Research Article

Electrospinning of Cross-Linked Magnetic Chitosan Nanofibers for Protein Release

Ehsan Tayerani Nicknejad,¹ Seyyed Mohammad Ghoreishi,^{1,3} and Neda Habibi²

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Abstract. A poly(vinylalcohol) (PVA) electrospun/magnetic/chitosan nanocomposite fibrous cross-linked network was fabricated using *in situ* cross-linking electrospinning technique and used for bovine serum albumin (BSA) loading and release applications. Sodium tripolyphosphate (TPP) and glutaraldehyde (GA) were used as cross-linkers which modified magnetic-Fe₃O₄ chitosan as Fe₃O₄/CS/TPP and Fe₃O₄/CS/GA, respectively. BSA was used as a model protein drugs which was encapsulated to form Fe₃O₄/CS/TPP/BSA and Fe₃O₄/CS/GA/BSA nanoparticles. The composites were electrospun with PVA to form nanofibers. Nanofibers were characterized by field emission scanning electron microscopy (FESEM) and Fourier transform infrared spectroscopy (FTIR). The characterization results suggest that Fe₃O₄ nanoparticles with average size of 45 nm were successfully bound on the surface of chitosan. The cross-linked nanofibers were found to contain uniformly dispersed Fe₃O₄ nanoparticles. The size and morphology of the nanofibers network was controlled by varying the cross-linker type. FTIR data show that these two polymers have intermolecular interactions. The sample with TPP cross-linker showed an enhancement of the controlled release properties of BSA during 30-h experimental investigation.

KEY WORDS: cross-linker; electrospun; magnetite; mano-composite; protein loading.

INTRODUCTION

Recently, electrospinning technology has emerged as a versatile process of fabricating nanofibers. Different methods are reported to produce biomimetic non-woven materials comprising a large network of interconnected fibers and pores (1,2). A large number of biomacromolecules, such as poly(ϵ -caprolactone), poly(lactic acid), poly(lactide-co-glycoside), collagen, and gelatin have been successfully fabricated into non-woven mats by the electrospinning method (3–5). Especially, a series of electrospun nanofibers based on chitin and chitosan have gained a great attention owing to their extensive biomedical applications in such as tissue engineering scaffolds and drug delivery (6). Electrospun nanofibrous membranes are considered to be of great potential in the field of tissue regeneration, since they can closely mimic the extracellular matrix architecture (7–11). The electrospinning process also provides operational flexibility for incorporating multiple components into nanofiber, such as metal nanoparticles and inorganic nanoparticles (12–17). Magnetic nanoparticles (NPs) are of enormous importance for their prospective biomedical applications such as magnetic resonance imaging

(MRI), tissue repair, immunoassay, detoxification of biological fluids, hyperthermia, and drug delivery. As well, electrospinning technique had been successfully used in fabricating magnetic fibrous scaffolds (18). Wei *et al.* applied magnetic chitosan electrospun nanofibers for bone regeneration purposes (19). In the field of drug delivery, Fe₃O₄ nanoparticles are used for their ability to be driven by external magnetic force and thus achieving direct drug targeting. Moreover magnetic nanoparticle once been exposed to an alternate magnetic field could be used for controlled release of drug components embedded inside a polymer. Therefore, incorporation of magnetic particles in electrospun nanofibers could offer enhanced drug delivery efficacy (19–29).

It is difficult to disperse magnetic Fe₃O₄ nanoparticles homogeneously in a polymer matrix because of a difference in density and polarity between nanoparticles and polymer. On one hand, surface modification of magnetic particles before integration in chitosan (CS) nanofibers prevents aggregation of particles allowing a uniform distribution in fibers. On the other hand, the modification could increase the chemical stability of the Fe₃O₄ NPs and could improve the biocompatibility of the NPs. In addition, using cross-linker allows enhanced loading properties for functional molecules.

In this work, Fe₃O₄ NPs were modified with CS nanoparticles by using glutaraldehyde (GA) and sodium tri polyphosphate (TPP) as cross-linkers (Fig. 1). Using cross-linker allows enhanced loading properties for functional molecules and also proteins in addition to providing a uniform distribution of magnetic nanoparticles in the fibers. The two terminal aldehyde

¹Chemical Engineering, Isfahan University of Technology, Isfahan, 84156-83111, Iran.

²Nanotechnology and Advanced Material Institute, Isfahan University of Technology, Isfahan, Iran.

