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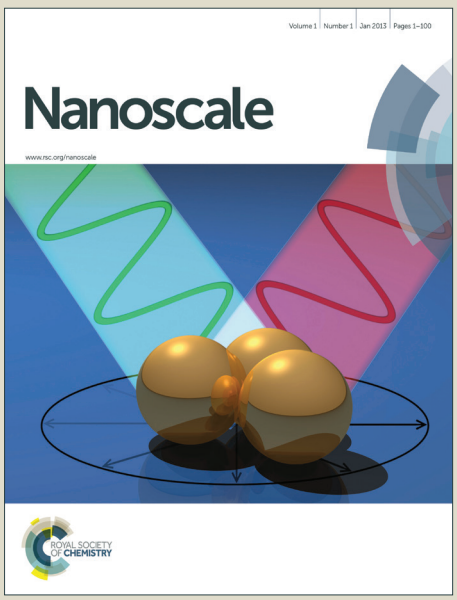

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Emerging Chitin and Chitosan Nanofibrous Materials for Biomedical Applications

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Abstract

Over the past several decades, we have witnessed significant progress in chitosan and chitin based nanostructured materials. The nanofibers from chitin and chitosan with appealing physical and biological features have attracted intense attentions due to their excellent biological properties in relation to biodegradability, biocompatibility, antibacterial activity, low immunogenicity and wound healing capacity. Various methods, such as electrospinning, self-assembly, phase separation, mechanical treatment, templating, ultrasonication and chemistry treatment were employed to prepare chitin and chitosan nanofibers. These nanofibrous materials have tremendous potential to be used as drug delivery systems, tissue engineering scaffolds, wound dressing materials, antimicrobial agents, and biosensors. This review article discusses the most recent progress in the preparation and applications of chitin and chitosan based nanofibrous materials in the biomedical fields.

Keywords: chitin, chitosan, nanofibers, biomedicine

1. INTRODUCTION

Nanofibrous materials are generally defined as fibers with diameters less than 100 nanometers and an aspect ratio of more than 100.[1, 2] Biopolymeric nanofibers have attracted intense attentions due to their larger surface area, flexible surface functionalities, extremely small pore dimensions and the superior biocompatible performance.[3-5] Until now, biopolymeric nanofibers fabricated from collagen, silk, cellulose, hyaluronic acid, alginate, chitosan, and chitin have been widely used in biomedical field.[6-8]

Chitin is a natural polysaccharide found in the exoskeleton of crustaceans such as crabs, shrimp, insects, and other arthropods.[9] It has a highly-organized crystalline microfibrils structure and is made up of an aggregation of nanofibers with a diameter of 2-5 nm and a length of about 300 nm.[10] Chitin nanofibers can be obtained through simple mechanical treatment or ultrasonication in acid condition.[11, 12] Chitin can be dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) and nanofibers can be produced in this solvent through electrospinning and self-assembly.[13-15] However, the processes have some drawbacks such as the solvent used is toxic, the conditions used are harsh, and degradation and deacetylation of the chitin chains occurred.[16] As the developments of the chitin chemistry, some newly developed solvents such as ion liquid and NaOH/urea have been employed to fabricate chitin nanofibers.[17-21]

Chitosan is the N-deacetylated derivative of chitin and can be dissolved in acidic solutions when the pH is below its pKa (~6.3) (the structures of chitin and chitosan are shown in Fig. 1).[22] Compared to the process for producing chitin nanofibers, the

conditions to fabricate chitosan nanofibers were milder since chitosan can be dissolved in aqueous solution.[23] Pure chitosan nanofibers can be produced in concentrated acetic acid. Moreover, chitosan nanofibers blended with other biodegradable and biocompatible polymers can be easily prepared in order to reach a balance between mechanical strength and biocompatibility.

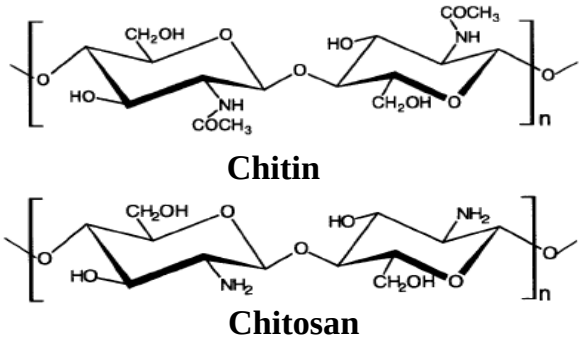

Figure 1. The structures of chitin and chitosan.

As one of the most frequently used biomaterials, chitin and chitosan based nanofibers can be potentially applied in areas such as antibacterial filtrations, drug release, tissue engineering, wound dressing, cosmetics, biosensors and medical implants.[24, 25] Microcontact printing,[13] direct-drawing,[26] self-assembly,[27] phase separation,[28] and electrospinning[16] have been well investigated to produce chitin and chitosan based nanofibers. Recently, some chitin and chitosan based nanofibers with special structures were fabricated by non-electrospinning methods to pursue certain applications. In this review, we reported the advances in the fabrication of chitin and chitosan originated nanofibrous materials. Furthermore, the emerging biomedical applications of chitin and chitosan based nanofibers were discussed in detail.

2. DIFFERENT CHITIN/CHITOSAN NANOFIBERS

Nanofibrous materials based on chitin and chitosan have been studied for decades due to their excellent biological properties.[29, 30] Different kinds of chitin and chitosan based nanofibers have been produced and used as functional biomaterials.

2.1. Pure Chitin/Chitosan Nanofibers

Chitin can be dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) and fabricated into nanofibers through methods such as self-assembly, microcontact printing and electrospinning.[13, 27, 15, 31] The ionic liquids have been regarded as a "green" solvent that can be used to dissolve cellulose, chitin, starch and lignin.[32-34] Recently, pure chitin nanofibers with high molecular weight were successfully electrospun through a one-pot process in 1-ethyl-3-methylimidazolium acetate.[19] Physical methods such as mechanical treatment and ultrasonication can also be applied to produce chitin nanofibers.[10, 35, 36, 11]

Chitosan nanofibers can be obtained in acetic acid or trifluoroaceticacid (TFA) solution.[37-39] Ohkawa *et al.* electrospun pure chitosan nanofiber using trifluoroaceticacid (TFA) as a solvent (Fig 2).[37, 40] Most recently, Haider *et al.* successfully prepared random and highly aligned bead-free chitosan nanofibers (NFs) via electrospinning.[41] Pure chitosan nanofibers can also be obtained in dilute acetic acid solution via freeze-drying method.[42]

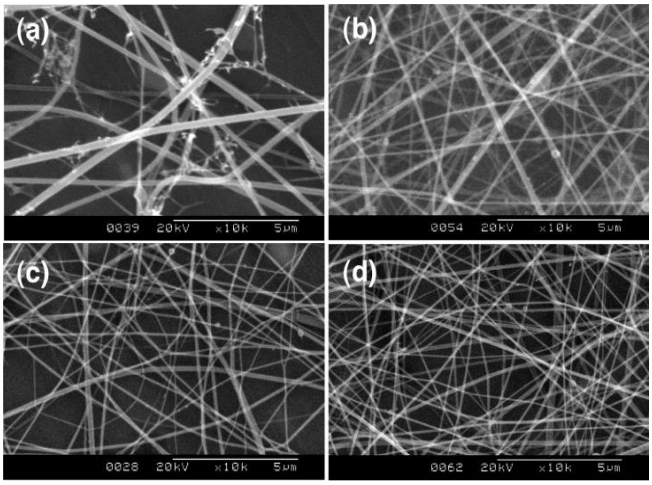

Figure 2. SEM of the electrospun nanofibers produced using different kinds of chitosan at various concentrations: (a) chitosan10 at a concentration of 8.0 wt%, (b) chiotsan100 at a concentration of 4.25 wt%, (c) chitosan500 at a concentration of 3.25 wt%, (d) chitosan1000 at a concentration of 2.0 wt%. Reproduced with permission from ref. [40]. Copyright 2006 American Chemical Society.

2.2. Chitin/Chitosan Originated Blend Nanofibers

In the last decades, many attempts have been paid to electrospin chitin/chitosan based blended nanofibers. Synthetic polymer, such as Poly(ethylene oxide) (PEO), Poly(vinyl alcohol) (PVA), Poly(L-lactide) (PLA), poly (glycolic acid) (PGA), Polyvinylpyrrolidone (PVP), natural polymers (silk, cellulose, collagen, alginate, zein and agarose) and mineral (hydroxyapatite) have been blended with chitin and chitosan to form nanofibers. Chitin/chitosan based blended nanofibers may enhance both physical and biological functionality as it can take advantage of favorable properties (strength/durability, enhancement of cell attachment) of both components.[43]

2.2.1. Chitin Based Binary Blend Nanofibers

Chitin based binary blend nanofibers are normally fabricated using HFIP as solvent. Park *et al.* prepared chitin/poly (glycolic acid) blend nanofibers by using HFIP as a solvent via electrospinning.[44] The resulted blend nanofibers have an

average diameter of 140 nm. Using the same solvent, chitin/silk blend nanofibers were fabricated as well.[45] Upon increasing the content of chitin in the blend compositions, the diameters of the nanofibers decreased from 920 to 340 nm.

