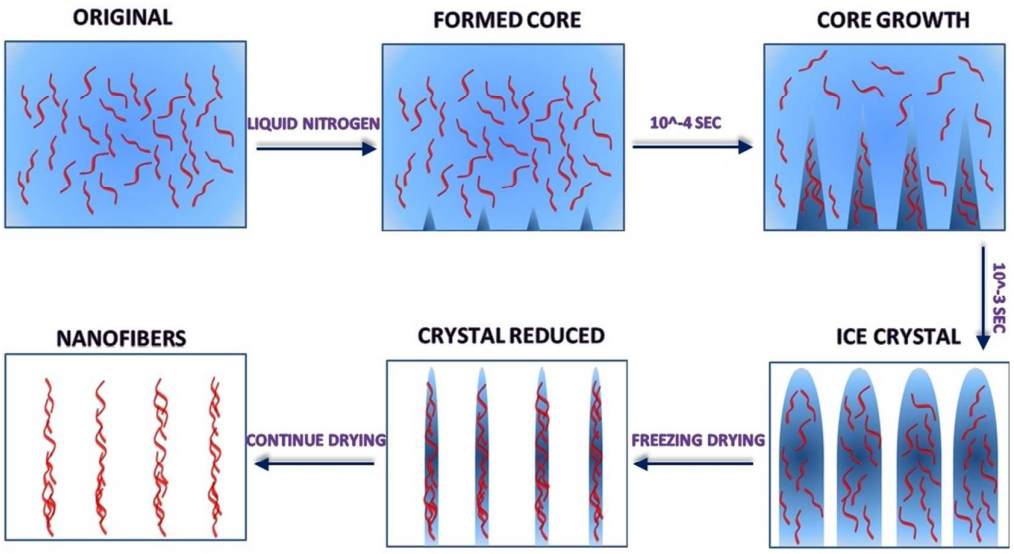
RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

The formation mechanism polyelectrolyte complex nanofibers during the process of freeze drying.



Crosslinked polyelectrolyte complex fiber membrane based on chitosan-sodium alginate by freeze-drying

Chengling Jiang, Zhiliang Wang, Xueqin Zhang, Xiaoqun Zhu, Jun Nie, Guiping Ma*

State Key Laboratory of Chemical Resource Engineering, Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology, Beijing 100029, P R China

Tel(Fax):+86-01064421310

magp@mail.buct.edu.cn

Abstract:

Polyelectrolyte complex (PEC) membrane of cationic chitosan and anionic sodium alginate with fiber structure were prepared by freeze-drying method. Chitosan and sodium alginate were blended in different concentration and frozen at different temperature. Freeze-dried fiber membrane were extensively characterized for their inter-molecular interaction, the solution property, morphology and biocompatibility by using FTIR, XRD, Zeta (ζ) potentials, SEM and Cytotoxicity assay, respectively. The study of swelling property showed that PEC membrane with the fiber structure cross-linked with glutaraldehyde exhibited pH and ionic strength-dependent swelling in aqueous media, which might have a potential application in tissue engineering or drug controlled release. Furthermore, chitosan-sodium alginate samples showed better cell adhesion and proliferation than pure chitosan. The results indicated that two natural polyelectrolyte complex nanofibers were prepared by freeze-drying method and fitted for tissue engineering or drug carrier.

Keywords: freeze-drying; fiber membrane; chitosan; sodium alginate

1. Introduction

Being both polymers and electrolytes, polyelectrolyte complex (PEC) has both the properties of macromolecules and the charge possibilities of electrolytes, which has been studied broadly for its fundamental and application interests. The PEC has many applications,

mostly related to dialysis and ultrafiltration membranes [1], carriers and microcapsules for controlled release [2-4], biosorbent [5] and biomimetic application [6]. The PEC formation based on the strong coulomb interaction between oppositely charged polyelectrolyte, which leads to spontaneous aggregation after mixing of the component solutions [7]. PEC doesn't need to use chemical cross linking agents for electrostatic interaction between oppositely charged polyelectrolyte, which can reduce the possible toxicity and other undesirable effects of the reagents and has potential to be used in tissue engineering or drug carrier. There are three types of PEC, including soluble, colloidally stable, and coacervate complexes, respectively. The type of complex formed is corresponding to charge density, salt, polyelectrolyte concentration, pH and temperature, which were characterized by using turbidimetric or light scattering techniques [8, 9].

Chitosan and sodium alginate are widely studied in tissue engineering and drug delivery applications for their nontoxicity, biodegradability [10], biocompatibility [11, 12] and easy commercial accessibility [13, 14]. Chitosan is a natural cationic polysaccharide obtained from the N-deacetylation of chitin, and alginate is an anionic polysaccharide with the property of degradation in vivo [15]. Upon mixed, they form alginate-chitosan polyelectrolyte complex through electrostatic interaction very quickly, which has the property of limiting the release of encapsulated materials more effectively than either alginate or chitosan alone [16].

Currently, most available methods for nanofiber fabrication are electrospinning [17, 18], self-assembly [19] and phase separation [20]. Electrospinning is a common and efficient method to produce nanofibers. Jamil A. Matthews et al. [21] demonstrated the preparation of collagen nanofibers by electrospinning used for tissue-engineering scaffolds and the results suggested that electrospun collagen might represent a nearly ideal tissue engineering scaffold. Self-assembly, just as its name implies, is the autonomous organization of molecules into different structures and occurred on hydrophobic surfaces from different solvents. Liu et al. [22]

reported the self-assembly of polyphenylene dendrimer into nanofibers on various substrates. Phase separation is usually used to prepare 3-D tissue engineering scaffolds. Thermal-induced phase separation and non-solvent-induced phase separation are two kinds of phase separation, which are induced by changing temperature and by adding nonsolvent to the polymer solution, respectively. Hua FJ et al. [23] reported a biodegradable poly(L-lactic acid) (PLLA) macroporous scaffold fabricated from a PLLA-dioxane-water ternary system by use of thermal induced phase separation process. However, these three methods of producing nanofibers ether are limited in producing fibers with toxic organic solvents, or require much more complicated procedures and extremely elaborate techniques, or produce with low productivity. Freeze-drying, known as lyophilisation, bases on the self-assembly of polymer in the interface of ice crystal, has been explored widely as a route to prepare porous materials in tissue engineering [24-26]. Also freeze-drying is a novel route to fabricate nanofibers under appropriate condition. Compared with other three methods, freeze-drying has many advantages. For example, water is often used as solvent to produce nanostructure, which is green and environment-friendly. Moreover, ice crystal is sustainable and facile. Here we report synthesis and characterization of polyelectrolyte complex nanofibers, which are synthesized from natural polyelectrolyte complex of chitosan and sodium alginate by the method of freeze-drying. Using a simple method to prepare a more complex nanostructure with high specific surface area, good thermostability, water-absorb quality and biocompatibility, is an originality of this article.

2. Experimental Section

2.1 Materials.

Chitosan (Mw 100000, degree of deacetylation (DDA) 87%) and sodium alginate (SA, 1.28Pa s for a 2wt% aqueous solution at 30℃) were purchased from Zhejiang Golden-Shell Biochemical Co., Ltd. (Zhejiang, China) and Sinopharm Chemical Reagent Co., Ltd. (Shanghai,

China), respectively. Glutaraldheyde solution was purchased from Sinopharm Chemical Reagent Co.,Ltd(Beijing, China). Other reagents and solvents were of analytic grade and purchased from Beijing Chemical Reagent Company. Ultrapure water with specific resistance around 18MΩ cm was used to prepare the solution. All materials were used without further purification.

