *Escherichia coli*, and then isolated and purified. The absorption ratio at the wavelength of 260 nm was measured for evaluation of plasmid concentration and purity.

Preparation of CS-PAA nanofibers and 3-D nanofibrous scaffold

Adipic derived CS-PAA nanofibers were prepared in a similar manner to our literature procedure.[10] The effect of individual experimental parameters on the preparation of the nanofibers was assessed while all other variables were kept constant. Briefly, CS flakes were measured and dissolved in adipic acid (0.137*M*) at a concentration of 0.01, 0.1, and 0.2% wt %. Solutions of high molecular weight (HW) and low molecular weight (LW) CS were combined with solutions of PAA. The pH of the PAA and CS solutions was adjusted to pH = 3, 3.6, 3.8, and 4 using adipic acid, and stirred for 1 day at 25°C. CS solution was then added into PAA solution in a volume ratio of 5 : 1, 4 : 1, 3 : 1, 2 : 1, 1 : 1, and 1 : 2 at a dropping rate of 1 drop/s using a syringe needle connected to a peristaltic pump. To address the effect of incubation time and temperature on the size of CS-PAA nanofibers, the CS-PAA nanofibers were prepared by adding CS solution into PAA solution of different temperatures and then incubated at a different time. The final reaction mixture was solidified in liquid nitrogen (−70°C) and freeze-dried for 2 days. The frozen complex was then dipped into a 0.01*M* sodium hydroxide solution to remove the remaining adipic acid. After being washed with deionized distilled water, the precipitates were kept at −55°C for 1 day and freeze-dried again. Then the CS-PAA nanofibrous scaffold can be obtained.

SEM observation

Morphology of the CS-PAA nanofibrous structure was obtained using a scanning electron microscope (SEM, S-4100, Hitachi, Japan) operating at 15.0keV, in which the specimens were Pt-coated before examination.

FTIR–ATR analysis

Fourier Transform Infrared Spectroscopy–attenuated Total Reflectance Spectrometer (FTIR–ATR, Nicolet Nexus 470, Thermo Nicolet Corporation, USA) was used to determine the chemical interaction between CS and PAA. The spectra were collected from 4000 to 400 cm^{-1} with a 4 cm^{-1} resolution over 40 scans.

Size distribution and zeta potential analysis

Mean diameter and size distribution of the CS-PAA nanofibers were determined by measuring the diameters of nanofibers at 100 different points in a 511 × 479 SEM image. Image processing software ImageJ (NIH) was used to measure the fiber diameter from the SEM micrographs in this study. Measurements were done for three different images for each sample. Surface charges of the nanofibers were determined by Malvern Zetasizer 3000 HS (Herrenberg, Germany). Each sample of the nanofiber suspension was adjusted to a concentration of 0.01% (w/v) in deionized water or in 0.01 mol/L NaCl solution in the case of zeta potential examination. All analyses were triplicated and the results were the average of three runs. To determine the zeta potential of the scaffold, the Electrokinetic Analyzer (EKA, Anton Paar KG, Graz, Austria) based on the streaming potential method was used. PBS was supplied upstream of the scaffold, at which the substrate potential was stable due to the reduction of the concentration polarization. The streaming potential was measured at 25°C by two Ag/AgCl reference electrodes. The zeta potential can be calculated from the measured streaming potential with the Helmholtz−Smoluchowski relationship.[11]

Equilibrium swelling study

Swelling behaviors of nanofibrous scaffold have been investigated in sterilized phosphate buffered saline (PBS, pH 7.4) solution at 37°C. Nanofibrous scaffold with various CS concentration were used, in order to observe the integrity of the nanofibrous structure in aqueous solution. The samples were immersed in the solution, taken out from it at various time intervals, and then weighted with an electronic balance. The experiment was continued until samples reached equilibrium. The degree of swelling (S) for each sample at time t was calculated using the relation $((W_S\text{-}W_0)/W_0)$, Where W_s and W_0 are the weights of the swollen and dry nanofibrous scaffold, respectively.

Evaluation of cytotoxicity

To evaluate the cytotoxicity of the scaffold toward NIH3T3 fibroblasts, the prepared scaffold was deposited on cell cultures. CS-PAA scaffolds prepared by different CS concentration (0.01, 0.1, and 0.2 wt %) sterilized in 75% alcohol overnight and rinsed with PBS solution were placed in 6-welled tissue culture polystyrene plates (Costar, USA). After aspiration of phosphate buffered saline (PBS), 2 mL medium of cell suspension at a density of 1 × 105 cells/mL, DMEM was placed on each well and maintained in an incubator with 5% CO_2 at 37°C. After cell culturing for 6 days, the prepared scaffolds were washed twice with a sterile PBS solution to eliminate dead cells. Then, a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) test[12] was carried out to quantity the viability of the fibroblasts. Scaffold without cells was expressed as control. All experiments were repeated four times, and

results were expressed as mean ± standard deviation of mean.

Fabrication of CS–PAA nanofibrous scaffolds containing DNA

CS were dissolved in adipic acid solution at a concentration of 0.01 wt % and the final pH value was adjusted to pH 3, and stirred for 1 day at 25°C. The CS solution was then added into PAA solution (1%) in a volume ratio of 3 : 1 at a dropping rate of one drop/s using a syringe needle connected to a peristaltic pump. The mixed solution was poured into a stainless steel mold and then frozen in liquid N_2(97K), and freeze-dried for 48 h to get scaffolds of columned shape with a diameter of 5 mm. The bulk porous cylinders were soaked in a 0.01*M* NaOH, followed by washing with DIW and immersed in 75% ethanol solution for 12 h for sterilization, and then lyophilized again to get neutral scaffolds.

A sterilized phosphate buffered saline (PBS, pH 7.4) containing pEGFP-N_2 plasmid through a syringe filter for sterilization was dropped onto the dried CS-PAA cylinders. The DNA scaffolds (μg/mg) was controlled as 0.5, 1, 2, 4, 5, and 10, and then kept at 4°C overnight for full incorporation of CS-PAA complex with DNA. These DNA complexes were then frozen by immersion into liquid nitrogen and lyophilized for cell culture use.