2.2.2. Chitosan Based Binary Blend Nanofibers

Synthetic polymers such as PVA, PLGA, PEO and PGA have been widely used to blend with chitosan to produce nanofibers.[44, 46-48] Highly aligned nanofibers with controllable size may have important application as functional biomaterial and tissue engineering scaffolds.[49] Recently, a favorable technology named continuous near-field electrospinning was developed to prepare nanofibers.[50] Fig. 3 (A) shows the schematic of the nanofiber formation using near-field electrospinning.[51] Well-aligned parallel and arc patterns of chitosan/poly(ethylene oxide) (PEO) nanofibers can be fabricated using this technology.[26, 52] Fig. 3 (B) shows the parallel chitosan/PEO nanofiber with an average diameter of 722.26 nm and controlled 100 μm spacing. Arched chitosan/PEO nanofibers with an average diameter of 720.31 nm can be prepared and showed in Fig. 3 (C).

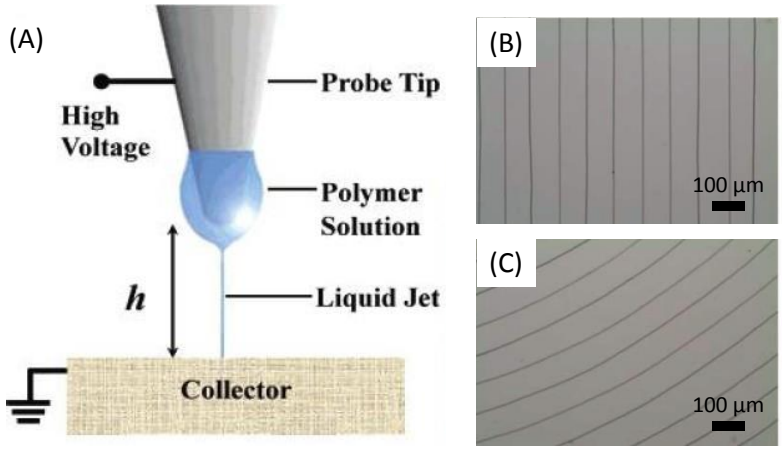

Figure 3. (A) Schematic of near-field electrospinning. Reproduced with permission from ref. [51]. Copyright 2006 American Chemical Society. (B) Parallel chitosan/PEO nanofibers with an average diameter of 722.26 nm and controlled 100 μm spacing. (C)

Arched nanofibers with an average diameter of 720.31 nm and spacing of 100 μm. The scale bars are 100 μm. Reproduced with permission from ref. [52]. Copyright 2013 Springer.

Biopolymers such as silk, cellulose, collagen, hyaluronic acid, alginate, and zein have been electrospun with chitosan.[53-56] More recently, native cellulose/chitosan composite nanofibers were successfully produced.[57] Hydroxyapatite is the major inorganic component in natural bone, which can enhance the proliferation and mineralization of cultured osteoblasts.[58] Hydroxyapatite has been blended with chitosan to produce nanofiberous scaffolds for tissue engineering.[59, 60]

2.2.3. Chitin/Chitosan Based Ternary Blend Nanofibers

Recent developments of chitin/chitosan based nanofibers scaffolds aimed to produce ternary composite nanofibers blended with synthetic polymers, biopolymers and inorganic substances.[61-66] Nanofibers scaffolds composed of chitosan, collagen and poly(L-lactic acid-*co*-ε-caprolactone) with different blend ratio were electrospun to form vascular graft.[67] The composite nanofiberous scaffolds showed better mechanical properties and biocompatibility. Other chitosan/synthetic polymer/nature polymer ternary blend nanofibers such as carboxymethyl chitosan/poly(vinyl alcohol)/silk fibroin,[68] chitosan/lysozyme/poly(vinyl alcohol),[69] poly (lactic acid)/chitosan/collagen[70] collagen/chitosan/hermoplastic polyurethane[71] were also fabricated. Some inorganic substances such as silica, Au nanoparticles, Ag nanoparticles, multiwalled carbon nanotube, and hydroxyapatite were incorporated into chitosan/synthetic polymers (PVA and PEO) nanofibers to enhance the mechanical properties and confer the composite nanofibers properties, such as

antibacterial activity, protein adsorption ability, attachment and proliferation of cells.[28, 72-76]

2.3. Modified Chitin/Chitosan Nanofibers

Chemical modification of chitin/chitosan based nanofibers can further expand the application of the nanofibers in biomedicine.[77, 78] Original chitin nanofibers can be heterogeneously acetylated from the surface to the core.[79, 80] Fig.4 showed the SEM images of the chitin nanofibers with degree of substitutions of 0.99, 1.81 and 2.96. The acetylated chitin nanofiber based nanocomposites showed high transparency, as shown in Fig. 4 (d). Other strategies such as graft polymerization and self-assembly were also employed to modify the chitin/chitosan based nanofiber surface.[81-83]

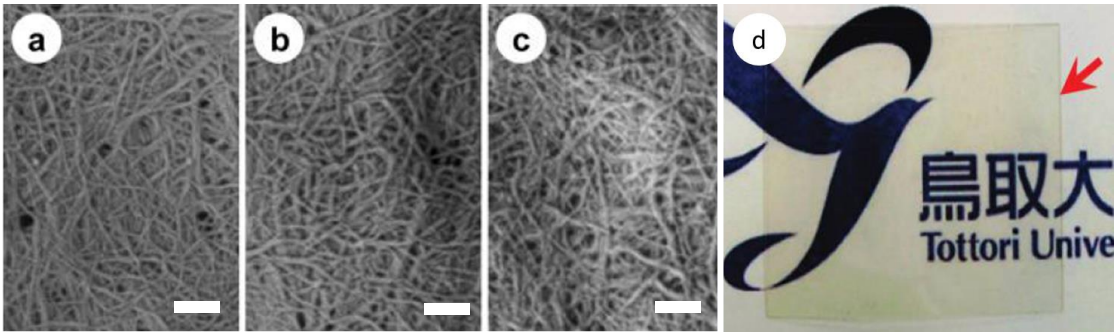

Figure 4. SEM morphologies of acetylated chitin nanofibers with degree of substitution of (a) 0.99, (b) 1.81, (c) 2.96 and (d) optical image of acetylated chitin nanofibers based membrane. Reproduced with permission from ref. [79]. Copyright 2010 American Chemical Society.

2.4. Chitin/Chitosan Derivative Based Nanofibers

Chitin derivative such as carboxymethyl chitin and dibutyrylchitin have been blended with other substances to form nanofibers.[84-87] Compared to chitin derivative, chitosan derivative is easier to be obtained for the highly active amino groups in the

chains and the good solubility in aqueous solution. Carboxymethyl chitosan was one of the most frequently used chitosan derivatives to obtain nanofibers using electrospinning method. In order to enhance the spinning capacity, some synthetic polymer such as PVA and PEO was blended with carboxymethyl chitosan to form nanofibers.[68, 88, 89] Quaternized chitosan was another chitosan derivative which was chosen to form nanofibers for its excellent antibacterial activity and biocompatibility.[90, 91, 92, 93] Table 1 summarized the functional chitin/chitosan derivatives based nanofibers by the method of eletrospinning and their biomedical application.

Table 1. Chitin/chitosan derivatives based nanofibers prepared by electrospinning for various biomedical applications

Polymer	**Additives**	**Application**	**Reference**
Carboxymethyl chitosan	PEO	Antimicrobial	88
	PVA	Wound dressing	94
	PVA/silk fibroin	Wound dressing	68
	Silver nanoparticles	No	95
	PVA/Ag nanoparticles	Antibacterial	89
	Hydroxyapatite	Cell culture	60
Quaternized chitosan	coPLA/DOX	Antitumor	96
	PVA	Antibacterial	92, 97
	PLA	Antitumor	93
	PLA	Wound dressing	98

	PVP	Antibacterial	99
	Graphene	Virus removal	100
	Organic rectorite	Antibacterial	61
Chitosan-LA	—	No	101
	—	Cell culture	102
PEG-grafted chitosan	PEO	No	103
	PLGA	Drug release	104
Poly-ε-caprolactone -grafted chitosan	Poly-ε-caprolactone	Skin tissue engineering	105
Poly(chitosan-g-DL-lactic acid)	—	Cell culture	106
Iminochitosan	—	Wound healing	107
Galactosylated chitosan	—	Hepatocytes culture	108
	—	Bioreactor	109
Chitosan oligosaccharide	PVA	No	110
	PVA/clay	No	111
Hexanoyl chitosan	—	Cell culture	112
Cyanoethyl chitosan	—	Wound dressing	113
N-methylene phosphonic chitosan	—	Bone grafting	114
Hydroxypropyl chitosan	Organic rectorite	Antibacterial	115
Acylated chitosan	PVP	No	116

Carboxymethyl chitin	organic rectorite/PVA	No	[84]
Carboxymethyl chitin	PVA	Tissue engineering	[85]
Dibutyrylchitin	PLA	Wound healing	[86]
Dibutyrylchitin	—	No	[87]

—: means that no other substance was added
No: means that no potential application was reported in the article
Abbreviations: PEO: Poly(ethylene oxide), PVA: Poly(vinyl alcohol), PEG: Poly(ethylene glycol), PLGA: Poly(D,L-lactide-co-glycolide), PLA: Poly(L-lactide), coPLA: poly(L-lactide-co-D,L-lactide), PVP: Polyvinylpyrrolidone, PCL: Poly-ε-caprolactone.