2.2 Nanofibers preparation

2.2.1 Stock solution

At room temperature, stock solution of sodium alginate and chitosan at different concentrations (0.025g/100mL, 0.05g/100mL, 0.075g/100mL, 0.1g/100mL) was prepared by dissolving each material (25mg, 50mg, 75mg, 100mg) in 100mL of deionized water and 100mL of 1% (V/V) acetic acid, respectively. All the stock solutions were stirred with magnet for about 30 minutes. The pH of sodium alginate and chitosan solution was 7.4 and 2.8, respectively.

2.2.2 Preparation of polyelectrolyte complex membrane

Chitosan and sodium alginate polyelectrolyte solutions were mixed together by adding chitosan solution dropwisely to sodium alginate solution. Then the mixed solution was stirred about 20 minutes and freeze dried. The freezing of solution was done by slowly immersing a shallow glass beaker containing chitosan-sodium alginate solution into a bath with liquid nitrogen for about 25 minutes. Then the frozen samples were freeze-dried in a freeze drier (LGJ-10) for 48 h, the vacuum was 25 Pa and temperature of cold well was -56℃. Chitosan-sodium alginate PEC was fabricated by varying the freezing temperature (the different temperature was obtained by using cryostat)、initial concentration of chitosan and sodium alginate solutions and the proportion of two polyelectrolyte solutions(5:1, 3:1, 1:1). To have a good cell growth on the surface of chitosan-sodium alginate PEC, the membrane needed to be crosslinked with 25% glutaraldheyde solution, using steam method.

2.3 Cross-linking of the fibers Membranes

The process of cross-linking was carried out as follows. Briefly, in a sealed desiccator containing 20 mL of aqueous 25% glutaraldehyde solution, the membrane were placed on a holed ceramic shelf in the desiccator and were cross-linked in the glutaraldehyde vapor at room temperature for 2 days. After cross-linking, the samples were immersed in deionized water for 1 day by changing the water every 2 h. Finally, the cross-linked fiber membrane was dried at 50°C in vacuum for 12 h.

2.4 Characterization of polyelectrolyte complex membrane

Zeta (ζ) potentials were determined by zeta potentiometric analyzer (MaiKeMo instrument co., LTD), the concentrations of two polyelectrolyte solution were 0.075g/100mL. FT-IR spectra of chitosan, sodium alginate and chitosan-sodium alginate (0.075g/100mL，1:1) PEC pelletized with KBr powder were recorded on Nicolet Spectra 5700 spectrometer (Nicolet Instrument, Thermo Company, Madison, USA). The crystallinity of chitosan, sodium alginate and chitosan-sodium alginate(0.075g/100mL，1:1) PEC were identified by using a Rigaku D/Max2500VB_2^+/Pc diffractometer (Rigaku Company, Tokyo, Japan) with 40 kV and 50mA with Cu Kα radiation (λ= 0.154nm). The scanning scope of 2θ was 5-50° and the scanning rate was 5°/min. The microstructure of PEC was observed by scanning electron microscopy using a Hitachi S-4700 microscope, it fixed on stubs with sputter coated with gold before observation.

2.5 Swelling properties study

At room temperature, water uptake by the cross-linked reinforced chitosan sodium alginate (0.075g/100mL，1:1)membrane was determined by accurately controlling the weight and size (2 × 2) of films immersed in 100mL aqueous medium, which had different pH values including 1.3, 4.0, 7.7 and 9.6, respectively. The swollen PECs were processed with filter paper to remove adsorbed water on the surface of membranes. The swelling percentage was calculated from the following equation.

$$E_{sw} = [(w_t - w_o)/w_o] \times 100$$

Where w_t is the weight of the sample at the time t, and w_o is the initial weight of the sample. In order to make the experiments more accurate, experiments had been repeated for three times, and the average value was taken as the final swelling percentage value.

2.6 Cytotoxicity assay

To evaluate cytotoxicity of chitosan-sodium alginate PEC, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay was used [27]. All samples used in cytotoxicity assay were previously sterilized by high temperature in a hermetically-sealed instrument. After sterilization, chitosan-sodium alginate PEC samples were put into 200 μL DMEM (Dulbecco's Modified Eagle Medium) medium with 10% FBS (fetal bovine serum) for 48h at 37℃ and different concentrations of their extract liquid were obtained from the medium. Then the mouse fibroblast L929 cells were cultured onto a 96-well plate in the DMEM medium. After 24h incubation, fresh media (10%FBS) with different concentration of PEC was used to remove the media and incubated for another 4h. After that, the medium in each well has been removed with fresh medium and incubated for 24h.Then 20 μL MTT(5mg/mL in PBS) stock solution was added to each well and incubated for 4h. The medium was removed with 150 μL DMSO to dissolve the precipitated fomazan crystals which was produced by live cells. The absorbance was measured by using a microplate reader at a test wavelength of 490nm. The cell viability was estimated in the form of percentage of the absorbance with respect to the control experiment without using PEC.

3. Results and discussion

3.1 Zeta (ζ) potentials analysis

Fig. 1

The alternating ζ-potentials are an excellent indicator of the different ratios between chitosan and sodium alginate to form PEC. The ζ-potentials alternated from 38.18 mV to 57.69 mV and from -60.19mV to -38.81mV with the ratios variation, as shown in Fig.1 (a). The ζ-potentials observed with each step suggested that the charge in the mixed solutions changed and PEC formed with the main driving force of electrostatic adsorption [28, 29]. When neat chitosan or sodium alginate solution was mixed with another solution, the ζ-potentials changed. This was connected with the stability of the solution. Upon mixed, the solution appeared amount floccules, leading to the decrease of absolute values of ζ-potentials. As shown in Fig.1 (b) and (c), the ζ-potentials affected nanofibers formation. The redundant positive charge in the solutions was not beneficial to fiber formation while the solutions of electric neutrality or partial electro negativity were helpful for fiber formation.

3.2 FT-IR analysis

Fig.2

The FT-IR spectra of materials including chitosan (a), sodium alginate (b) and polyelectrolyte complex fiber membrane (c) are shown in Fig.2. The spectrum of chitosan showed the characteristic absorption. 3438 cm^{-1} is ascribed to the stretching vibration of the N-H and O-H group. The peaks located around 1636 cm^{-1} and 1596 cm^{-1} are related to amide I and amide II bands from N-H group, respectively. The peak at 1422 cm^{-1} is associated with the C-H symmetrical deformation and the peak at 1384 cm^{-1} is corresponding to the stretching C-N vibration, the peak at 1082 cm^{-1} is attributed to C-O-C stretching vibration [30]. For sodium alginate, the characteristic peaks appeared at 3430 (-OH stretching), 1616 (-CO- stretching), 1417 (-COOH stretching), 1092 (C-O stretching) and 1030 cm^{-1} (C-O-C stretching), which are characterized by its saccharide structure [31]. In the spectrum of polyelectrolyte complex

membrane, the amide peak at 1636cm^{-1} disappeared, which was assigned to $-NH_3^+$ deformation [32]. Two small peaks at 1595 and 1383cm^{-1} appeared, which were responding to the NH_3^+ and $-COO^-$, respectively, revealing the strong electrostatic interaction between negatively charged carboxylic acid salts ($-COO^-$) on sodium alginate and the positively charged amino groups ($-NH_3^+$) on chitosan.