Gel retarding analysis

Complex formation was evaluated by agarose gel electrophoresis. The CS-pEGFP-N_2 nanofibrous scaffold were prepared at DNA/scaffolds (μg/mg) ratios of 0.5, 1, 2, 4, 5, and 10, respectively, by varying the weight of scaffold. The nanofibrous scaffolds and the naked plasmid were loaded onto a 0.8% w/v agarose gel. The samples were run on the gel at 80V for 60min; then the gel was stained with ethidium bromide and photographed using an UV illuminator (Eagle EyeP, Stratagene, La Jolla, CA) to document the mobility of the DNA.

Cell culture and transfection

Transfection studies were conducted using the human dermal fibroblast (HDF) cell line. Cells were suspended in medium at a concentration of 1×10^6/mL and seeded by pipetting cell suspension dropwise onto a $5 \times 5 \times 3$ mm^3 piece of dry scaffold in a 24-well plate. Scaffolds were transferred out of the plate into 1.5 mL/well of medium in wells of a 24-well plate after 1 day seeded. Culture medium was changed every 3 days. HDF seeded scaffolds were incubated in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco, USA). Cells from scaffolds were trypsinized from the scaffold on day 14 and GFP expression was quantified using flow cytometry in a FACS Calibur System from Becton-Dickinson and the Lipofectamine with plasmid DNA were used as positive control. Control transfections with Lipofectamine reagent were done according to the recommended procedure described by Sullivan et al.[13] Each transfection experiment was carried out in triplicate.

RESULTS AND DISCUSSION

Effect of CS to PAA ratio and chitosan molecular weight

As reported by Schatz,[14] chain length of CS is a major parameter affecting the dimensions of the complexes. In our case, nanofibers were prepared by HW (600000) and LW (400000) Chitosan with PAA at six different CS/PAA ratios to see the effect of their molecular weight on the resulting nanofiber size. The reaction pH was kept constant at a pH of 3, which is according to our previous study.[10] The morphology of the scaffold and resulting mean diameter sizes distribution as determined by SEM images. Morphology of CS-PAA scaffolds prepared by LW Chitosan with 1 : 1, 2 : 1, and 3 : 1 CS/PAA volume ratio at a pH of 3 are shown in Figure 1. Figure 1 shows that fibers were easily processed into a three-dimensional fibrous scaffold using a dropping method known as needle-dropping process. A macroscopic view of the scaffold suggests that it possesses high porosity. Random entanglements between fibers provide the physical reinforcement that holds the scaffold together, and the pores between fibers are on the order of hundreds of nanometers to a few microns in diameter. Figure 2 shows that with increasing the CS/PAA volume ratio, the mean diameter of nanofibers decreased except that the ratio over than 3. The same trend can be observed for both molecular weight combinations, with the smallest diameter obtained when the CS/PAA ratio is close to 3 : 1. This result suggests that the ratio of CS to PAA influences the diameter size of nanofibers. When the ratio of CS/PAA increased from 2 to 3, the smallest diameter size distribution of the scaffold can be obtained. Table I shows that the surfaces of CS-PAA nanofibers have positive charges of about 15–40 mV as the CS/PAA ratio is higher than 1. The positive charged surface of CS-PAA nanofibers is same as to other CS nanofibers prepared by electrospinning because of the cationic characteristic of CS. Meanwhile, as the ratio of CS/PAA increases, the zeta potential of CS–PAA nanofibers increases monotonously and undergoes a transformation from a negative potential to a positive potential. It indicates that as the content of CS

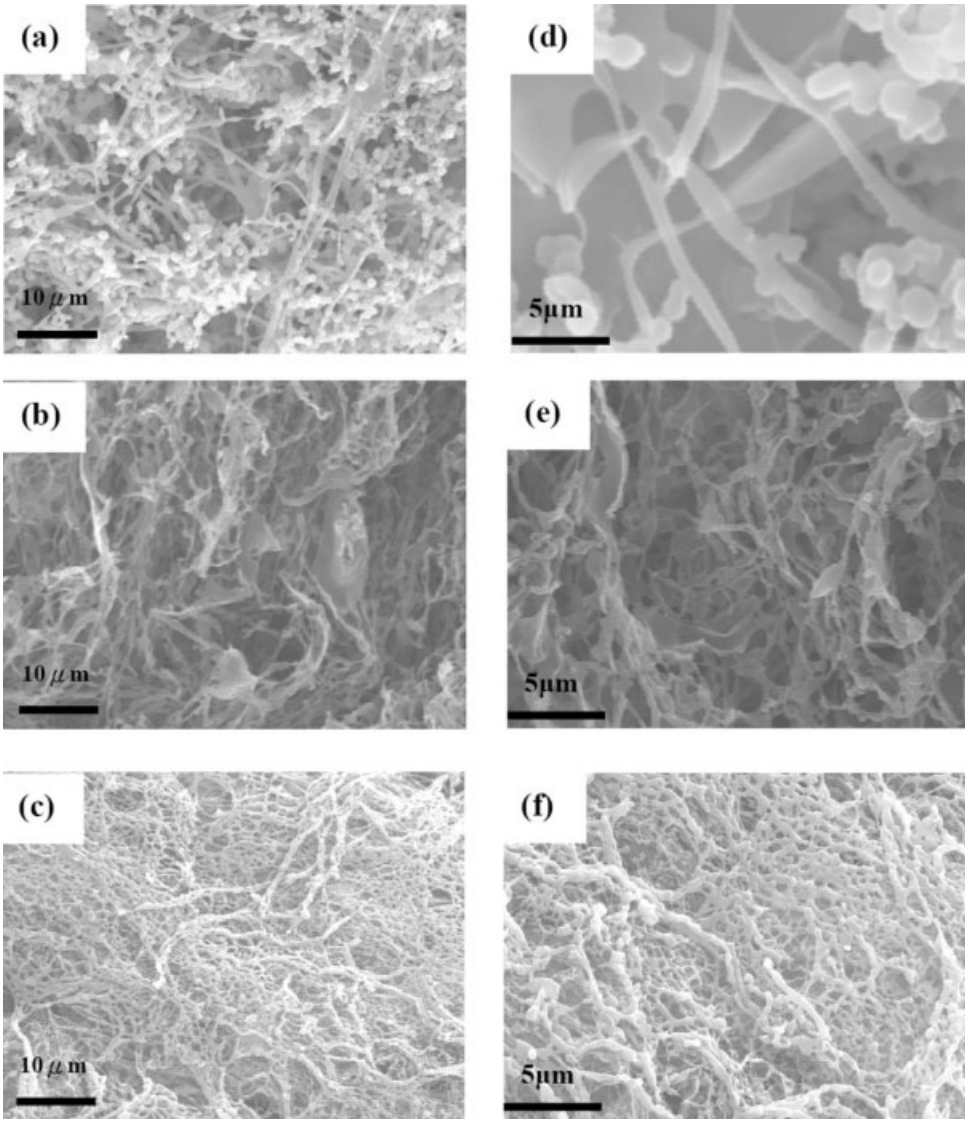

Figure 1 SEM morphology of CS–PAA scaffolds prepared by LW chitosan with different CS/PAA volume ratio (a) 1 : 1, (b) 2 : 1, and (c) 3 : 1 at a pH of 3. (d), (e), and (f): higher magnification of (a), (b), and (c).