3. FABRICATION METHODS OF CHITIN/CHITOSAN NANOFIBERS

Many approaches, including electrospinning, printing, self-assembly, phase separation and template synthesis have been developed to produce nanofibers. With respect to chitin/chitosan nanofibers fabrication, electrospinning, self-assembly and phase separation were the most widely used methods. In addition, other new methods, such as templating, simple mechanical treatment, ultrasonication and 2,2,6,6-Tetramethylpiperidinooxy (TEMPO) mediated oxidization were also used to produce chitin/chitosan nanofibers.

3.1. Electrospinning Method

Electrospinning, namely utilizing electrostatic forces to generate polymer fibers, has been used for more than 60 years.[117, 118] It still attracts many interests for its application in protective clothing, catalysis, electronics, filtration, agriculture, and biomedicine.[3, 119-121] The main advantages of this top-down nanotechnology includes: (1) low cost; (2) the resulted nanofibers are uniform and do not need expensive

purification; (3) the manufactured nanofibers are continuous compared to other bottom-up methods.[6, 40, 122-124]

Electrospinning method can produce continuous polymer nanofibers from polymer solutions or melts under high electric fields. Fig. 5 shows the schematic diagram of polymer nanofibers fabrication process using electrospinning method.[77] Electrospinning setup normally consists of syringe pumps, grounded target, a high voltage power supply and a spinneret. The electrospinning process is influenced by many parameters.[120, 123, 125] These parameters include (1) system parameters such as the types of the polymer and properties of polymer solution (conductivity, viscosity and surface tension); (2) process parameters such as electric potential, the distance between the tip and the collector, flow rate and concentration; (3) ambient parameters such as humidity, solution temperature, and air velocity in the chamber.

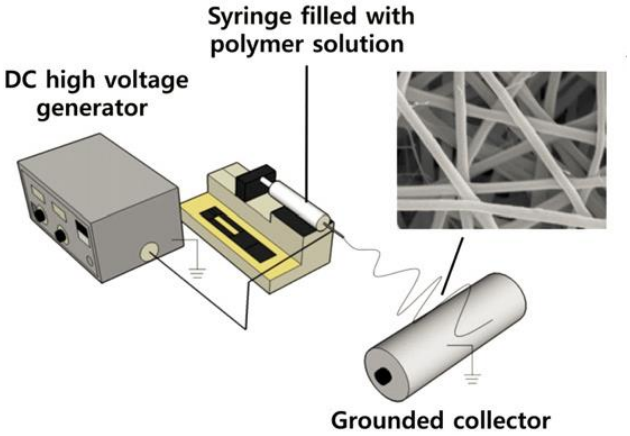

Figure 5. Schematic diagram of polymer nanofibers formation using electrospinning method. Reproduced with permission from ref. [77]. Copyright 2009 Elsevier.

In the case of formation of chitin/chitosan based nanofibers, electrospinning was the most frequently used method. Randomly oriented or aligned chitosan/poly-ε-caprolactone blend nanofibers with diameters of 200 nm and 400 nm

were produced by electrospinning, as shown in Fig. 6.[126] Cells cultured on the nanofibrous substrates showed elongation and alignment along the orientation of aligned fibers as early as 24 h and up to 120 h of culture. Due to the poor nanofiber formation capacity of chitin and chitosan by electrospinning, the chitin/chitosan nanofibers were always formed with other substances such as synthetic polymer, biopolymer and inorganic particles.[16] The chitin/chitosan based nanofibers formed by electrospinning have been introduced in detail in section 2.

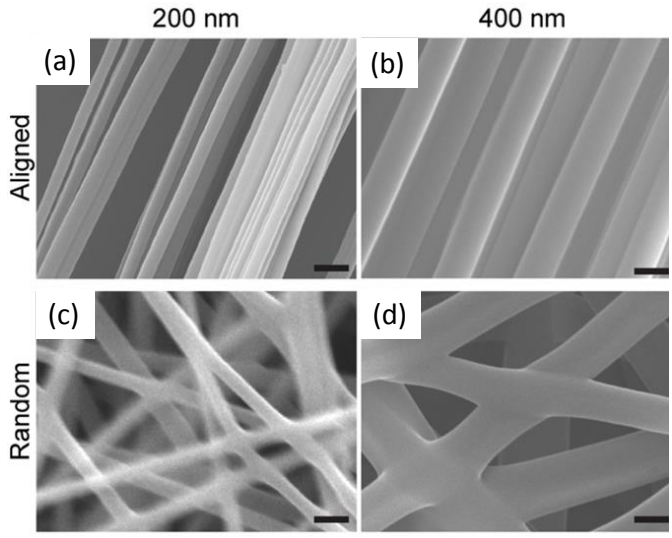

Figure 6. SEM images of aligned chitosan/PCL nanofibers with diameters of 200 nm (a) and 400 nm (b) and random-oriented nanofibers with diameters of 200 nm (c) and 400 nm (d). Reproduced with permission from ref. [126]. Copyright 2013 WILEY-VCH Verlag GmbH & Co.

3.2. Self-assembly

Molecular self-assembly is a powerful approach to fabricate novel materials. Molecular self-assembly is mediated by notably hydrogen bonds, weak noncovalent bonds, ionic bonds, van der Waals interactions, hydrophobic interactions, and water-mediated hydrogen bonds.[127, 128] Biomolecules, including peptides and proteins, were introduced to form nanofibers using self-assembly method.[129]

Chitin nanofibers with diameters of 3 nm have been fabricated in HFIP through a mild, facile self-assembly strategy.[13, 27, 130, 131] Fig.7 (a) showed the schematic of chitin nanofiber formation using self-assembly method. The self-assembly process was initiated through evaporation of HFIP. Fig.7 (b) showed the topographic AFM image of the chitin nanofibers. The diameter of a single chitin nanofiber is 3 nm. In addition, the diameter of the chitin nanofiber is regardless of the concentration of the solution. Although chitin nanofibers have been prepared in HFIP using self-assembly, the HFIP is toxicity. Efforts are still needed to develop chitin nanofibers in some "green" solvent such as ionic liquids and urea/NaOH mixture.[17, 18, 20, 132-134]

Figure 7. (a) Schematic of chitin nanofiber formation using self-assembly method. Reproduced with permission from ref. [13]. Copyright 2011 WILEY-VCH Verlag GmbH & Co. (b) Topographic AFM image of the chitin nanofibers. Reproduced with permission from ref. [131]. Copyright 2013 WILEY-VCH Verlag GmbH & Co.

3.3. Phase Separation

The phase separation technology is based on thermodynamic demixing of homogeneous polymer-solvent solution into a polymer-rich phase and polymer-poor phase.[135] Nanofibers prepared by phase separation always involve five basic steps as follows: polymer dissolution, phase separation and gelation, solvent extraction from the gel with water, freezing, and then freeze-drying under vacuum. Various parameters

influenced nanofiber formation, including: gelation temperature, polymer concentration, type of solvent, type of polymer and thermal treatment.[135, 136]

Chitosan based nanofibers can be obtained by method of phase separation.[28, 137, 138] Uniform chitosan nanofibers with diameters of 50–500 nm were obtained with chitosan concentration of 0.05% (w/v), acetic acid 0.025% (v/v) and liquid nitrogen by freeze-drying technique, as shown in Fig 8. The concentration of chitosan, phase separation temperature and concentration of acetic acid were investigated to influence the formation of nanofiber structure.

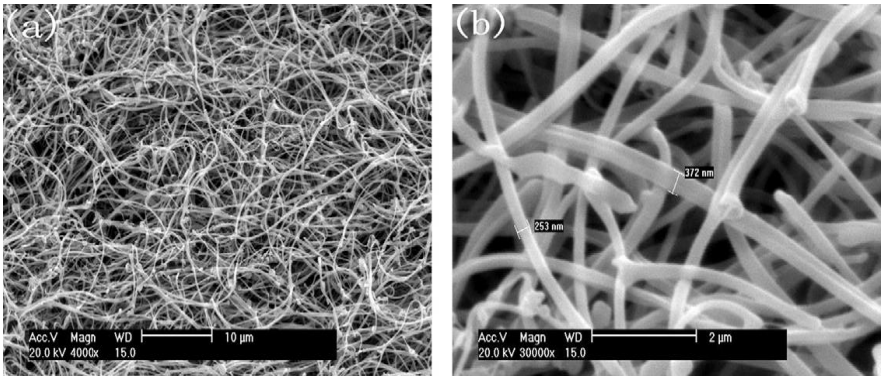

Figure 8. SEM images of freeze-dried chitosan nanofibers prepared with chitosan concentration of 0.05% (w/v), acetic acid 0.025% (v/v) and liquid nitrogen: (a) ×4000; (b) ×30,000. Reproduced with permission from ref. [138]. Copyright 2011 Elsevier.