3.3 XRD analysis

Fig.3

Fig.3 shows the wide-angel X-ray diffractograms of chitosan (a), sodium alginate (b) and the polyelectrolyte complex fiber membrane (c). Chitosan exhibited four typical peaks at around 2θ =12, 21, 23 and 26°. Sodium alginate showed four diffraction peaks at about 2θ =13, 21, 23 and 26° while the polyelectrolyte complex membrane appeared more amorphous morphology than sodium alginate. When the polyelectrolyte complex membrane formed between chitosan and sodium alginate, a crystalline peak of chitosan at 2θ =12° (110) disappeared. The reason was that the hydrogen bonding between amino groups and hydroxyl groups in the chitosan was broken by complexation [33].

3.4 Morphology and fiber formation

Fig. 4.

The proposed mechanism for formation of fiber membrane under the condition of freezing drying with liquid nitrogen is illustrated in Fig. 4. Freeze drying is one of methods for preparing nanoparticles, porous materials or nanofibers, which had been reported that freezing drying technique could fabricate and prepare polymeric nanoparticles or nanofibers under the designed conditions[34-36]. It could overcame the disadvantages of the electrospinning such as requirement

for toxic organic solvents or highly concentrated acetic acid as the solvent, When the technique used to prepare the nanofibers with diameters in the range of 300–700 nm from chitosan solutions, or poly(l-lactic acid) [37-39]. According to the different forming process that reported before by utilizing other materials, their structure formation mechanisms had been established in different way[40]. While the liquid nitrogen was poured into the chitosan-sodium alginate solution, the chitosan-sodium alginate solution likely leaded to precipitate into many and tiny ice nuclei during the initial freezing process immediately. During the next freezing step, ice nuclei grew up and became longer, the solute molecules were fixed and included in the frozen solvent. Next, the ice crystals were directly sublimated into water vapor, at the same time, the solute molecules attempted to stick together slowly. Finally, until the sample was completely freezing drying, the fiber could be formed by that molecules agglomeration.

Fig. 5

The complexation occurred once two polyelectrolytes mixed. The entropy was the driving force for the complexation and such complexation process appeared spontaneously with the release of counter ions. Fig.5 shows SEM images of uncross-linked and cross-linked PEC membrane prepared from concentration of 0.075g/100mL of each polyelectrolyte solution and freeze dried at -196℃. As seen, the uncross-linked PEC was interconnected and composed of interconnected fiber. The fiber structure was attributed to ice crystals. When the solution was frozen, ice crystals grew and the polyelectrolytes molecules were excluded by these crystals to form fiber structure. The phenomenon of ice crystals growing was explained by Mullins-Sekerka instability, according to which, the primary ice-template structure was dependent on the destabilizing polyelectrolytes interfacial concentration gradient and the surface energy that opposes cell formation [41-43]. While cross-linked PEC membrane had a

rather smooth surface, which was related to cross-link between glutaraldehyde and hydroxyl of chitosan or sodium alginate. The structure of the PEC was connected with relative molecular weights of the polyelectrolytes and the strength of mutual electrostatic interaction. What is more, other parameters such as pH, ionic strength, initial solution concentration and freeze temperature could affect the structure of the PEC. Here how initial concentration and freeze temperature affected the fibers formation was laid emphasis on in this article.

Fig. 6

To understand how initial concentration affected the fiber formation of PEC, we got PEC by varying initial concentration of each polyelectrolyte and keeping freeze-drying temperature constant. The SEM images of PEC membranes with concentration of 0.025, 0.05, 0.075 and 0.1g/100mL frozen at -196℃ are shown in Fig.6. The PEC fiber membrane with concentration of 0.025g/100mL was found to have sheet-like structures, and almost no structure of fiber. When the solution increased to concentration of 0.05g/100mL, the PEC membrane had few fiber and many holes connected with fibers or sheet-like structure. When the concentration of the solution increased to 0.075g/100mL, the fiber structure appeared and they were continuous and interwoven together. PEC membrane exhibited an intermediate morphology at a concentration of 0.1g/100mL. The SEM results indicated that at even lower concentration (0.05g/100mL), the PEC membrane have no obvious and special structure besides disorder sheet-like. The reason was that the concentration of polyelectrolytes was too low to maintain its three dimensional structure, and form the continuous nanoparticles. So if the concentration of the solution was low, it could be got the fiber membrane.

Another parameter, freeze temperature had a significant effect on structure of PEC formation. PEC with the same concentration of 0.075g/100mL frozen at different temperatures

-60□, -50□, -40□, and -20□ were prepared by using freeze-drying method. It could be found that the nanofibers structure increased with the decrease of temperature, as shown in Fig. 6. When the freezing process was conducted with a cold source of higher temperature -20□, materials with disorder of sheet-like or stratiform structure were produced. When the aqueous solution was frozen at -60□, the extremely low temperature resulted in rapid formation of ice nuclei and the growth of small ice crystals. The process of freeze-drying took small solvent crystal away and small pores formed in the space originally occupied by solvent, which led to fibers structure. However, ice nucleation was slow and the nuclei tended to grow into larger ice crystals at a higher freeze temperature (e.g -20□), which led to the production of membranes with sheet-like structure. Controlling the structure of PEC membrane became possible because of the ability of varying the structure of membranes from sheet-like to fiber by adjusting freeze-drying temperature and the concentrations of two natural polyelectrolytes.

3.5 Swelling properties study

Fig. 7

As seen from Fig.7, the pH values of solution and ratios between chitosan and sodium alginate had protuberant effects on the swelling percentage. The cross-linked PECs showed a higher swelling degree at pH 1.3 and slightly lower swelling degree at pH 4.0. At pH 1.3, the amine group of chitosan was protonated by hydrogen ions from acid, resulting in the increase of electrostatic repulsion and hydrophily, which facilitated the increase of swelling degree. The lower swelling degree at pH 4.0 was attributed to the mixed solution being closed to isoelectric point. At pH 7.7, swelling percentage had a relatively increase, while swelling degree decreased at pH 9.6. The reason was that at pH 7.7, the electrostatic attraction between the $–NH_3^+$ and $–COO^-$ groups in the membrane had a significant effect. Deprotonation of the amino groups at

pH 9.6 reduced the number of ionic bonds between the two functional groups, which contributed to the improvement of swelling ability. The general trend of the swelling degree decreased following the increase of the amount of sodium alginate in the PEC, which lied in the ionic strength in the surrounding solution increased to make the osmotic pressure between the floccules and the solution different. Swelling percentage depended on the pH of solutions and ratios between chitosan and sodium alginate included. Ionic strength of the solution affected the swelling degree, much stronger ionic strength was, much stronger binding force the charge between ion and floccules had. This made the molecule chains of hydrogel crimp, leading to the decrease of swelling degree. With the increase of ionic strength, the osmotic pressure between the floccules and the solution decreased, resulted in the decrease of outer water molecules getting into floccules and lowering the swelling percentage.

3.6 Biocompatibility studies

Fig. 8

The cytocompatibility of PEC membranes was characterized through in vitro studies. SEM analysis and cell fluorescence images (Fig. 8) were used to characterize cell adhesion and proliferation in the presence of the membranes. The cell fluorescence images showed that more and more cells grew along the direction of fibers with extension of time. The cross-linked PEC was stained with live/dead L929 cells and the cells proliferated well. After 48h, cells exhibited a fusiform morphology and had the tendency of keeping growing, which was indicative of the membranes' biocompatibility. With a great difference, uncross-linked membranes had only few cells grown after 48h, which presented a good growing condition yet. The reason was that uncross-linked membranes were so loose that the cells were washed away in the process of cultivation or many cells were grown inside the membranes, resulting in only few cells on the

surface. The cytotoxicity of the materials was further characterized through a MTT assay. The results showed that the complex membranes had higher cell viability than the positive control (red bar graph). A statistically significant difference between negative control and cells exposed to the membranes was observed, including 24h, 48h and 72h of incubation, respectively. But there were some differences between the results. When the content of chitosan increased, the viable cells decreased, revealing more live cells on chitosan-sodium alginate than on pure chitosan.