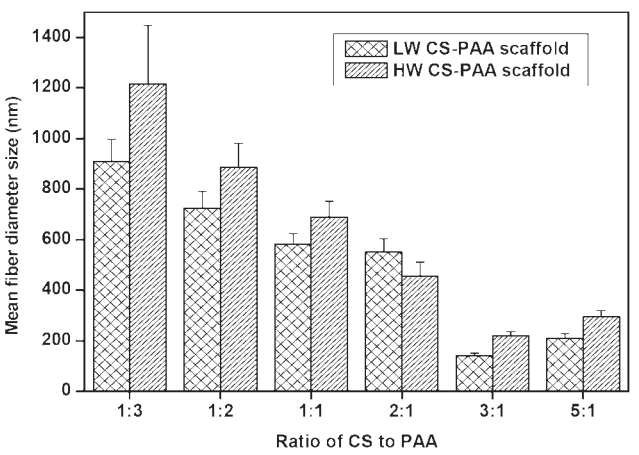

Figure 2 Effect of the ratio of CS to PAA and CS molecular weight on mean diameter size at a pH of 3.

excesses than PAA, some of the excessive CS will be absorbed onto the surface of CS-PAA nanofibers, which will increase the surface charges of CS-PAA nanofibers and resulting in the increase of zeta

TABLE I
Fiber Diameters and Zeta Potential of CS–PAA Nanofibrous Scaffold Prepared by LW Chitosan

CS : PAA (wt : wt)	Diameter range (nm)	Averaged diameter (nm)[a]	Zeta potential (mV)
1 : 3	800–1700	910	−21.5 ± 3.1
1 : 2	600–960	724	−11.5 ± 2.2
1 : 1	110–920	580	6.1 ± 1.5
2 : 1	80–960	550	16.2 ± 1.8
3 : 1	30–300	140	31.5 ± 1.4
5 : 1	150–420	210	39.1 ± 1.3

[a] Fiber diameter was characterized at pH of 3

potential. On the contrary, as the content of PAA close to CS, the surface charges of the sample decreased due to the charge neutralization.

The effect of CS molecular weight on nanofibers size was also shown in Figure 2. Generally, the LW Chitosan resulted in smaller nanofibers for most ratios of CS/PAA. These results indicate that molecular weight of CS is a major parameter, which affects the size of resulting CS-PAA nanofibers greatly. According to Chen et al.'s study,[15] When CS solution was dropped into PAA solution, the size of CS-PAA was determined by the initial dispersion extent of CS in PAA solution, that is, the higher the dispersion extent of CS in PAA solution, the smaller the size of the CS-PAA nanofibers. The dispersion extent of CS in PAA solution was mainly determined by the viscosity of CS solution or CS molecular weight. When using higher molecular weight CS specimen, the CS solution became more viscous, which restricted the dispersion of PAA in it and caused the increase in size of resulting CS-PAA nanofibers. This may stem from the ability of LW Chitosan to diffuse more readily in the PAA gel matrix to form smaller, more homogeneous fibers. On the contrary, HW–CS may bind to the surface of PAA matrices, forming an outer membrane and increasing fiber size.

Effect of pH on the mean diameter and morphology of CS–PAA nanofibrous scaffold

The effect of pH (pH = 4, 3.8, 3.6, and 3) on the morphology and mean diameter of CS–PAA nanofibrous scaffolds prepared by LW Chitosan with volume ratio of CS/PAA = 3 were investigated by SEM and Zetasizer. Table II shows the result of the mean diameters of the CS–PAA nanofibrous scaffolds under different pH values. From Table II, it can be seen that, the diameter of the nanofibers increases with the increase of pH value from 3 to 4. Figure 3 shows the SEM photographs of CS–PAA nanofibrous scaffold prepared at various pH environments. The nanofibrous scaffold, which were derived from adipic solution at a pH value of 4 [Fig. 3(a)], exhibit irregular and consistent sheet-like porous structure. However, the nanofibrous scaffold derived from adipic acid at a pH of 3 shown in Figure 3(d) exhibit a well-connected network structure with diameters in the range of 30–300 nm. Figure 4 also shows the FTIR-ATR spectra of untreated and pH-treated CS-PAA nanofibrous scaffold. The term "untreated" and "pH-treated" CS-PAA nanofibrous scaffold is refers to the CS-PAA nanofibrous scaffold prepared without and with solution pH adjustment, respectively. Untreated CS-PAA nanofibrous scaffold have new absorption band around 1690 cm^{-1}, which is assigned to C=O stretching vibration of carboxyl groups. The two characteristic peaks of CS (amide I band at 1665 $cm^{-1}$and the amide II band at 1580 cm^{-1}) decrease dramatically. The broad peak at 2500 cm^{-1} also confirms the presence of NH_3^+ in CS-PAA nanofibrous scaffold. Additionally, two peaks around 1465 and 1421 cm^{-1} were observed due to asymmetric and symmetric stretching of COO^- groups, respectively. These results confirm that the carboxylic groups of PAA are dissociated into COO^- groups, which complex with protonated amino groups from CS through electrostatic interaction during the formation of carboxylic acid-derived CS-PAA complex.[16] Figure 4(d) shows that the pH 3 treated CS-PAA nanofibrous scaffold spectra except that the peak at 1690 cm^{-1}moved to a higher wave number 1693 cm^{-1}, coupled with the peak at 1465 cm^{-1} moved to a higher wave number 1524 cm^{-1}. In contrast with spectra Figure 4(e,f) (pH 3.6 and 4 treated CS–PAA nanofibrous scaffold), a significant peak at 1524 cm^{-1} increased with the decrease of peaks at 1693 cm^{-1} in the spectra when the reaction pH increase from 3 to 4. It implied that the electrostatic interaction between CS and PAA was favorable in pH 3 environment. Moreover, COO^- groups were favor formed at pH 4 environment resulted in decreasing the ionic interaction between CS and PAA.