In another study, a simple and environmental-friendly method to prepare chitosan/PVA and chitosan nanofibers through freeze-drying dilute aqueous solutions was reported.[42] Chitosan nanofibers with diameters ranging from 100 to 700 nm were prepared from dilute chitosan solutions (less than 0.1 wt%). Interestingly, aligned chitosan nanofibers with diameters of 100-500 nm were produced when the concentration of chitosan solution was 0.02 wt% due to the rapid freezing process. The author also fabricated chitosan/poly (vinyl alcohol) blend nanofibers by

freeze-drying in order to test feasibility of this method.

3.4. Microcontact Printing

Microcontact printing is a powerful technique that has been used for fabricating nanostructured biological molecules such as dendrimers, peptides and conducting polymers.[139-141] Chitin nanofibers were obtained using microcontact printing method by dissolving chitin in HFIP.[13] The high evaporation rate of HFIP allowed the formation of chitin nanofibers. The procedure of microcontact printing is illustrated in Fig.9. PDMS stamp was placed on the top of a container filled with chitin nanofiber ink (200 μ L) for 30s. Then, PDMS stamp was contacted with a substrate to print the chitin nanofiber ink. As characterized by AFM, chitin nanofibers with a width of 30 nm and a height of 20 nm were successfully obtained. Chitin nanofibers prepared by microcontact printing method have advantages as follows: (1) it can manufacture 2D and 3D chitin nanofibers ranging from micrometers to sub 50 nm in one step (2) the condition used is mild without any heating, vacuum, or ultrasonication (3) it can be combined with other fabrication technology to produce more complex structures.

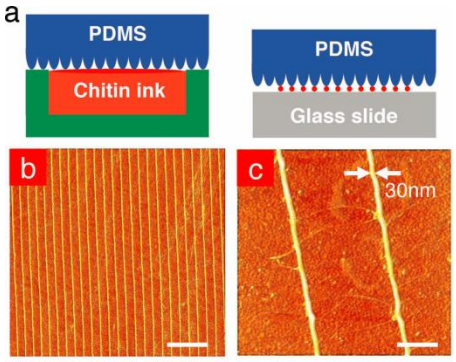

Figure 9. (a) Processes of chitin nanofibers formation using microcontact printing method, (b) AFM images of chitin nanofibers prepared from 0.05% (w/v) chitin solution. Scale bar: 4 μ m, (c) AFM images of two chitin nanofibers. Scale bar=400

nm. Reproduced with permission from ref. [13]. Copyright 2011 WILEY-VCH Verlag GmbH & Co.

3.5. Mechanical Treatment

Recently, it was reported that the chitin nanofibers can be fabricated through a simple mechanical treatment.[10, 12, 35, 36] The crab shell was made up of an aggregation of chitin nanofibers, as illustrated in Fig. 10 (A). The aggregated chitin nanofibers can be disintegrated by a grinder. Chitin was firstly treated with NaOH and HCl to remove the proteins and minerals. The purified chitin with 1 wt% concentration in water was passed through a specially designed grinder. Chitin slurry formed a gel after grinder treatment, suggesting that disintegration was accomplished. Highly uniform chitin nanofibers with a width of 10–20 nm were successfully obtained, as shown in Fig. 10 (B) and (C). The disaggregation of original chitin nanofibers under acidic condition is the key point in the mechanical treatment. This simple method offers a way to obtain chitin nanofibers in large amounts. In addition, it is a "green" method without any toxic organic solvent involved.

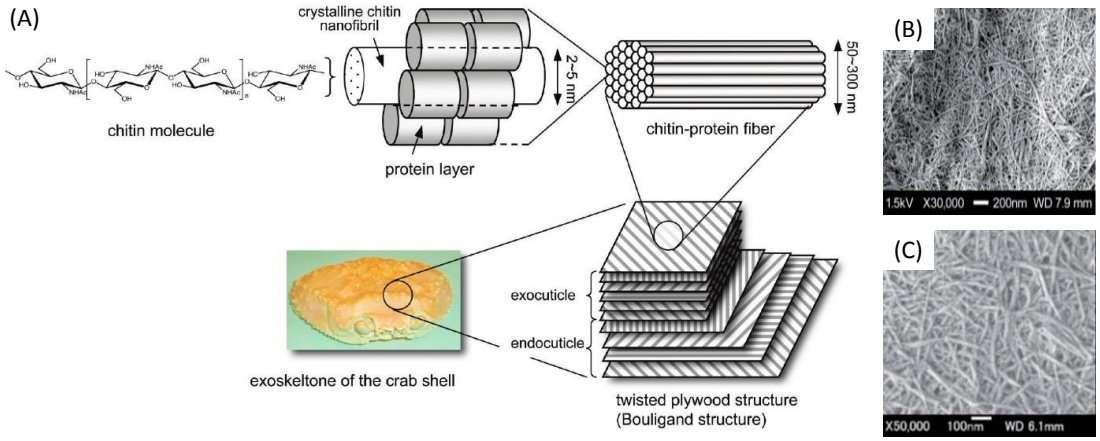

Figure 10. (A) Schematic illustration of exoskeleton structure of crab shells. (B) FE-SEM images of chitin nanofibers extracted from crab shells after mechanical treatment. (C) A higher magnification of the chitin nanofibers. The scale bars were 200 nm (B) and 100 nm (C). Reproduced with permission from ref. [12]. Copyright

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3.6. Ultrasonication

Ultrasonication has been widely used to prepare individualized chitin and chitosan nanofibers. This method is simple and without needs of any chemical modification. Chitin individualized nanofibers with a width of 3-4 nm in cross-section and at least a few microns in length were prepared through ultrasonication.[11, 142] The optimal condition for the formation of chitin nanofibers were as follows: (1) the raw material used was β-chitin, (2) chitin was dispersed in water with a pH of 3-4, (3) concentration was 0.1-0.3%, (4) the time was 2 minutes. It was reported that protonation of amino groups under acid conditions was the most important factor for preparing the nanofibers via ultrasonication. Fig. 11 (A) showed the photographs of the β-chitin nanofibers prepared by using Squid pen and Tubeworm as raw materials. The ultrasonication treatment was performed in water (pH 4) for 2 min. As shown in Fig. 11 (B), the chitin nanofibers extracted from Squid pen possessed a diameter of 3-4 nm characterized by transmission electron micrograph (TEM).

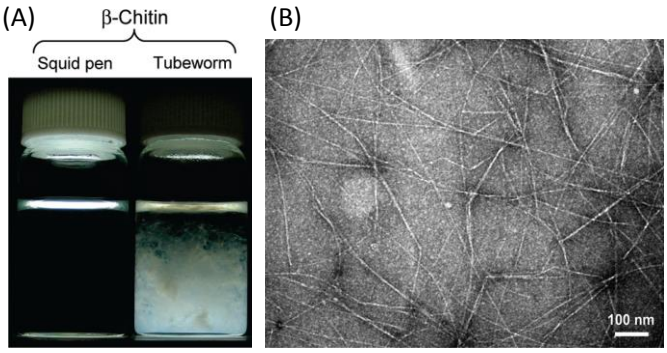

Figure 11. (A) Optical images of β-chitin nanofibers prepared by ultrasonication. (B) TEM images of the chitin nanofibers extracted from Squid pen. Reproduced with permission from ref. [11]. Copyright 2008 American Chemical Society.

Recently, Lu *et al*. fabricated chitin nanofibers via a simple high intensity ultrasonic treatment under neutral conditions (60 KHz, 300 W, pH = 7) using α-chitin as raw material.[143] By adjusting the ultrasonication time, chitin nanofibers with diameter of 20–200 nm were produced. α-chitin nanofibers with a width of 20 nm were successfully prepared under high intensity ultrasonication (300 W, 60 KHz) for 30 mins.

3.7. TEMPO-mediated Oxidation

TEMPO-mediated oxidation method has been used to fabricate cellulose nanofibers. Chitin has the similar structure as cellulose except that the substituted moiety in C2 was acetamido. Chitin nanofibers were successfully prepared by Fan *et al*. through TEMPO-mediated oxidation.[144] The raw materials used were β-chitins originating from tubeworm and pH was maintained at 10 by adding 0.5 M NaOH. Fig. 12 (A) showed the photographs of the chitin nanofibers extracted from Tubeworm by TEMPO-mediated oxidation with 0 mmol/g and 10 mmol/g of NaClO consumed. Chitin nanofibers can be obtain with a width of 20–50 nm and several microns in length by controlling the addition level of NaClO used as the primary oxidant in the oxidation, as shown in Fig. 12 (B). Chitin nanofibers prepared by TEMPO-mediated oxidized method were not continuous nanofibers. This kind of chitin nanofibers can be used as injectable nanomaterials for controlled drug release.