Conclusions

In this study, we explored some factors of chitosan-sodium alginate nanofibers formation and managed to fabricate chitosan-sodium alginate nanofiber membranes at the interface of ice crystals by varying concentration and temperature based on freeze-drying method. The nanofibers formation appeared with the concentration of 0.075g/100mL of mixed solution and the freezing temperature below -60℃, the diameter of PEC was about 200~600nm. The higher or lower concentration and the higher temperature facilitated sheet-like and stratiform structure. The study of swelling property showed that the general trend of the swelling degree decreased following the increase of the amount of sodium alginate in the PEC. The results indicated that the PEC membrane with the nanofibers structure cross-linked by glutaraldehyde exhibited pH and ionic strength-dependent swelling in aqueous media, which may have a potential use in tissue engineering or drug controlled release. Furthermore, MTT assay showed the PEC membranes were nontoxic and chitosan-sodium alginate samples showed better adhesion and proliferation than pure chitosan.

Acknowledgment

The author would like to thank the project supported by the National Natural Science Foundation of China (Grant No.21304005) and Natural Science Foundation of Jiangsu Province (Grant No.BK20131145) for its financial support. This study was also supported by Open Fund

from State Key Laboratory of Chemical Resource Engineering.

Reference

[1] Richau, K., Schwarz, H. H., Apostel, R., & Paul, D. Dehydration of organics by pervaporation with polyelectrolyte complex membranes: some considerations concerning the separation mechanism. Journal of Membrane Science, 1996, 113(1), 31-41.

[2] Lee, K. Y., Park, W. H., & Ha, W. S. Polyelectrolyte complexes of sodium alginate with chitosan or its derivatives for microcapsules. Journal of Applied Polymer Science, 1997, 63(4), 425-432.

[3] Liao, I. C., Wan, A. C., Yim, E. K., & Leong, K. W. Controlled release from fibers of polyelectrolyte complexes. Journal of Controlled Release, 2005,104(2), 347-358.

[4] Li, J.J., Zhu, D.W., Yin, J.W., Liu, Y.X., Yao, F.L., &Kangde, Y. Formation of nano-hydroxyapatite crystal in situ in chitosan–pectin polyelectrolyte complex network. Materials Science and Engineering: C, 2010, 30(6), 795–803.

[5] Ali, A., Richard, A., Venditti, J. J., Pawlak, A.S., &Martin, A.H. Novel Hemicellulose−Chitosan Biosorbent for Water Desalination and Heavy Metal Removal. Sustainable Chemistry & Engineering. 2013, 1 (9), 1102–1109.

[6] Sun, Z.W., An, Q.Fu., Zhao, Q., Shangguan, Y.G., &Zheng, Q. (2012). Study of Polyelectrolyte Complex Nanoparticles as Novel Templates for Biomimetic Mineralization. Crystal Growth & Design, 12(5), 2382-2388.

[7] Dautzenberg, H. Polyelectrolyte Complex Formation in Highly Aggregating Systems. 1. Effect of Salt: Polyelectrolyte Complex Formation in the Presence of NaCl. Macromolecules, 1997, 30(25), 7810-7815.

[8] Biesheuvel, P. M., &Cohen Stuart, M. A. Cylindrical Cell Model for the Electrostatic Free Energy of Polyelectrolyte Complexes. Langmuir, 2004, 20(11), 4764-4770.

[9] Fredheim, G. E., & Christensen, B. E. Polyelectrolyte Complexes: Interactions between

Lignosulfonate and Chitosan. Biomacromolecules, 2003, 4(2), 232-239.

[10] Yamamoto, H., & Amaike, M. Biodegradation of Cross-Linked Chitosan Gels by a Microorganism. Macromolecules, 1997, 30(13), 3936-3937.

[11] Chen, S.H., Yung Chang, Kueir-RarnLee, Juin-Yih Lai. A three-dimensional dual-layer nano/microfibrous structure of electrospun chitosan/poly(D,L-lactide) membrane for the improvement of cytocompatibility. Journal of Membrane Science, 2014, 450, 224-234.

[12] Zhang, M., Li, X. H., Gong, Y. D., Zhao, N. M., & Zhang, X. F. Properties and biocompatibility of chitosan films modified by blending with PEG. Biomaterials, 2002, 23(13), 2641-2648.

[13] Chaussard, G., & Domard, A. New Aspects of the Extraction of Chitin from Squid Pens. Biomacromolecules, 2004, 5(2), 559-564.

[14] Lamarque, G., Viton, C., & Domard, A. Comparative Study of the First Heterogeneous Deacetylation of α- and β-Chitins in a Multistep Process. Biomacromolecules, 2004, 5(3), 992-1001.

[15] Mi, F.-L., Sung, H.-W., & Shyu, S.-S. Drug release from chitosan–alginate complex beads reinforced by a naturally occurring cross-linking agent. Carbohydrate Polymers, 2002, 48(1), 61-72.

[16] Yan, X. L., Khor, E., & Lim, L. Y. Chitosan-alginate films prepared with chitosans of different molecular weights. Journal of Biomedical Materials Research, 2001, 58(4), 358-365.

[17] Meng, L., Arnoult, O., Smith, M., & Wnek, G. E. Electrospinning of in situ crosslinked collagen nanofibers. Journal of Materials Chemistry, 2012, 22(37), 19412-19417.

[18] Zhao, Z.G., Zheng, J.F., Wang M.J., Zhang, H.Y., &Charles, C. H.High performance ultrafiltration membrane based on modified chitosan coating and electrospun nanofibrous PVDF scaffolds. Journal of Membrane Science, 2012,394-395, 209-217.

[19] Chae, W.S., An, M.J., Lee, S.W., Son, M.S., Yoo, K.H., & Kim, Y.R. Templated Carbon

Nanofiber with Mesoporosity and Semiconductivity. The Journal of Physical Chemistry B, 2006, 110(13), 6447-6450.

[20] Nam, Y. S., & Park, T. G. Biodegradable polymeric microcellular foams by modified thermally induced phase separation method. Biomaterials, 1999, 20(19), 1783-1790.

[21] Matthews, J. A., Wnek, G. E., Simpson, D. G., & Bowlin, G. L. Electrospinning of Collagen Nanofibers. Biomacromolecules, 2002, 3(2), 232-238.

[22] Liu, D., Feyter, S. D., Cotlet, M., Wiesler, U.-M., Weil, T., Herrmann, A., Müllen, K., & De Schryver, F. C. Fluorescent Self-Assembled Polyphenylene Dendrimer Nanofibers. Macromolecules, 2003, 36(22), 8489-8498.

[23] Hua, F. J., Nam, J. D., & Lee, D. S. Preparation of a Macroporous Poly(L-lactide) Scaffold by Liquid-Liquid Phase Separation of a PLLA/1,4-Dioxane/Water Ternary System in the Presence of NaCl. Macromolecular Rapid Communications, 2001, 22(13), 1053-1057.

[24] Gutiérrez, M. C., Ferrer, M. L., & del Monte, F. Ice-Templated Materials: Sophisticated Structures Exhibiting Enhanced Functionalities Obtained after Unidirectional Freezing and Ice-Segregation-Induced Self-Assembly. Chemistry of Materials, 2008, 20(3), 634-648.