TABLE II
Fiber Diameters of CS–PAA Nanofibrous Scaffolds Prepared by LW Chitosan at Various pH Values

pH values	Diameter range (nm)	Averaged diameter (nm)[a]
4	_[b]	_[b]
3.8	1500–8400	2800
3.6	240–1500	340
3	30–300	140

[a] diameter was characterized at CS/PAA = 3.
[b] Porous structure.

The CS–PAA nanofibrous scaffolds were formed by ionic interaction between positively charged CS and negatively charged PAA in this study. The p*K*a values of PAA and CS are 4.43 and 6.5,[16] respectively. Under pH 3 condition, CS are completely ionized. The partly ionized PAA and CS can form compact polyelectrolytes complex by ionic interaction. The interaction between $NH3^+$ and COO^- in the CS–PAA nanofibers could be disrupted by the adipic acid of small molecules, which leads to chain branch of CS and PAA and resulted in a fibrous structure.[17] When pH values increased from 3 to 3.8, the charge density of the PAA molecules significantly increased. Thus, the electrostatic repulsive forces of PAA molecules increased, resulting in the increase of swelling degree of PAA and the increase of the mean diameter of these CS–PAA nanofibers. When

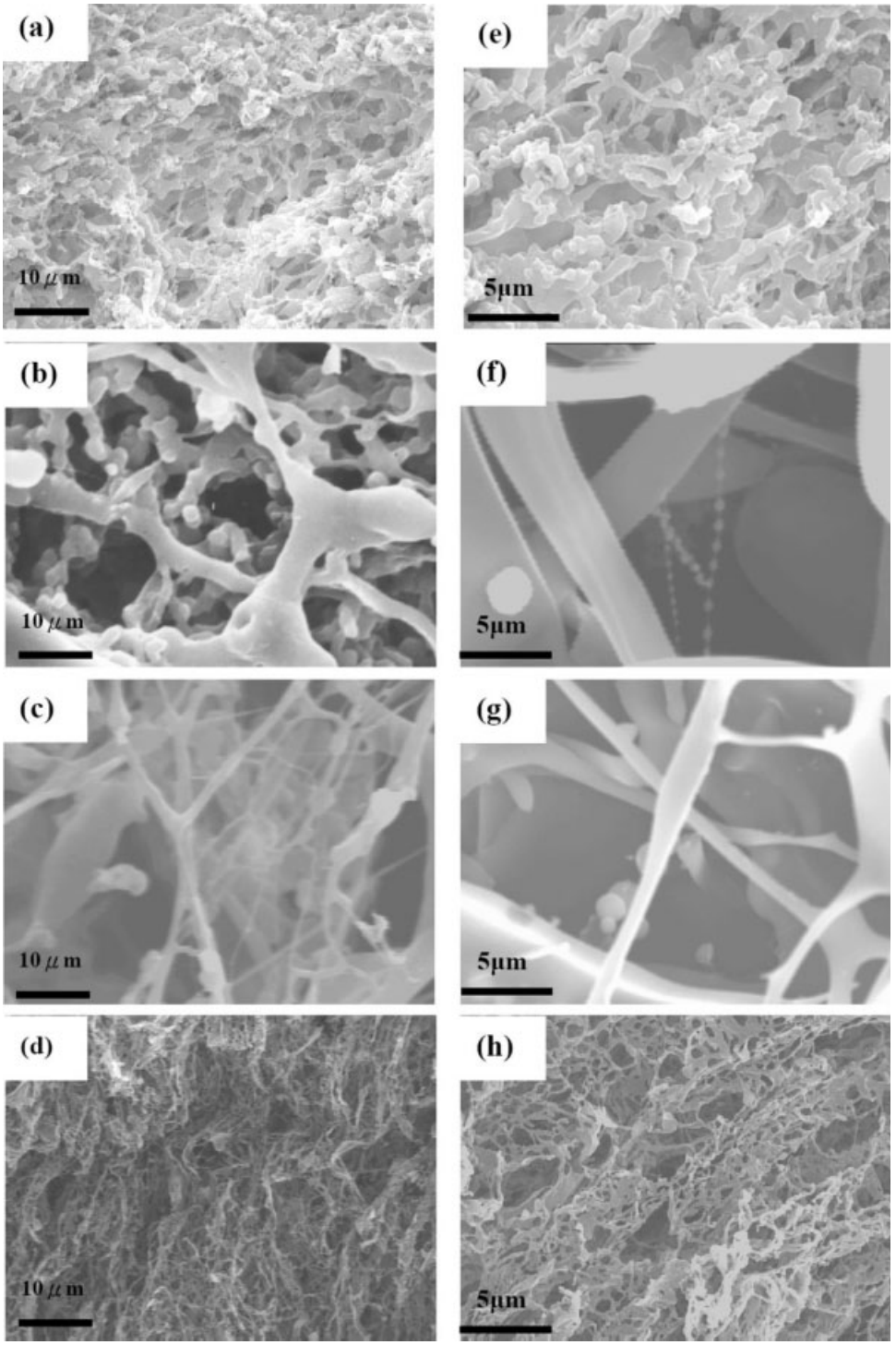

Figure 3 CS-PAA scaffold obtained from LW chitosan dissolved at (a) pH 4, (b) pH 3.8, (c) pH 3.6, and (d) pH 3. (e), (f), (g), and (h): higher magnification of (a), (b), (c), and (d). (preparation conditions: CS/PAA = 3).

these CS–PAA nanofibrous scaffolds were derived from adipic acid in pH 4 condition, the morphology of nanofibrous scaffold was changed to sheet-like structure because of lower solubility of CS. At this pH value, PAA was highly swollen while CS was low solubility, which results in the phase separation of nanofibers. Hence, the sheet-like structure was formed in these CS–PAA scaffolds as shown in Figure 4(a).