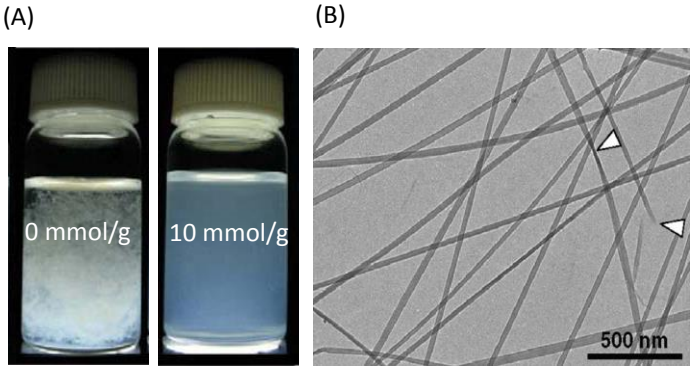

Figure 12. (A) Optical images of β-chitin nanofibers prepared through TEMPO-mediated oxidation by using Tubeworm as raw material. The amount of NaClO consumed was 0 mmol/g (left) and 10 mmol/g (right). (B) Transmission electron micrograph of the TEMPO-mediated oxidized chitin nanofibers extracted from Tubeworm. Reproduced with permission from ref. [144]. Copyright 2009 Elsevier.

4. BIOMEDICAL APPLICATIONS OF CHITIN/CHITOSAN NANOFIBERS

Chitin and chitosan based nanofibers with certain physical and biological properties in relation with biocompatibility, biodegradability, cellular binding capability, wound healing, antitumor and antibacterial have been widely applied in biomedical fields.[16, 23, 30, 145] In this section, biomedical application of chitin and chitosan based nanofibrous such as tissue engineering, drug delivery, wound dressing, antimicrobial treatment, and biosensors would be discussed.

4.1. Tissue Engineering

Tissue engineering is defined as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function".[146-149] The design of polymeric scaffolds with certain biological and mechanical properties similar to native extracellular matrix (ECM) for modulating cellular behavior is one important aspect of tissue engineering.[5] Chitin/chitosan nanofibers have been widely applied in

tissue engineering for the structure is similar to glycosaminoglycans in the ECM.[150, 151] Their morphological properties are also similar to fibrous collagen structures in the ECM on the scale of nanometers. Some recent applications of chitin and chitosan nanofibers demonstrate their remarkable potential on tissue engineering.

4.1.1. Bone Tissue Engineering

Bone is an important organ playing key roles in human body. The traditional way to treat bone-defect includes autografting and allografting.[152-154] However, these methods may cause problems, such as supply limitation, donor scarcity, immune rejection and pathogen transfer. Bone tissue engineering has become a rapidly expanding research area because it offers a new way to repair bone.[155-157] Bone tissue engineering approaches always involve the usage of scaffold in combination with cells and growth factors.[158, 159] Scaffolds suitable for bone tissue engineering must possess properties of high quality, sustainability, reliability and cost inexpensive.[160-162]

In recent years,.[155, 163] Chitin and chitosan based nanofiberous materials have attracted considerable attention in bone tissue engineering due to their interesting characteristics such as they can attach and proliferate osteoblast cells, support the formation of mineralized bone matrix, easy to be molded in various geometries, and properties of biocompatibility, biodegradability and antibacterial property.[151, 164-166] In the case of application of chitin and chitosan based nanofibers in bone tissue engineering, hydroxyapatite (HA) is normally combined with the chitin and chitosan scaffold to improve the activity and viability of cells, as well as enhance the mechanical and cell-attachment properties of the scaffolds.[167, 168]

Chitosan nanofibers incorporated with hydroxyapatite have been used in bone tissue engineering for the similar structure to the ECM in native bone tissue.[72, 169, 170] Chitosan nanofiber containing hydroxyapatite was electrospun to test its ability to regenerate bone.[59] More recently, hydroxyapatite-containing chitosan nanofibers were electrospun and crosslinked with genipin to facilitate the formation of bone.[171] The crosslinked chitosan nanofibers have the similar Young's modulus to periosteum. Bioactive properties of the scaffolds were investigated *in vitro* using 7F2 osteoblast-like cells. After 14 days' culture, cells present a more rough texture on the chitosan-hydroxyapatite-genipin crosslinked composite scaffolds (CTS-HA-GP) (Fig. 13 a and b) than that on the chitosan-genipin crosslinked scaffolds (CTS-GP) (Fig. 13 c and d). This difference was attributed to that the hydroxyapatite can enhance the maturation of 7F2 cells. In the meantime, the metabolic activity decreased in cells cultured on HA-containing scaffolds which were caused by the differentiation cease proliferation of cells. The HA containing chitosan nanofibrous materials crosslinked with genipin may be applied in bone tissue engineering.

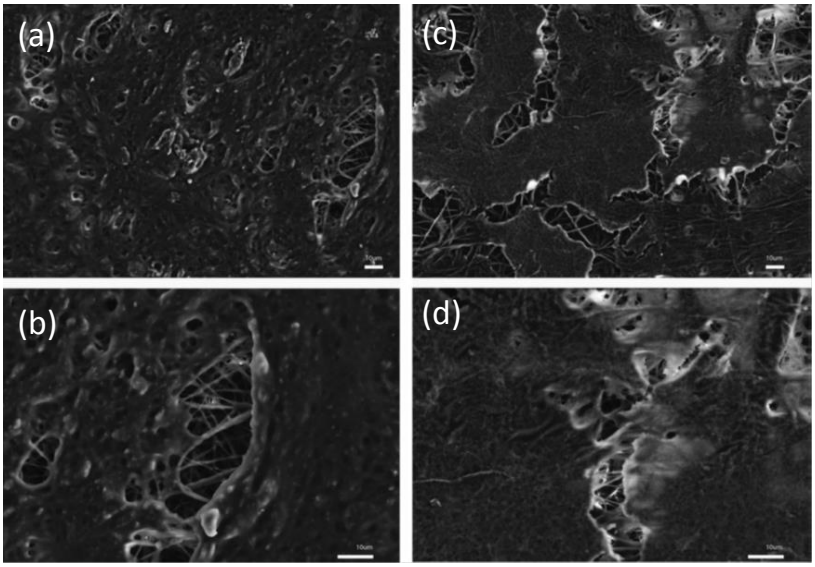

Figure 13. SEM of 7F2 cells on CTS-GP (a 500×, b 1000×) and CTS-HA-GP (c 500 ×, d 1000×) nanofibers scaffold after 14 days' culture. Reproduced with permission from ref. [171]. Copyright 2012 Elsevier.

4.1.2. Vascular Tissue Engineering

Cardiovascular diseases is the leading cause of death and disability worldwide.[172, 173] The traditional way to treat cardiovascular diseases is to implant conduits. Artificial grafts, allografts, autologous tissues and xenografts are the frequently used graft materials. However, these materials may cause problems such as thrombogenicity and infection. Vascular tissue engineering offers an alternative way to treat cardiovascular diseases.[174-179] Various research groups investigated the cardiovascular diseases treatment using vascular grafts.[180, 181] The aims of vascular tissue engineering are to produce a vascular construction that similar to a native vessel by using a biodegradable scaffolds with the autologous cells.[173, 182, 183] Scaffold materials used should be biodegradable, biocompatible, non-immunogenic and supporting the attachment and proliferation of the cells.[172, 184-187] In recent years, nanostructured scaffolds materials fabricated by electrospinning have been widely used in vascular tissue engineering.[183, 188, 189] Synthetic polymers and biopolymers such as PGA, PLA, PCL, collagen, silk, and chitosan in the form of nanofibers have been investigated to apply in the vascular tissue engineering.[190-193]

Chitosan based nanofibers were eletrospun and employed as scaffolds in vascular tissue engineering.[194] In order to prevent thrombosis, a 3D gradient heparinized chitosan/poly-ε-caprolactone nanofibrous scaffold with vascular endothelial growth factor (VEGF-Hep-gCS/PCL) and uniform CS/PCL nanofibrous scaffold with VEGF

(VEGF-Hep-uCS/PCL) were produced.[195] After 72 h cultures of human umbilical vein endothelial cells (HUVEC), the cells expressed abundant f-actin which suggested the formation of actin filaments around each cell periphery and in the networks (Fig. 14 a and c). Furthermore, HUVEC expressed more Von Willebrand factor (vWF) on the lumen surface of gradient scaffold than on the uniform scaffolds (Fig. 14 b and d). Thus, the proliferation of HUVEC was enhanced by gradient CS/PCL and induced rapid endothelialization. In addition, the release behaviors of VEGF from gradient CS/PCL scaffold were more stable and continuous compared to the uniform CS/PCL scaffold.