[25] Zhang, H., & Cooper, A. I. Aligned Porous Structures by Directional Freezing. Advanced Materials, 2007, 19(11), 1529-1533.

[26] Zhang, H., Hussain, I., Brust, M., Butler, M. F., Rannard, S. P., & Cooper, A. I. Aligned two- and three-dimensional structures by directional freezing of polymers and nanoparticles. Nat Mater, 2005, 4(10), 787-793.

[27] Yin, Y. J., Yao, K. D., Cheng, G. X., & Ma, J. B. Properties of polyelectrolyte complex films of chitosan and gelatin. Polymer International, 1999, 48(6), 429-432.

[28] Caruso, F., Yang, W., Trau, D., & Renneberg, R. Microencapsulation of Uncharged Low Molecular Weight Organic Materials by Polyelectrolyte Multilayer Self-Assembly†. Langmuir, 2000, 16(23), 8932-8936.

[29] Schüler, C., & Caruso, F. Decomposable Hollow Biopolymer-Based Capsules. Biomacromolecules, 2001, 2(3), 921-926.

[30] Davidenko, N., Carrodeguas, R.G., Peniche C, Solís Y, Cameron RE. Chitosan/apatite composite beads prepared by in situ generation of apatite or Si-apatite nanocrystals. Acta Biomaterialia, 2010, 6(2), 466–76.

[31]Sartori, C., Finch, D.S., Ralph, B., Gilding, K. Determination of the cation content of alginate thin films by FTIR spectroscopy. Polymer, 1997, 38(1), 43-51.

[32] Zang, S., Gonsalves, K.E.. Synthesis of Calcium carbonate-chitosan composites via biomimetic processing. Journal of Applied Polymer Science. 1995, 56(6), 687-695.

[33] Kim, J. H., & Lee, Y. M. Synthesis and properties of diethylaminoethyl chitosan. Polymer, 1993, 34(9), 1952-1957.

[34] Wu, J., Meredith J. C. Assembly of Chitin Nanofibers into Porous Biomimetic Structures via Freeze Drying. ACS Macro Letters, 2014, 3(2),185–190.

[35]Han, J.Q., Zhou C.J., Wu, Y.Q., Liu, F.Y., Wu, Q.L. Self-Assembling Behavior of Cellulose Nanoparticles during Freeze-Drying: Effect of Suspension Concentration, Particle Size, Crystal Structure, and Surface Charge. Biomacromolecules, 2013, 14(5):1529–1540.

[36] Grant, N., Zhang, H.F. Poorly water-soluble drug nanoparticles via an emulsion-freeze-drying approach. Journal of Colloid and Interface Science, 2011, 356(2), 573–578

[37] Ma, P.X. Zhang, R.Y. Synthetic nano-scale fibrous extracellular matrix. Journal of Biomedical Materials Research, 1999, 46(3):60–72.

[38] Liu X, Won Y and Ma PX, Porogen-induced surface modification of nano-fibrous poly(l-lactic acid) scaffolds for tissue engineering. Biomaterials, 2006, 27(21),3980–3987.

[39] Qian, L., Willneff, E., Zhang, H.F. A novel route to polymeric sub-micron fibers and their use as templates for inorganic structures. Chemical Communications, 2009, (26),3946–3948

[40] Kim, M.Y., Lee, J.H., Chitosan fibrous 3D networks prepared by freeze drying. Carbohydrate Polymers, 2011, 84(4), 1329–1336.

[41] Butler, M. F. Growth of Solutal Ice Dendrites Studied by Optical Interferometry. Crystal Growth & Design, 2001a, 2(1), 59-66.

[42] Butler, M. F. Instability Formation and Directional Dendritic Growth of Ice Studied by Optical Interferometry. Crystal Growth & Design, 2001b, 1(3), 213-223.

[43] Butler, M. F. Freeze Concentration of Solutes at the Ice/Solution Interface Studied by Optical Interferometry. Crystal Growth & Design, 2002, 2(6), 541-548.

Figure legends

Fig.1 Zeta (ζ) potentials of solutions with different ratios between two polyelectrolytes(a), SEM images of polyelectrolyte complex membranes and their Zeta (ζ) potentials were ζ = -45.08mV(b), ζ = +43.2mV(c), respectively .

Fig. 2 FT-IR spectra of chitosan(a), sodium alginate(b), and polyelectrolyte complex membranes (c).

Fig. 3 XRD pattern of chitosan (a), sodium alginate (b), and polyelectrolyte complex membranes (c).

Fig. 4. The formation mechanism of polyelectrolyte complex nanofibers during the process of freeze drying.

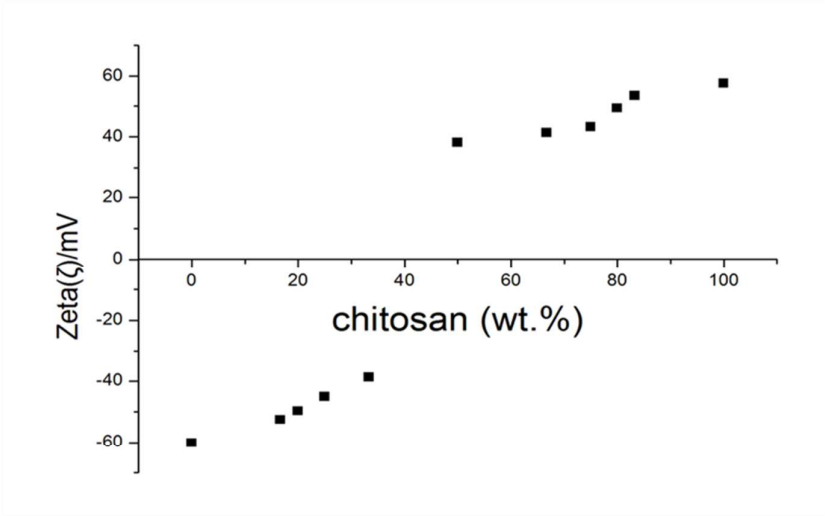
Fig. 5 SEM images of uncross-linked 0.075g/100mL chitosan-sodium alginate membrane (a_1, a_2), and cross-linked 0.075g/100mL chitosan-sodium alginate membrane (b_1, b_2)

Fig. 6 SEM images of 1:1 chitosan-sodium alginate membrane synthesized from solution with concentrations of 0.025g/100mL (a_1), 0.05g/100mL (b_1), 0.075g/100mL(c_1), 0.025g/100mL (d_1), the freezing temperature of all solutions were -196℃. SEM images of 1:1 chitosan-sodium alginate membranes synthesized from a solution with concentration of 0.075g/100mL at freezing temperature of -20℃(a_2), -40℃(b_2), -50℃(c_2), -60℃(d_2).