Effect of CS concentration on the mean diameter of nanofibrous structure

The similar results of effect of CS concentration on the fibers morphology and diameter were obtained

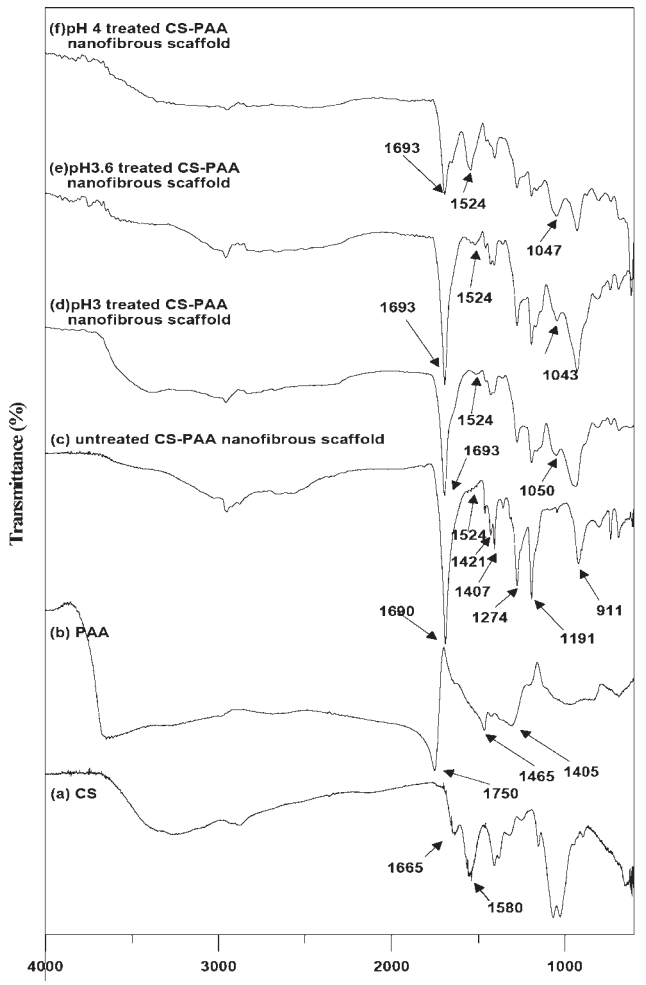

Figure 4 FTIR–ATR spectra of untreated and pH-treated CS-PAA nanofibrous scaffold (preparation conditions: LW chitosan, CS/PAA = 3).

from the various volume ratios of CS to PAA at a pH of 3. Figure 5 shows SEM images of the nanofibrous scaffolds as a function of concentration (preparation condition: CS/PAA = 3, pH 3). It was found that the morphology of the nanofibrous scaffold changed gradually from the uniform fiber-structure to the entangled fiber-structure with increasing concentration of the solution. Figure 5(d) shows the average diameter of the adipic derived CS-PAA nanofibrous scaffold as a function of CS concentration. The average diameter of the nanofibers increased with increasing the concentration. When the concentration of CS solution was more than 0.2 wt %, the self-assembly process was hard to maintain due to the high viscosity of the solution. Hence, the size of CS-PAA fiber cannot shrink to nano scales.

Effect of incubation time and incubation temperature

The effects of the incubation time and the incubation temperature on CS-PAA nanofibers size (preparation conditions: LW Chitosan, CS/PAA = 3, pH 3) were shown in Figure 6. The same trend can be observed, in which the size of CS-PAA nanofibers becomes smaller when incubation time increased even though the reduced magnitude in size is small. It implied that the size of CS-PAA nanofibers would be changed in a modulation process during the incubation time.[15] Otherwise, the temperature of CS solution has a great influence on the size of CS-PAA nanofibers. With increasing the temperature of CS solution, the small size of nanofibers can be obtained. According to Buscher's study,[18] increasing temperature of CS solution can reduce the viscosity of CS solution and increase the interaction parameter between polyelectrolytes and solvent, which results in a reduction in the fiber size. Figure 7 also show that the TEM images of CS-PAA nanofibers prepared under different incubation time and the incubation temperature. As shown in Figure 7(a), the solid CS-PAA nanofibers with clear contrast are observed when the incubation time was 0.5 hr at 25°C. However, after the nanofibers were incubated for 3 hr at 25°C, a core-shell separation is occurred, there is a space between the core and shell. Based on the positive zeta potential of these CS-PAA nanofibers, we attribute the bright ring in the outer shell to CS chains, while the dark area in the center of nanofibers to CS-PAA complex.[19] Otherwise, when increasing temperature of CS solution to 70°C for 0.5 hr, the smaller size of nanofibers can be obtained as shown in Figure 7(c). It indicates that in addition to molecular weight of CS used, the size of CS-PAA nanofibers can also be adjusted by controlling the temperature of CS solution.

Formation mechanism of the CS–PAA nanofiber networks

To further understand the formation mechanism of the CS-PAA nanofiber networks, the influence of the molecular weight of CS, the concentration of CS, the volume ratio of CS/PAA and the reaction pH value on the morphologies of the CS-PAA nanofibers have been investigated. The results are briefly summarized as follows: (1) if carboxylic acid with high crystallized structure is used in the reaction solution, fiber-like CS-PAA complexs are found; (2) when the content of CS excesses than PAA, the fibers scale have dramatically shrinking. (3) As the concentration of CS is between 0.01 and 0.2 wt %, CS-PAA nanofibers can be obtained; (4) when the reaction pH decreasing to 3, the morphology of CS-PAA also transforms into nanofibers. These changes in morphology of the synthesized CS-PAA nanofibers with the synthesis conditions suggest that the carboxylic acid type and reaction pH have great influence on the formation of the CS-PAA nanofiber networks.