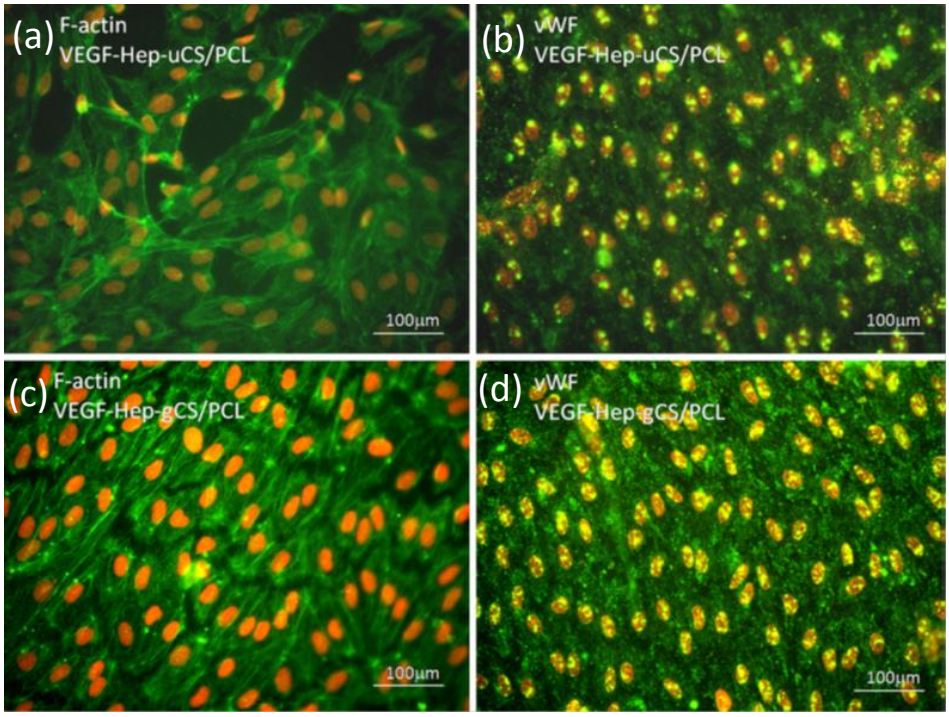

Figure 14. Morphological characterization of HUVECs cultured for 72h on the VEGF-Hep-gCS/PCL and VEGF-Hep-uCS/PCL scaffolds. The cells were stained with Alexa Fluor 488 phalloidin and anti-vWF antibody. (a, b) F-actin and vWF expressions of cells on VEGF-Hep-uCS/PCL. (c, d) F-actin and vWF expressions of cells on VEGF-Hep-gCS/PCL. Reproduced with permission from ref. [195]. Copyright 2012 Elsevier.

4.1.3. Neural Tissue Engineering

The nervous system contains the peripheral nervous system (PNS) and the central nervous system (CNS).[196] The injuries or traumas in the nervous system introduce pain to the patients.[196, 197] The treatment is of the nervous system complex for the poor regeneration ability. Treatments such as autografts, allografts, xenografts and autologous may cause problems such as immunological rejections and disease transfer.[198-200] Advances in the neural tissue engineering provide a new approach for neural regeneration. In the neural tissue engineering, a biodegradable, biocompatible scaffold with high-porosity and proper mechanical strength is required to facilitate neural regeneration.[198, 199] Nanofiberous materials fabricated by electrospinning and self-assembly were applied in neural regeneration for the structural similar to the extracellular matrix (ECM).[197, 200-203]

In recent years, chitosan nanofibers have been produced to open new routes for the development of scaffolds in neural tissue engineering and regenerative medicine.[26, 41, 49, 204, 203] Cooper *et al.* fabricated randomly oriented and aligned chitosan/poly-ε-caprolactone (chitosan/PCL) fibrous scaffolds and investigated the potential use in nerve regeneration.[205] Fig. 15 (A) shows the SEM images of the randomly oriented nanofibers with mean diameters of 405.0±59.8 nm and aligned nanofibers with mean diameters of 408.2±76.6 nm. Neuron-like PC-12 cells were selected to investigate the potential usage of the chitosan/PCL nanofibers in nerve regeneration. As shown in Fig. 15 (B), the PC-12 cells adhered well on both randomly oriented and aligned chitosan/PCL nanofibers after cultured for 7 days. However, as

shown in Fig. 15 (B) (b), the cells on aligned chitosan/PCL nanofibers showed a parallel growth along the nanofibers direction compared to randomly oriented nanofibers, with longer neuritis parallel to the nanofibers. In addition, gene expressions of β-tubulin and neurofilament-200 (NF-200) by PC-12 cells on nanofibers were tested. As shown in Fig. 15 (C), PC-12 cells expressed almost the same amount of NF-200 on aligned and randomly oriented nanofibers, suggesting that the cells differentiated well on the nanofibers. PC-12 cells expressed almost three times of β-tubulin on the aligned nanofibers compared with the randomly oriented nanofibers, demonstrating that the neurite extended longer on the aligned nanofibers.

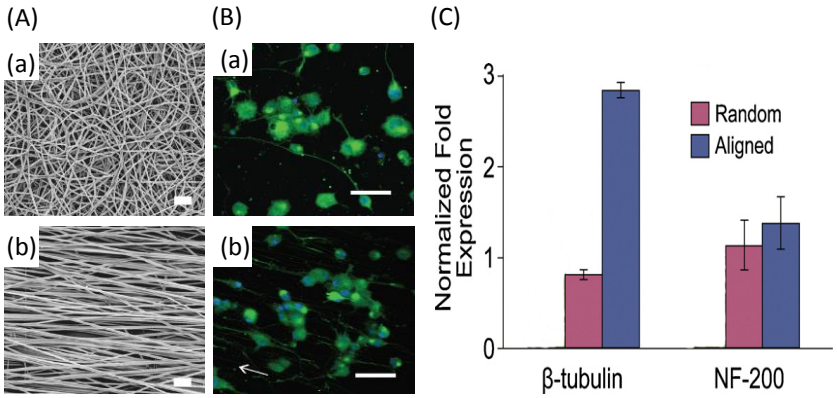

Figure 15. (A) SEM images of chitosan/PCL nanofibers randomly orientation (a) and aligned orientation (b). The scale bar is 5 μm. (B) Fluorescent images of PC-12 cells cultured on randomly orientated nanofibers (a) and aligned nanofibers (b). (C) β-tubulin and NF-200 expression by PC-12 cells on nanofibers. Reproduced with permission from ref. [205]. Copyright 2011 Elsevier.

Chitosan nanofibers were also produced using technologies of self-assembly and deacetylation.[130] Chitosan nanofibers with diameter of 4 nm and 12 nm were coupled with poly-D-lysine (PDL) and were employed for mouse cortical neuron cultures to examine the capabilities to support cell attachment, neurite coverage and survival. Results showed that neurons placed on the 4 nm chitosan nanofibers scaffolds

displayed extensive neurite extension and arborization at day 3 compared to day 1, whereas no such further neurite elaboration was observed on the 12 nm nanofibers surface. After 7 days' culture, the 4 nm chitosan nanofibers with PDL supported 37.9% neuron viability compared to only 13.5% on traditional PDL surfaces, suggesting significantly improved long-term cell viability.

4.2. Controlled Drug Delivery

Nanotechnology has been widely used in controlled drug release.[206-209] Electrospun nanofibers were used as drug delivery vehicles due to their properties as follows: (1) high surface area to volume ratio; (2) the drug release behavior can be easily modulated by composition and morphology of the nanofibers; (3) bioavailability of a drug moiety can be easily controlled through designing various dosage forms; (4) drug encapsulation efficiency was higher than other nanotechnologies.[77, 210-213] Different kinds of therapeutic agents, for instance, anticancer drugs[214], antibiotics[215], proteins[216], and growth factors[217] were incorporated into nanofibers and be used as wound healing materials, tissue engineering scaffolds, and drug carriers.[77] The drug release from nanofiber is accompanied by complicated diffusion pathway and polymer degradation. Drug release behaviors can be controlled by various conditions such as composition of the nanofibers, polymer property, surface functionalization, and the state of drugs[218-220].

Randomly oriented and aligned PLGA/chitosan nanofibers were produced to test their potential application as drug release system.[221] Fig. 16 shows the SEM images of

PLGA/chitosan nanofibers with various PLGA/chitosan ratios (10/0, 9.375/0.625, 9/1 and 7/3). The releases of fenbufen were affected by the concentration of chitosan in the nanofibers and the morphology. As shown in Fig. 16 (B), the fenbufen released faster with more chitosan added. The nanofibers were more hydrophilicity with more chitosan added, which allowed drugs to release from the nanofibers easier. In addition, the drugs released faster from the aligned PLGA/chitosan nanofibrous scaffold than randomly oriented scaffold. This was attributed to the different density and pore size between aligned and randomly oriented nanofibers. It was noted that the aligned nanofibers have higher density and smaller poresize.