³To whom correspondence should be addressed. (e-mail: ghoreishi@cc.iut.ac.ir)

Fig. 1. Molecular structure of **a** chitosan (CS), poly-(D)-glucosamine, **b** glutaraldehyde (GA), **c** sodium triphosphate (TPP), and **d, e** cross-linking reaction of CS with TPP and GA

groups of glutaraldehyde react with amino groups, resulting in cross-linking of those chains through glutaraldehyde. Linkage of both terminal aldehyde groups with separate chitosan molecules results in cross-linking, while the linkage of only one aldehyde group results in the activation of surface for protein loading. On the other hand, TPP is a cross-linker which links the amide groups resulting in formation of CS nanoparticles and also allowing a linkage with other functional molecules. First surface of Fe_3O_4 nanoparticles were modified with TPP and GA cross-linkers and then CS were further added to form CS nanoparticles with cross-linkers absorbed on the surface of Fe_3O_4 . BSA was used as a model of protein drugs which was encapsulated and released from cross-linked nanofibers. Effect of cross-linker in magnetic NP distribution, nanofiber network morphology, and subsequently protein release was studied. This work seeks to explore efficient loading of drugs and the possibility to achieve controlled release from scaffold by means of magnetic properties and therefore offers an important biomedical material.

MATERIALS AND METHODS

Materials

Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) was purchased from Merck Co. Medium molecular weight chitosan (CTS, 75 and 85% deacetylated), bovine serum albumin (BSA), and acetic acid (TFA, 98%) were purchased from Sigma-Aldrich Co. The polyvinyl alcohol (PVA Z-410, Japan Synthetic Chemical Co.) had a degree of deacetylation of $97.0 \pm 0.5\%$ and a MW of $1.2 \times 10^5 \text{ g mol}^{-1}$.

Fe_3O_4 Nanoparticle Synthesis

Fe_3O_4 nanoparticles were prepared by modified coprecipitation route. $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Merck) was used as precursors. A solution of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ with molar ratio of 1.5:1 was prepared under ultrasonic agitation via ultrasonication (10 min, 100 W) to ensure homogeneous mixing, subsequently ultracentrifuged, and black

precipitate was produced immediately by adding sodium hydroxide. Dried nitrogen gas was inflated during synthesis in a closed system to prevent the oxidization of ferrous ions.

$\text{Fe}_3\text{O}_4/\text{CS}/\text{TPP}$ Nanocomposites

A suspension of Fe_3O_4 nanoparticles were prepared by adding 25 mg of Fe_3O_4 in 50-ml deionized water. 100 μL GA was dropped to the above solution with continual stirring at 500 rpm for 1 h to form an adsorption layer of GA around Fe_3O_4 nanoparticles. In order to remove unbounded GA, procedures of washing and dispersion were adopted. CS was dissolved in acetic acid and added to the reaction system in order to obtain a final CS solution of 2% w/v (0.4 g of CS in 20 ml acetic acid) followed by the coating process for 2 h. To cross-link the exterior of loaded CS layer, GA was dropped into the flask, the cross-linking reaction was completed after 2 h later, and the final products were gathered with a permanence magnet. Fe_3O_4 -CS nanoparticles were prepared by cross-linking with sodium tripolyphosphate (TPP). Specifically, 20 mg TPP were added to a solution of 500 ppm Fe_3O_4 and stirred for 1 h. The solution was washed and redispersed in deionized water. 0.4 g CS were dissolved in 20 ml of acetic acid and added to reaction system in order to cross-link with TPP absorbed on Fe_3O_4 nanoparticles. After stirring for 30 min, $\text{Fe}_3\text{O}_4/\text{TPP}/\text{CS}$ nanoparticles were collected and used to produce nanofibers by means of electrospinning process.

Fabrication of $\text{Fe}_3\text{O}_4/\text{CS}/\text{PVA}$ Nanofibrous Membranes by Electrospinning Process

Electrospinning of CS was performed with PVA solution. To this regard, 10% PVA were prepared and stirred for 4 h in 80°C . CS solution (2% w/v) was prepared and mixed with PVA in different ratios of 30:70 and 20:80. For fabrication of $\text{Fe}_3\text{O}_4/\text{CS}/\text{PVA}$, 500 ppm of prepared Fe_3O_4 was directly mixed with CS/PVA. Also, $\text{Fe}_3\text{O}_4/\text{GA}/\text{CS}$ and $\text{Fe}_3\text{O}_4/\text{TPP}/\text{CS}$ solutions were mixed with PVA at ratio of 30:70 and electrospun. Namely CS/PVA, $\text{Fe}_3\text{O}_4/\text{CS}-\text{PVA}$, $\text{Fe}_3\text{O}_4/\text{GA}/$

CS/PVA, and Fe_3O_4 /TPP/CS/PVA were fabricated by means of electrospinning. The polymer solution was delivered at a constant flow rate of 8 ml/h connected to a high-voltage power supply. Upon applying a high voltage (18 kV), a fluid jet was ejected from the capillary. The distance of collector from syringe was set to 15 cm as the jet accelerated towards a grounded collector, the solvent evaporated, and a charged polymer fiber was deposited on the collector in the form of a non-woven fabric.

Nanofiber Characterization

The magnetite nanoparticle sample was characterized by Fourier transform infrared spectroscopy (FTIR) and field emission scanning electron microscopy (FESEM) analysis. The FESEM micrographs were taken using a FESEM, Hitachi and model S-4160. FT-IR absorption spectra of selected samples were obtained using KBr disks on a FTIR 6300. Electrospinning was performed on Electroris®, Electroris-NL, and Fanavaran Nano-meghyas Co. Iran.