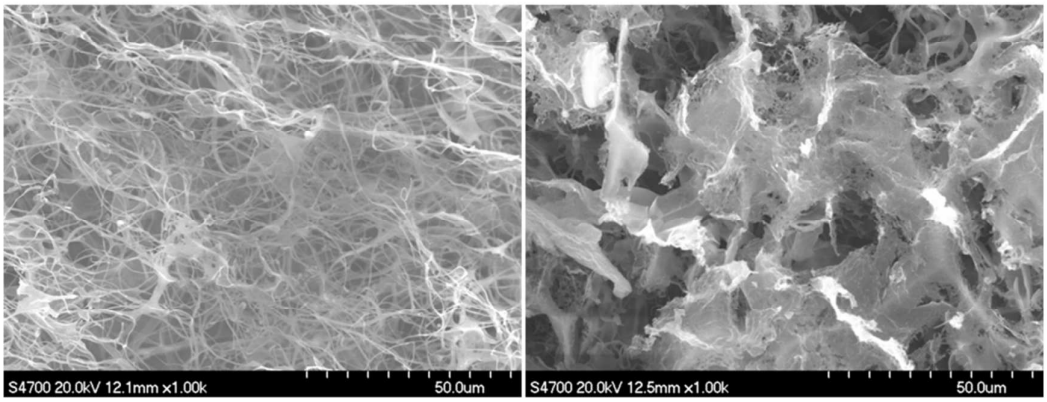
Fig. 7 Swelling degree of different ratios of chitosan-sodium alginate membranes at different pH

Fig. 8 Cell fluorescence images of membranes (a, 24h), (b, 72h), SEM images of cells adhered on the surface of uncross-linked PEC (c, 24h), (d, 72h) and cross-linked PEC (e,f). Evaluation of the cellular activity after 24h, 48h and 72h.

Fig. 1 Zeta (ζ) potentials of solutions with different ratios between two polyelectrolytes(a), SEM images of polyelectrolyte complex membranes and their Zeta (ζ) potentials were ζ = -45.08mV(b), ζ = +43.2mV(c), respectively .



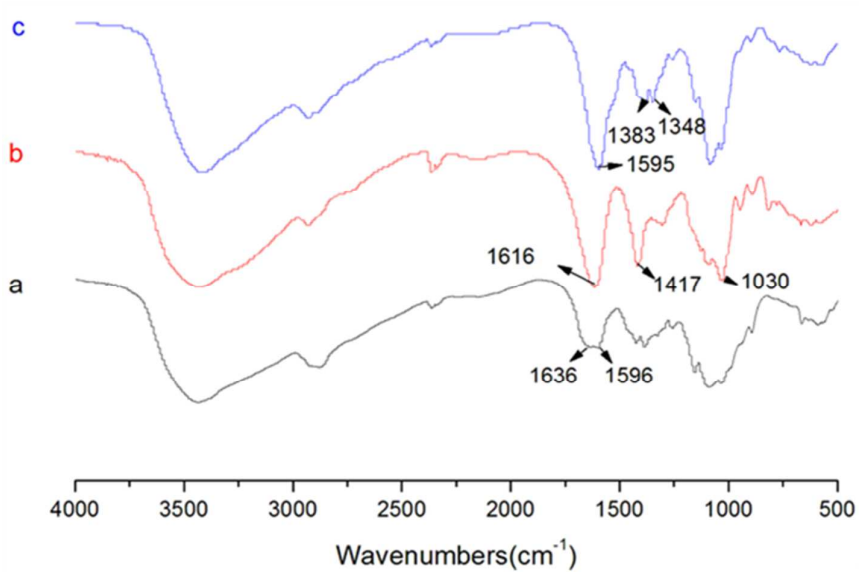
a



b c

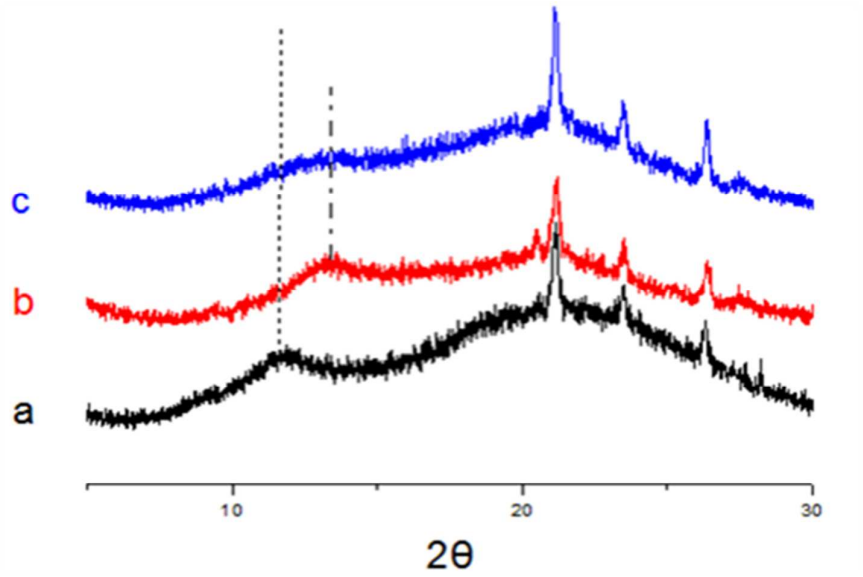
Chenling Jiang, Zhiliang Wang,Xueqin Zhang, Xiaoqun Zhu, JunNie,GuipingMa*

Fig.2 FT-IR spectra of chitosan(a), sodium alginate(b), and polyelectrolyte complex membranes (c).



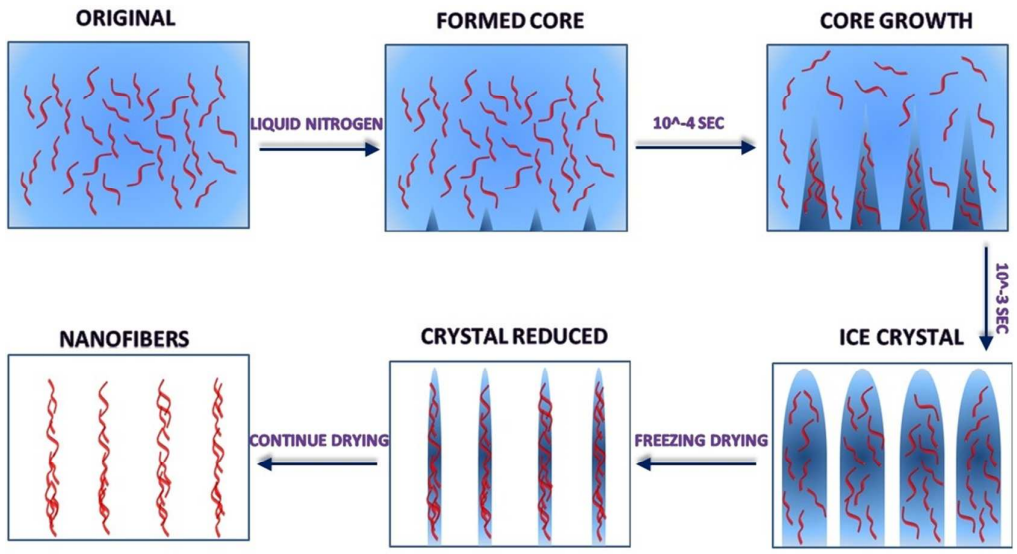
Chenling Jiang, Zhiliang Wang,Xueqin Zhang, Xiaoqun Zhu, JunNie,GuipingMa*

Fig.3 XRD pattern of chitosan (a), sodium alginate (b), and polyelectrolyte complex membranes (c).