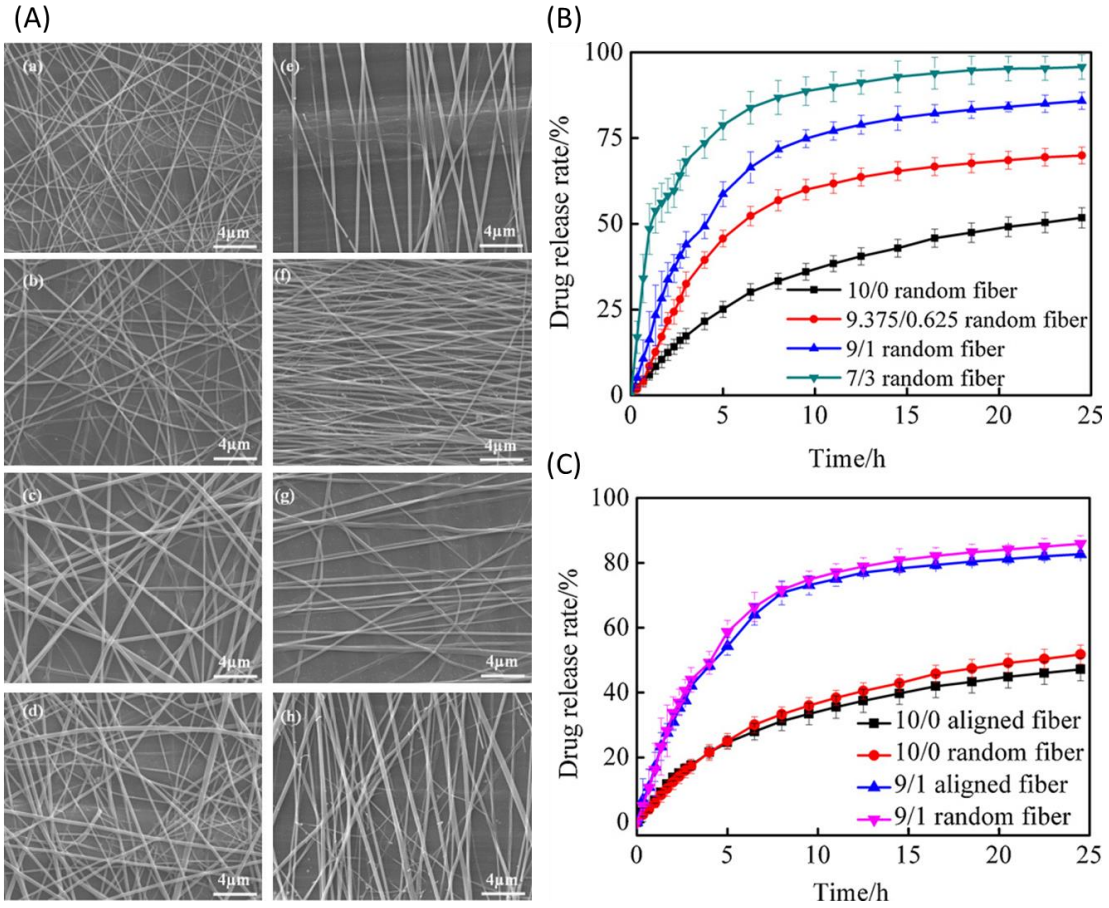

Figure 16. (A) SEM images of randomly oriented PLGA/chitosan nanofibers (a–d) and aligned nanofibers (e–h) with various PLGA/chitosan ratios (10/0, 9.375/0.625, 9/1 and 7/3). (B) Release profiles of PLGA and PLGA/chitosan nanofibers with different PLGA/chitosan ratios. (C) Release profiles of randomly oriented and aligned

PLGA/chitosan nanofibers with PLGA/chitosan ratios as 10/0 and 9/1. Reproduced with permission from ref. [221]. Copyright 2011 Elsevier.

Chitosan nanofibers produced by freeze-drying could also be used as drug carrier.[28] The drug release profiles were controlled by the structure of chitosan carriers. Concentration of the chitosan was a key factor to controlled structure. Sheet-like structure was observed at 1.0 wt% of chitosan. The nanofiber-like structure was mainly present at 0.02 wt% chitosan concentration (Fig 16 (A)). Fig 16 (B) showed the release profiles of curcumin into PBS at 37℃ from different structured chitosan carriers. The fastest release was observed from the chitosan nanofiber carrier. The fast release from the nanofibers was due to higher permeability, shorter diffusion distance and fast swelling activity.

4.3. Wound Dressing

Wound healing is one of the most important medical applications for chitin and chitosan. Hydrogels, membranes, fibers, and sponges derived from chitin/chitosan or their derivatives have been intensely prepared.[222] Among different forms of wound healing materials, chitin/chitosan based nanofibers have been widely used due to their excellent properties such as high levels of porosity, gas permeation, and high surface-to-volume ratio. These properties promoted cell respiration, skin regeneration, moisture retention, removal of exudates, and hemostasis.[223]

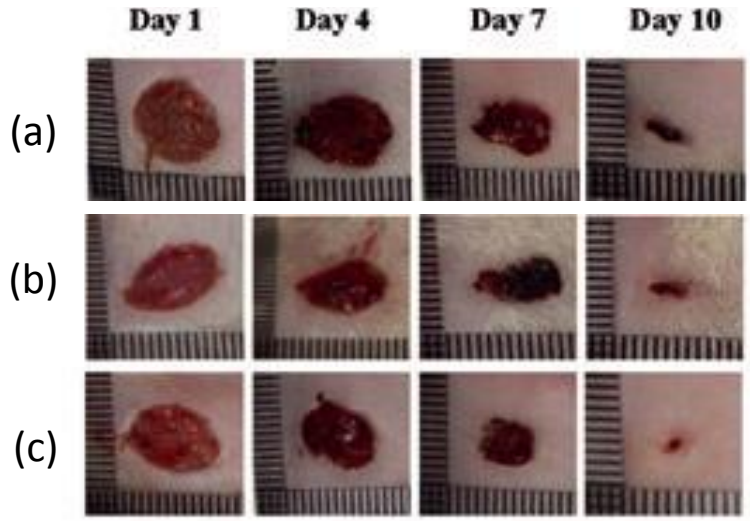

Figure 17. Healings of the wound treated with three kinds of wound dressings at day 1, 4, 7 and 10. (a) gauze (negative control), (b) 30/70 CS-EDTA/PVA nanofiber scaffold and (c) commercial wound dressing (Sofra-tulleregister) (positive control). Reproduced with permission from ref. [224]. Copyright 2012 WILEY-VCH Verlag GmbH & Co.

Chitosan (CS) blended with ethylenediaminetetraacetic acid (EDTA) and polyvinyl alcohol (PVA) (CS-EDTA/PVA) nanofibers scaffold were obtained by electrospinning.[224] The cytotoxicity assay indicated that the nanofibers mats were nontoxic to human fibroblast cells. The CS-EDTA/PVA nanofibers showed good antibacterial activity. In addition, wound healing test *in vivo* indicated that the CS-EDTA/PVA nanofiber scaffold performed better than gauze with reduced acute wound size during the 1st week (Fig. 17). More recently, the fruit hull of *Garciniamangostana* extracts and lysozyme were incorporated into CS-EDTA/PVA nanofibers respectively.[69, 225] The prepared nanofibers showed good wound healing ability and had great potential to be used as wound dressings.

4.4. Antibacterial Applications

Chitosan is a natural polymer of antimicrobial activity.[22] Antibacterial activities of

chitsoan based nanofibers were studied for the potential application in the field of wound dressing, antibacterial filter, and tissue engineering.[16, 24, 222, 226] The antibacterial mechanism of chitosan nanofiber has not been fully explained. Several mechanisms such as presence of amino groups from the dissolved chitosan nanofibers, molecular conformation, release of small chitosan oligomers, and formed chitosan layer around the bacterial cell may endow the chitosan nanofiber with antibacterial activity.[227]

The most studied antibacterial chitosan nanofiber consisted of chitosan or its derivatives, synthetic polymers and antibacterial substances.[55, 88, 99, 228] Chitosan/PVA/silver nanoparticles blended nanofibers were fabricated by electrospinning.[229] Silver nanoparticles were incorporated into the nanofibers through reduction of silver ions in polymer solution. Nanofibers contained silver nanoparticles showed excellent antibacterial activities. Chitosan/poly(ethylene oxide)/silver nanoparticles composite nanofibers were also prepared and showed higher antibacterial capacity than nanofibers without silver nanoparticles.[230] Fig. 18 showed the antibacterial activities of CS/PEO and Ag/CS/PEO nanofibers with the CS/PEO mass ratio of 1:1 against *E. coli*. The nanofibers containing Ag nanoparticles showed better antibacterial activity than the CS/PEO nanofibers. All bacterial inactivated within 10 and 6 h for Ag/CS/PEO nanofibers containing 1.1 and 2.2 wt.% nanoparticles, respectively. More recently, antibacterial chitosan/PCL nanofibrous membranes were produced for antibacterial water filtration.[231] Chitosan/PCL fibers with diameters ranging from 200 to 400 nm with chitosan contents of 25, 50 and 75

wt% were obtained by electrospinning. The chitosan–PCL fibrous membranes showed higher filter efficiency than the PCL fibrous membranes.

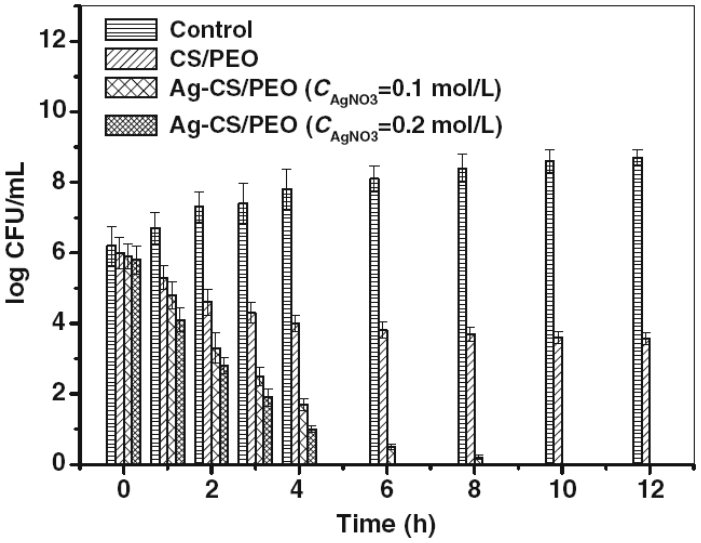

Figure 18. The antibacterial activity of CS/PEO and Ag/CS/PEO nanofibers against *E. coli*. The mass ratio of CS/PEO was 1:1. Reproduced with permission from ref. [230]. Copyright 2009 Springer.