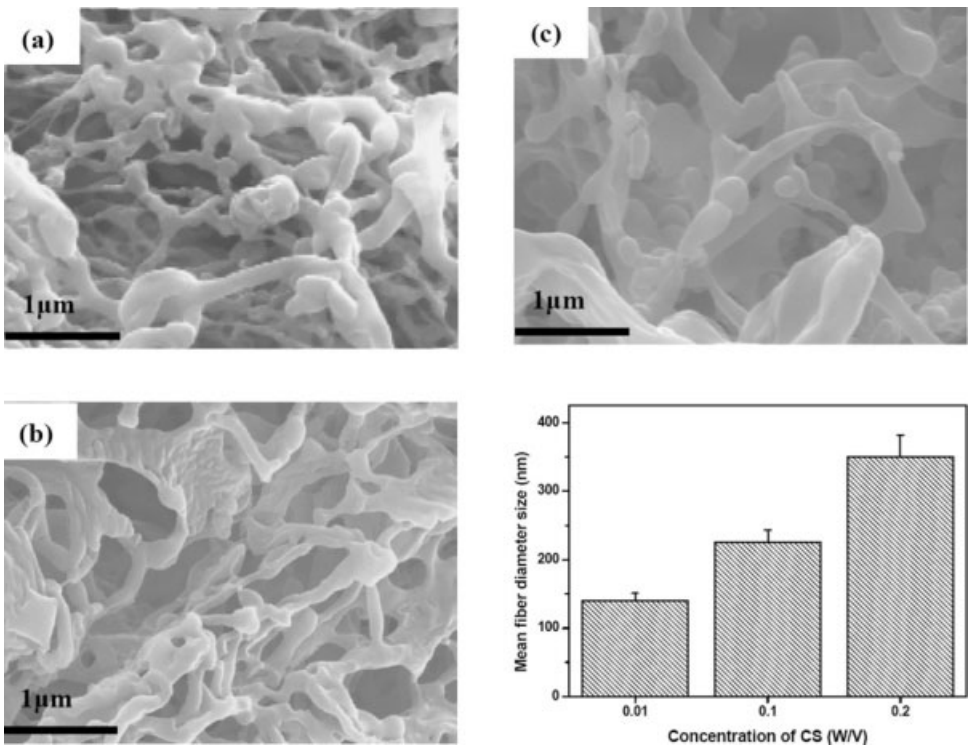

Figure 5 SEM morphology of CS-PAA scaffold at concentration of (a) 0.01 wt % CS, (b) 0.1 wt % CS, and (c) 0.2 wt % CS (d) diameter change of nanofibers as increasing the concentration of CS. (preparation conditions: LW chitosan, CS/PAA = 3, pH 3).

On the basis of the earlierresults, a model describing the mechanism of CS-PAA nanofiber formation using PAA is as follows: When CS drop into PAA gel, the PAA acts as a template and then CS with an amino group interact with the PAA with carboxylic groups. According to Keel et al.,[20] adipic acid is a two-protonated acid, which has very strong hydrogen bonds with other adipic acid molecules. It is also easily aligned and crystallized. Upon the adipic acid, the CS-PAA nanocomplexes could preferentially align to form nanofibers. In addition, PAA has many carboxyl groups and OH groups. It is reasonable to expect that hydrogen bond could be formed through the —OH group of PAA with the amine of CS. Thus, the structure of a junction between CS-PAA nanofibers could be constructed, and then a network of CS-PAA nanofibrous structure can be built up with limited parasitic branching by adipic acid.

Swelling behavior

Figure 8 shows the swelling behavior of CS-PAA nanofibrous scaffolds prepared with different CS concentrations (preparation conditions: LW Chitosan, CS/PAA = 3, pH 3). It indicates that little variation in water absorption was observed for all types of CS-PAA scaffolds with equilibrium swelling ratios in the range of 50–70% after 2 hr in PBS solution. The swelling ability of the CS-PAA nanofibrous scaffolds could be attributed to both of their hydrophilicity and the maintenance of their 3D-structure. As it is seen from Figure 8, swelling ratios of CS-PAA scaffolds of 0.01% composition were higher than that of other scaffolds prepared from 1 and 2% composition as the surface of those were more hydrophilic and pore interconnectivities were higher.[21] Although nanofiber scaffolds were swelled up to high ratio, they preserved their physical integrity upon

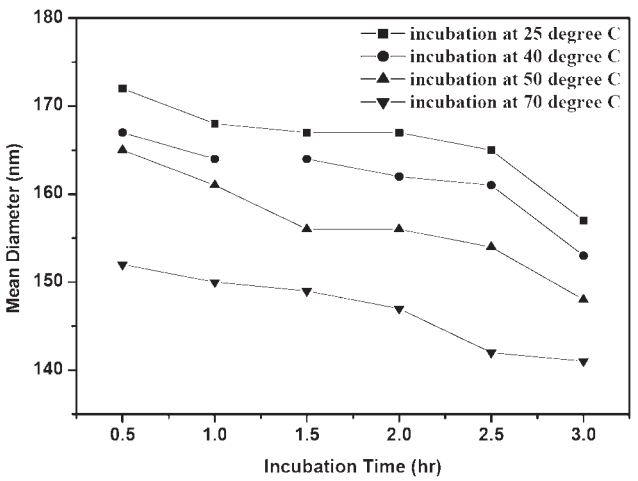

Figure 6 Effects of the incubation time and incubation temperature on CS-PAA nanofibers size. (preparation conditions: LW chitosan, CS/PAA = 3, pH 3).