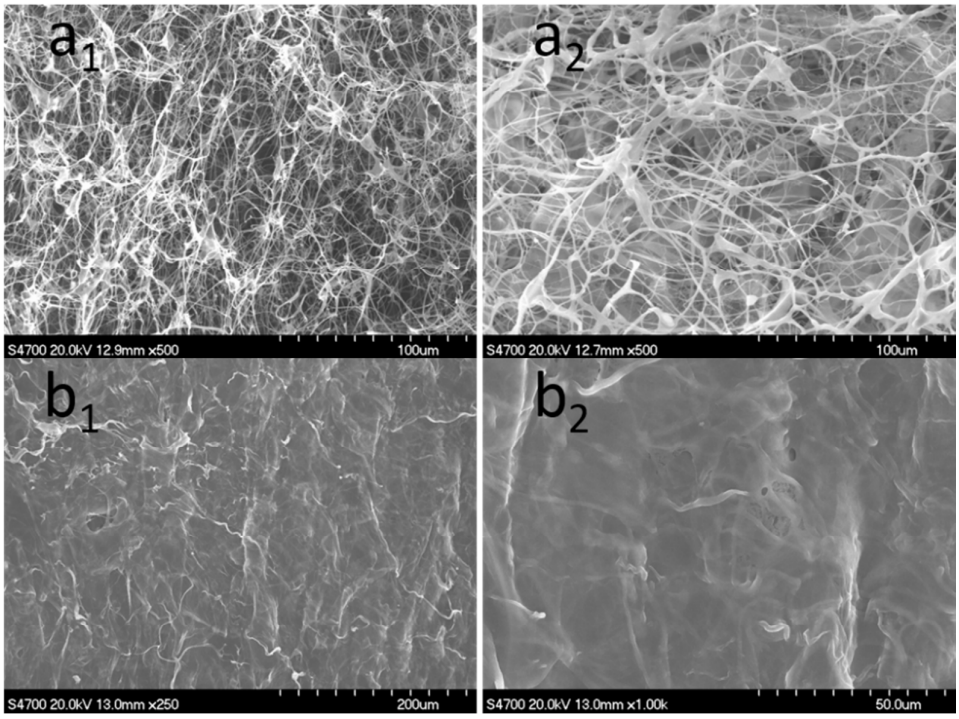
Chenling Jiang, Zhiliang Wang,Xueqin Zhang, Xiaoqun Zhu, JunNie,GuipingMa*

Fig. 4. The formation mechanismofpolyelectrolyte complex nanofibersduring the process of freeze drying.



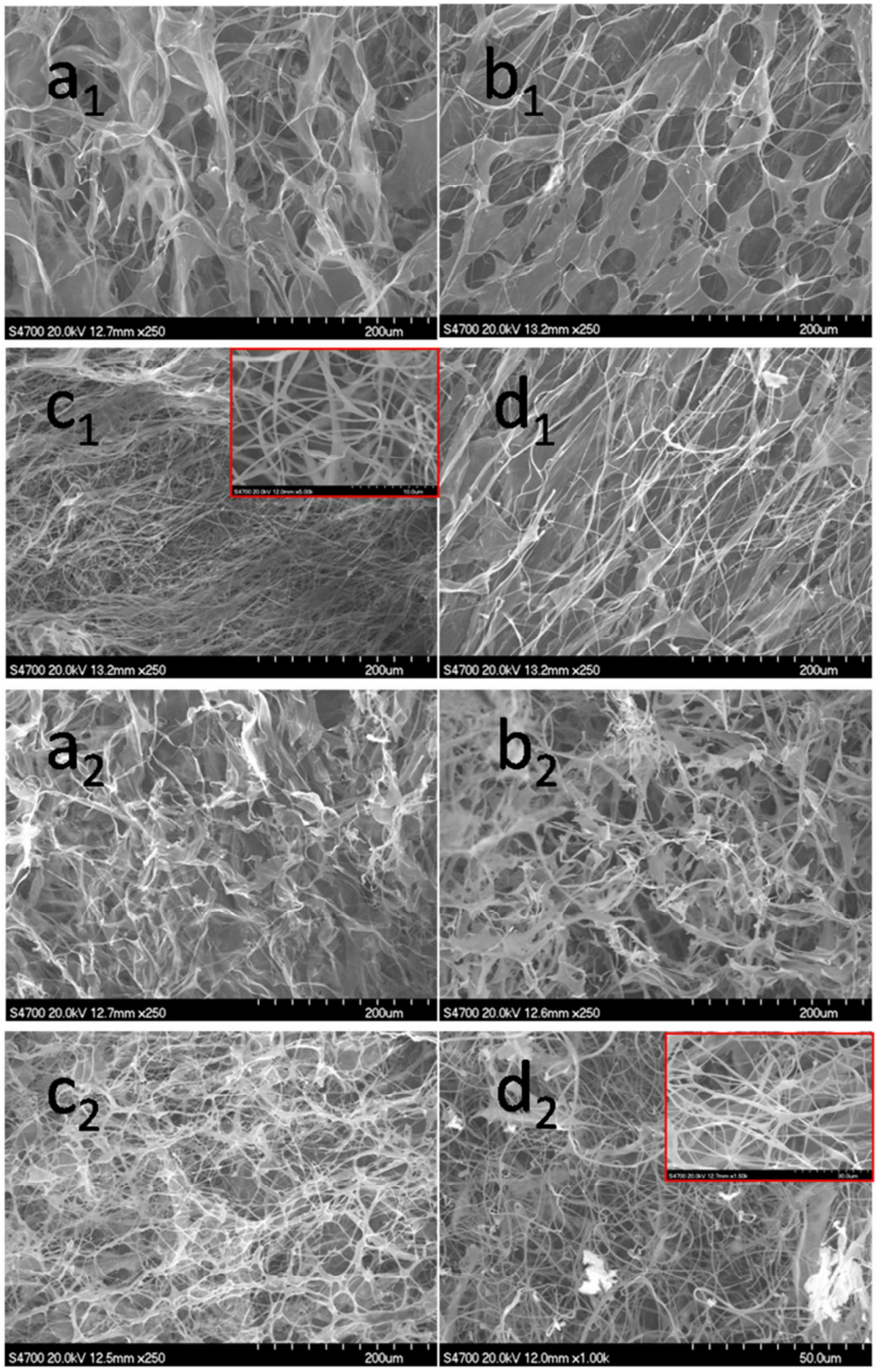
Chenling Jiang, Zhiliang Wang,Xueqin Zhang, Xiaoqun Zhu, JunNie,GuipingMa*

Fig.5. SEM images of uncross-linked 0.075g/100mL chitosan-sodium alginate membrane (a_1,a_2), and cross-linked 0.075g/100mL chitosan-sodium alginate membrane (b_1, b_2)



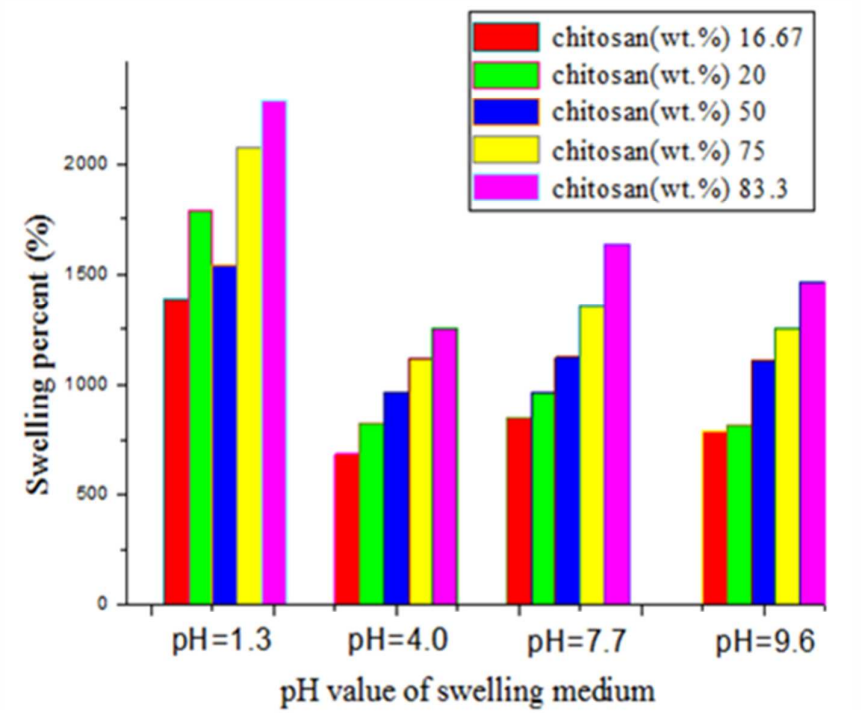
Chenling Jiang, Zhiliang Wang,Xueqin Zhang, Xiaoqun Zhu, JunNie,GuipingMa*