4.5. Biosensor and Diagnosis

Nanofiber-based biosensor is an ideal platform allowing for sensitive detection of clinical, environmental, and food safety analysts via unique characteristics of nanofibers.[232, 233] These unique properties include: (1) High surface area to volume ratio of nanofibers, which increases the number of binding sites available for biological recognition element immobilization; (2) Nanofibers have faster mass transfer rates, which results in lower limits of detection and faster analyte detection rates; (3) Nanofiber mats with specific sizes, shapes, pore sizes, and tensile strengths can be easily fabricated.[234]

Chitosan is an excellent substrate for enzyme immobilization and can be easily electrospun into high surface area nanofibrous mats. Chitosan nanofibers based

biosensors have been developed.[235, 236] Chitosan/PVA nanofibers were electrospun for lipase immobilization.[237] This chitosan nanofibrous mat showed a high enzyme loading of 63.6 mg/g with activity retention of 49.8%. The immobilized lipase was more stable than that using other technology, suggesting that chitosan nanofibers with excellent biocompatibility and high enzyme loading can be developed for biosensors. Biosensor based on chitosan nanofibers incorporated with cholesterol oxidase and gold nanoparticle was developed to detect cholesterol, [236] as shown in Fig. 19 (A). Oil/water emulsion method was applied to prepare the chitosan nanofiber coated on ITO electrode (50–100 nm). Then, gold nanoparticles were electrodeposited on the obtained nanofibers. Fig. 19 (B) showed the amperometric measurement of this electrode toward cholesterol at an applied potential of +0.3V (*vs.* Ag/AgCl). The currents increased once the cholesterol was injected at designed intervals. The curves in the inset of Fig. 19 (B) showed that the biosensor showed a linear response to cholesterol in the concentration ranging from 1 to 45μM. The limit of detection (0.5 μM) was also significantly lower than other cholesterol biosensors. In the meantime, the biosensor showed high sensitivity (1.02 μA/μM) and reproducible detection of cholesterol in real human serum samples.

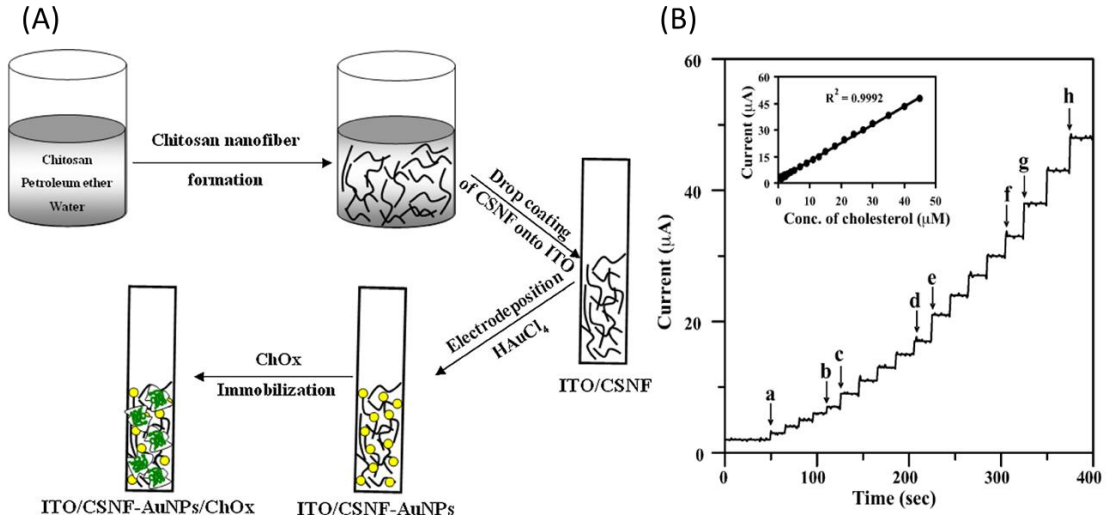

Figure 19. (A) Schematic illustration the process to fabricate the chitosan nanofiber/Au nanoparticles/cholesterol oxidase biosensor electrode. (B) Amperometric responses of chitosan nanofiber/Au nanoparticles/cholesterol oxidase biosensor electrode toward cholesterol (1–45 μM) at an applied potential of +0.3V (*vs.* Ag/AgCl). The inset was the calibration plot of cholesterol concentration *vs.* current response. Reproduced with permission from ref. [236]. Copyright 2011 Elsevier.

5. CLINICAL TRIALS

Despite many efforts have been paid to develop functional nanofibers to suit different biomedical applications, the clinical use of chitin and chitosan based nanofibers still remains challenges and needs further insights. Chitosan based nanofibrous members have been proved to promote the wound healing in the clinical trial. Kossovich *et al*. developed chitosan/PEO nanofiber using electrospinning method and applied as wound dressings for IIIa and IIIb degree burns.[238] Fig. 20 (A) showed the wound healings of patients with IIIa burns using chitosan nanofiber sheets with a thickness of 200 μm. Wound healings of patients with IIIb burns were shown in Fig. 20 (B). It was demonstrated that chitosan nanofiber dressings provided ventilation of the wound, protection from infection, effective absorption of exudate and stimulation of skin tissue regeneration on patients with IIIa and IIIb degree burns.

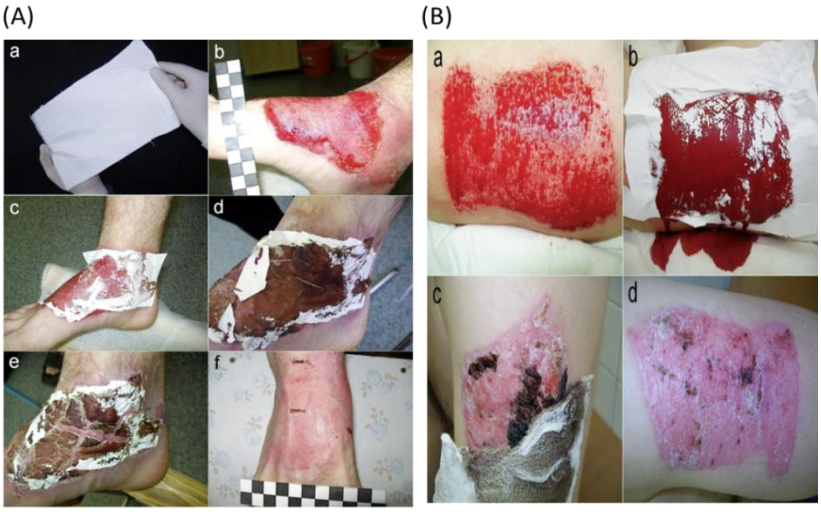

Figure 20. Healing processes of patients with IIIa and IIIb (B) burn. (A) IIIa burn, (a) the used dressing, (b) appearance of IIIa burn, (c) covered with the dressing, (d) covered the dressing for 5 days, (e) covered the dressing for 10 days (f) covered the dressing for 14 days. (B) IIIb burn, (a) appearance of IIIb burn, (b) covered with the dressing, (c) covered the dressing for 12 days, (d) covered the dressing for 14 days. Reproduced with permission from ref. [238]. Copyright 2010 Springer.

6. PERSPECTIVES

Advances in nanotechnology and biomedicine are enabling the preparations of chitin and chitosan nanofibers with multifunctions which are expected to have great potentials to be used as biomedical materials. Biomedical fields such as tissue engineering, wound dressing, drug release, antibacterial and biosensor are the most involved areas of chitin and chitosan based nanofibers due to their low cytotoxicity, biocompatibility, biodegradability, antibacterial activity, larger surface area to volume ratio, flexibility in surface functionalities and extremely small pore dimensions. Several drawbacks still need to be considered and solved to meet the practical criteria. For instance, more effective and environmental-friendly solvents of chitin and chitosan should be developed to meet the mass production of the nanofibers with lower toxicity. Some strategies need to be taken to enhance the mechanical strength of

chitin and chitosan nanofibers to perform some specific applications.

Future developments in chitin and chitosan nanofibrous materials would be investigated in the following aspects: (1) New technology is still required to facilely fabricate chitin and chitosan nonifibers; (2) Optimal parameters should be tested to meet the final practical applications of nanofibers; (3) Nanofibers with special structures, physical and biological features are needed to be fabricated to cater particular application; (4) Thorough toxicity studies needs to be conducted before human utilization; (5) Most of studies are still at the laboratory level and more clinical tests of the nanofibers should be carried out.

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