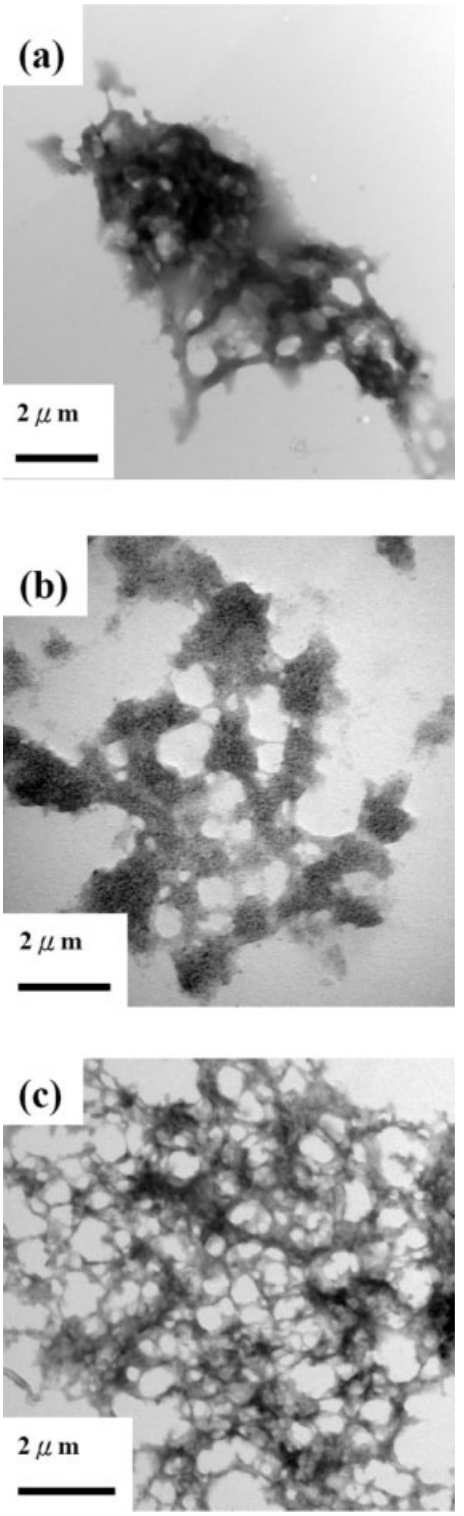

Figure 7 TEM images of CS-PAA nanofibers prepared under different incubation time and incubation temperature. (a) 0.5 hr at 25°C, (b) 3 hr at 25°C, and (c) 0.5 hr at 70°C (preparation conditions: LW chitosan, CS/PAA = 3, pH 3).

incubation in aqueous solution. This property of CS-PAA nanofibrous scaffolds may enable for easy handling of the scaffold material in practical clinical applications.

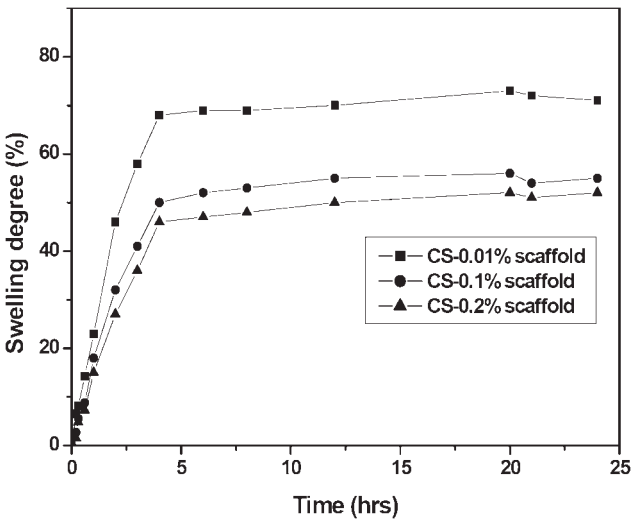

Figure 8 Swelling behavior of CS-PAA nanofibrous scaffolds prepared with different CS concentrations. (preparation conditions: LW Chitosan, CS/PAA = 3, pH 3).

Cell viability assay

The viability of fibroblasts on CS-PAA nanofibrous scaffolds with different CS concentrations (preparation conditions: LW Chitosan, CS/PAA = 3, pH 3) were evaluated through 10 days of incubation and the results were shown in Figure 9. After 1 day incubation period, fibroblasts start to proliferate on scaffolds throughout the attached cells. The results showed an increase in cell number for all scaffolds by the end of 7 days of culture period. Otherwise, with increasing the CS concentration, the cell viability also decreased. After 7 days of culture, cell numbers reaches plateau, possibly because cells may have occupied all available spaces in the prepared

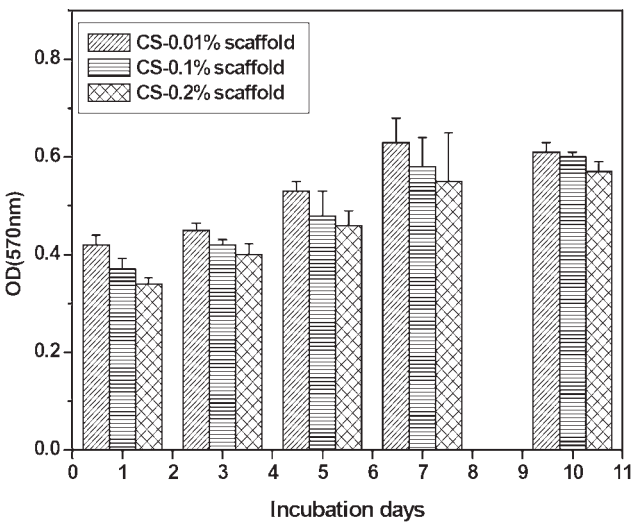

Figure 9 The viability of fibroblasts on CS-PAA nanofibrous scaffolds with different CS concentrations. (preparation conditions: LW Chitosan, CS/PAA = 3, pH 3).

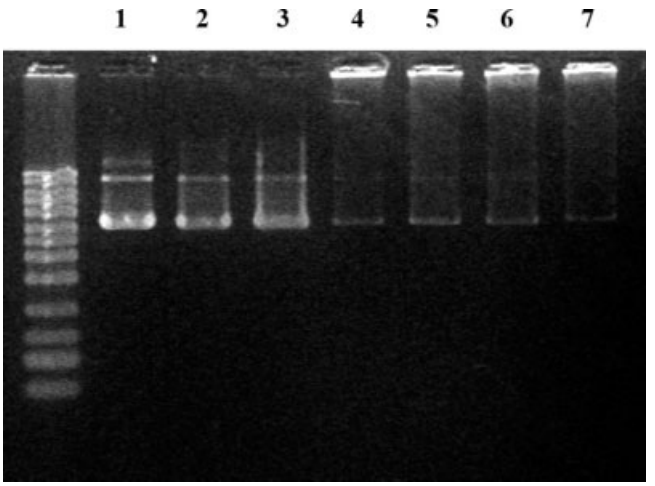

Figure 10 Gel retardation showing the coupling ability of CS-PAA nanofibrous scaffold for the coupling of plasmid DNA pEGFP-Control. Lane 1: Free DNA pEGFP-Control, 20μg; Lanes 2 and 3: DNA/scaffols in the ratio of 5 and 10 (μg/mg) respectively. Lanes 4, 5, 6, and 7: DNA/scaffolds in the ratio of 0.5, 1, 2, and 4 (μg/mg), respectively. (preparation conditions: LW chitosan, 0.01 wt % CS, CS/PAA = 3, pH 3).

scaffold. According to Li et.al.'s[22] study, when cells perform amoeboid movement to migrate through the pores, they can push the surrounding fibers aside to expand the hole as the small fibers offer little resistance to cell movement. This dynamic architecture provides cells with an opportunity to optimally adjust the pore diameter and grow into the scaffold even though some pores are relatively small. Therefore, some pores with smaller diameters in this structure may not hinder cell migration. In our case, CS-PAA scaffold with 0.01 wt % CS composition has highly porous structure and surface area to volume ratio among them. Hence, higher cell viability was observed. The obtained results suggest that CS-PAA nanofibrous scaffolds are nontoxic to fibroblasts and are good candidates for use as gene delivery vectors.