Fig. 6 SEM images of 1:1 chitosan-sodium alginate membranes synthesized from solutions with concentrations of 0.025g/100mL (a_1), 0.05g/100mL (b_1), 0.075g/100mL(c_1), 0.1g/100mL (d_1), the freezing temperature of all solutions were -196℃.SEM images of 1:1 chitosan-sodium alginate membranes synthesized from a solution with concentration of 0.075g/100mL at freezing temperature of -20℃(a_2), -40℃(b_2), -50℃(c_2), -60℃(d_2).



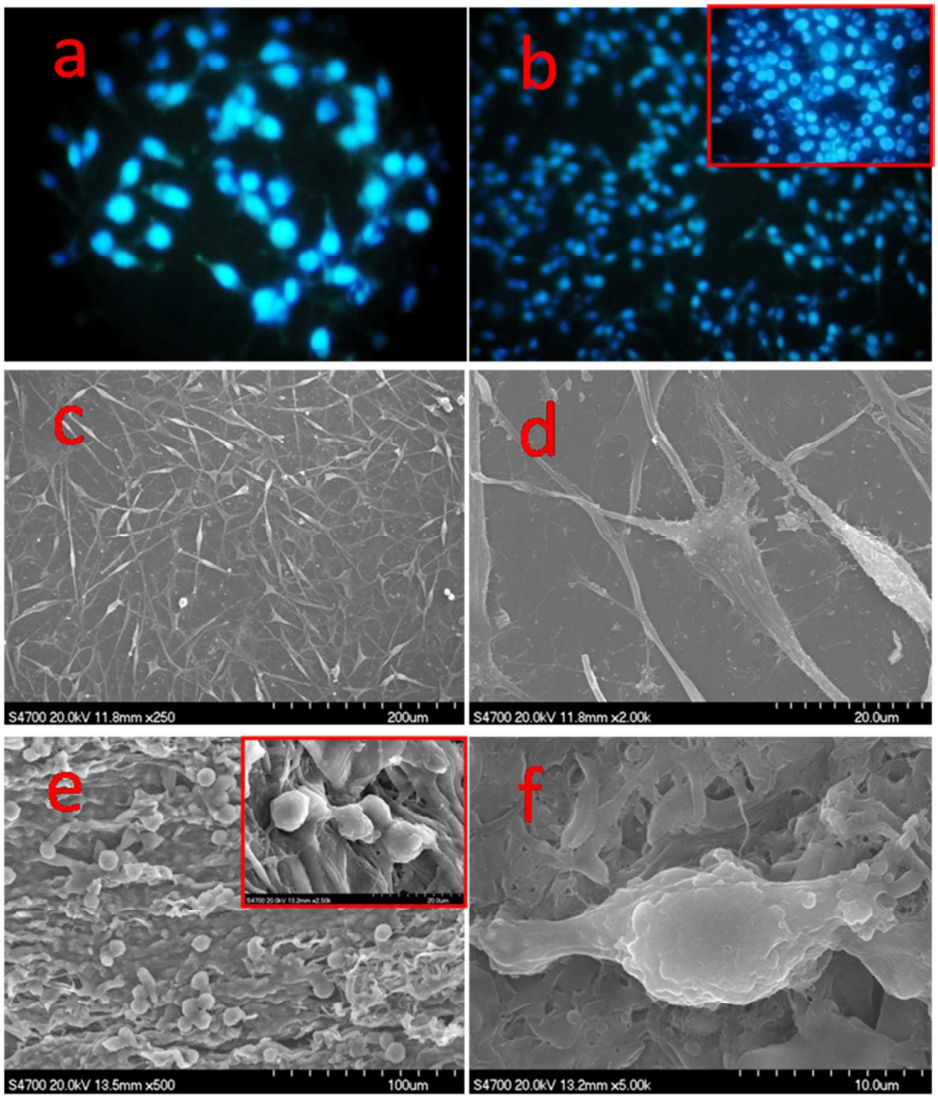
Chenling Jiang, Zhiliang Wang,Xueqin Zhang, Xiaoqun Zhu, JunNie,GuipingMa*

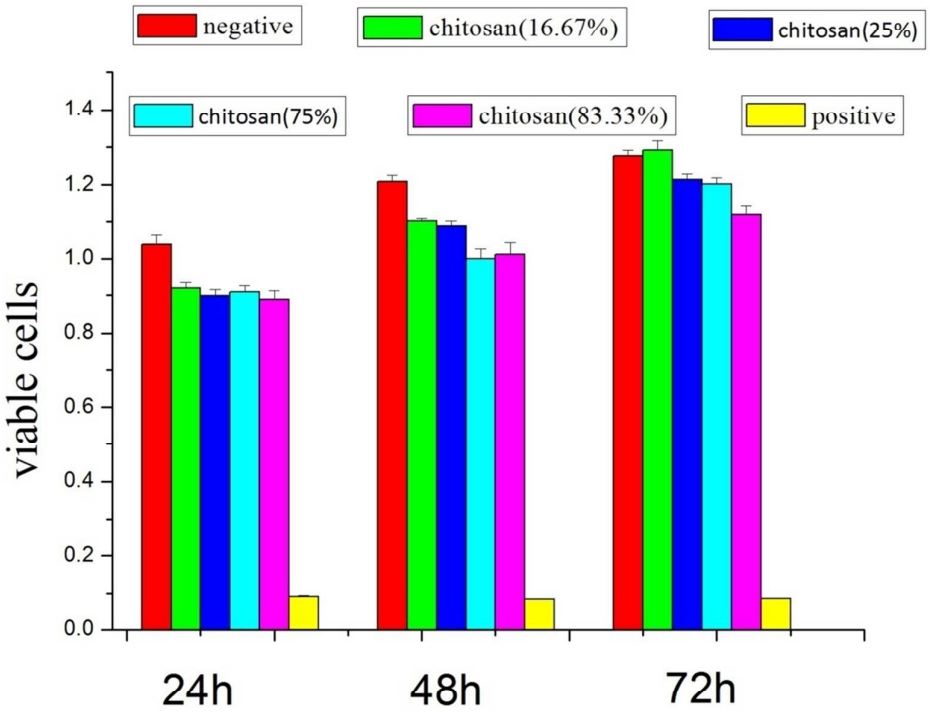
Fig. 7 Swelling degree of different ratios of chitosan-sodium alginate membranesat different pH



Chenling Jiang, Zhiliang Wang, Xueqin Zhang, Xiaoqun Zhu, Jun Nie, Guiping Ma*

Fig. 8 Cell fluorescence images of membranes (a, 24h), (b, 72h), SEM images of cells adhered on the surface of uncross-linked PEC (c, 24h), (d, 72h) and cross-linked PEC (e,f). Evaluation of the cellular activity after 24h, 48h and 72h.





Chenling Jiang, Zhiliang Wang,Xueqin Zhang, Xiaoqun Zhu, JunNie,Guiping Ma*