Gel retardation assay

Based on the description of formation mechanism of CS-PAA nanofibrous scaffold, it is realized that PAA molecules are located in the interior of CS-PAA nanofibers and the surface of nanofibers will be CS-rich phase. Thus, plasmid DNA can be encapsulated by CS-PAA nanofibrous structure by adding DNA into CS-PAA scaffold. To evaluate DNA binding ability of CS-PAA nanofibrous scaffold prepared at various DNA/scaffold ratios (μg/mg), the binding ability was analyzed using agarose electrophoresis. When DNA is encapsulated into these CS-PAA scaffolds, migration of DNA on an agarose gel is retarded because of the charge neutralization. Figure 10 shows the gel retardation result of CS-PAA nanofibrous scaffolds with increasing DNA/scaffold ratios (preparation conditions: LW Chitosan, 0.01 wt % CS, CS/PAA = 3, pH 3). It can be seen that no retention is observed for free DNA (Lane 1). For DNA encapsulated CS-PAA nanofibrous scaffold, DNA was totally retarded in a DNA/scaffold range without any leakage (Lane 4–7). However, when plasmid DNA was mixed with scaffolds above the ratio of 4 μg/mg, the naked DNA could be detected because of excess of DNA loading (Lane 2, 3). This result indicates that these CS-PAA nanofibrous scaffolds may serve as potential gene carriers for gene delivery.

Transfection efficiency of CS–PAA–pEGFP Nanofibrous Scaffolds in HDF cells

Several factors have great influence on the gene delivery potential of CS-based scaffold in mammalian cells, such as the cell types, pH transfection medium, serum, plasmid dosage, and molecular weight of CS.[23] In this study, we investigated the transfection efficiency of the nanofibrous scaffolds in HDF cells in different DNA/scaffold ratio (μg/mg) to find the optimal transfection condition. To quantify the extent of transfection, we trypsinized cells from nanofibrous scaffold and analyzed them using FACS.

Figure 11 shows the transfection efficiency of the naked plasmids and nanofibrous scaffolds contained DNA prepared by different DNA/scaffold ratio (preparation conditions: LW Chitosan, 0.01 wt % CS,

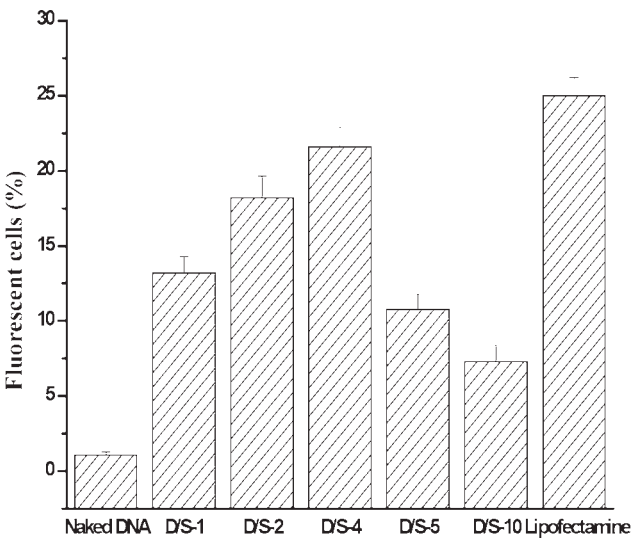

Figure 11 Transfection efficiency of the naked plasmids and nanofibrous scaffolds contained DNA prepared by different DNA/scaffold ratio. (D, DNA; S, scaffold; -number, DNA in scaffold (μg/mg)). (preparation conditions: LW chitosan, 0.01 wt % CS, CS/PAA = 3, pH 3).

CS/PAA = 3, pH 3). The naked plasmid and Lipofectamine as the positive control were used to transfect the cells at the same time. At DNA/scaffold ratio 4, the hightest transfection efficiency was achieved. However, when plasmid DNA was mixed with scaffold in the ratio of 5, the transfection efficiency drastic decreased. The increase of transfection efficiency from 1 μg/mg to 4 μg/mg may be related to their zeta potential as described earlier in gel retardation assay. However, the DNA overloading for DNA/scaffold ratio 5 resulted in low proton absorption capacity. According to Ishii et al.'s[24] study, the lack of proton adsorption capacity will lead to low transfection efficiency. Therefore, as the DNA/scaffold ratio changes, the gene delivery capacity will also changed.

CONCLUSION

In this work, a self-assembling process synthesized large quantities of CS-PAA nanofibers by dropping CS to PAA solution. This approach was without using any organic solvents and surfactants. It is an easy, inexpensive method to produce nanofibers with controllable average diameters in bulk quantities. It was found that the diameter of the CS-PAA nanofibers could be easily controlled by the CS molecular weight, the concentration of CS, the volume ratio of CS to PAA, the reaction temperature, the incubation time, and the final pH of the suspension. By using adipic acid as branch promoter, adjusting the pH value of the CS solution to 3, and then dropping CS into the PAA solution at a ratio of 3 : 1, yields nanofibers with diameters from 30 to 300 nm in the suspension. A nanofibrous structure with averaged diameter 140 nm is obtained after the suspension is freeze-dried through phase separation. Experimental results showed networks of CS-PAA nanofibrous structure could be built up with limited parasitic branching by crystallized adipic acid. Preliminary experiment shows that the nanofibrous scaffold was nontoxic and can bind plasmid DNA very well. Transgene expression in human dermal fibroblasts seeded on the nanofibrous scaffolds was significant after 14 days compared to lipofectamine controls. Such a fibrous scaffold can be used as a complementary technology to deliver gene vectors tailored for specific cell types.

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