Preparation of BSA-Loaded CS Nanofibers

Bovine serum albumin (BSA) was loaded into CS nanofibers by interaction onto CS-TPP absorbed on Fe_3O_4 nanoparticles. Specifically, after absorption of TPP on Fe_3O_4 nanoparticles, chitosan nanoparticles were added to cross-link via TPP coacervation, and the particle-containing solution was then mixed with solutions containing BSA at predetermined concentrations (3–10 mg). The mixed solutions were gently stirred for 60 min to allow protein adsorption on the nanoparticles. The solution was mixed with PVA and electrospun to form nanofibers. The concentration of BSA loaded into nanofibers was evaluated by Bradford assay. To this aim, surface of 1×1 cm of collected nanofibers were put into contact with 3 ml of Coomassie Brilliant Blue G-250. The absorbance value was detected UV spectrophotometer at 595 nm.

In Vitro Drug Release Experiments

Ten milligrams of BSA was dispersed into 10 g of Fe_3O_4 NPs containing PVA/CS solution with the weight ratio of 70/30, which was stirred for 24 h. The mixed solution was electrospun into nanofibers. The obtained BSA loaded hybrid nanofibers were taken off from the aluminum foil. The dried

nanofibers (8 mg) were immersed into 2 mL pH 7.4 phosphate buffer solution (PBS) at 37°C. At periodic interval, the release media was collected and another 2-mL fresh PBS solution was added. The amount of BSA was determined by measuring the adsorption at 595 nm and using calibration curves.

RESULTS AND DISCUSSION

Chitosan, poly-(D)-glucosamine is a favorable dispersant for many types of small particulates, including Fe_3O_4 magnetic nanoparticles and can be used in the preparation of fibers by electrospinning (30–33). For electrospinning, chitosan as a weak base was dissolved in acetic acid (weak acid) and the solution showed a good stability. Acetic acid evaporated completely after electrospinning which has no disadvantage in biomedical application of nanofibers. At the first stage, pure chitosan without magnetic nanoparticles was electrospun. Electrospinning of pure chitosan is limited since chitosan solutions are highly viscous which complicates its electrospinning (34). Furthermore, the formation of strong hydrogen bonds in a 3-D network prevents the movement of polymeric chains exposed to the electrical field. In order to produce nanofibers, PVA was added to chitosan solutions in ratios of 80:20 and 70:30. The microstructure measurement which is advanced image analyzer software was applied to the fabricated nanoparticles and nanofibers for size measurement. The size measurement was carried out for 10 different points in the synthesized sample and the average of those 10 measured sizes was calculated. Figure 2 shows the SEM image of nanofiber with PVA/CS ratio of 70:30 and 80:20 which shows average fiber thickness of 350 nm. The fiber thickness was decreased with the PVA/CS ratio of 80:20 with more uniform fibers. Electrospinning of chitosan nanofibers was achievable in the presence of PVA. Moreover, increasing PVA resulted in decreasing nanofibers thickness with homogenous nanofibers due to the reduced repulsive interaction between polycation groups.

Magnetic nanoparticles were added to the PVA/CS solution using three different methods. In the first method, magnetic nanoparticles were added directly to the PVA/CS solution. In the second and third methods, magnetic nanoparticles and chitosan were cross-linked using glutaraldehyde (GA) (Fig. 1e) or tripolyphosphate (TPP) (Fig. 1d) and added to PVA solution for electrospinning, respectively. Magnetic nanoparticles showed an average size of 45 nm (Fig. 3a). When glutaraldehyde or tripolyphosphate was used as cross-

Fig. 2. SEM image of electrospun CS-PVA nanofibers with **a** 70:30 and **b** 80:20 ratio of PVA to CS

Fig. 3. FESEM image of **a** Fe_3O_4 and **b** $\text{Fe}_3\text{O}_4/\text{CS}$ nanoparticles

linker to form CS layer, the average size of nanoparticle was 51 nm showing the absorbance of a layer on magnetic/CS nanoparticles (Fig. 3b).

The irreversible Schiff's base linking of aldehyde with amino group provides stability (35). The cross-linking reaction of chitosan in acetic acid proceeds with a high rate (36,37). In the case of GA, a "double-crosslinking" method was utilized to synthesize $\text{Fe}_3\text{O}_4/\text{GA}$. Firstly, GA as cross-linking agent was used to be absorbed onto the surface of Fe_3O_4 before CS loading. After CS loading, GA was used again to cross-link the adsorbed CS, so that the solvent resistance of as-obtained nanosphere would be enhanced.

The solution of $\text{Fe}_3\text{O}_4/\text{CS}/\text{GA}$ showed good stability which magnetic particles were uniformly distributed without aggregation. In this case, a uniform dispersion of magnetic nanoparticles in the nanofibers could be achieved.

On the other hand, TPP reacts with its negative site to the amide group of chitosan forming CS nanoparticles. A variety of functional molecules could be applied and absorbed onto the surface of $\text{CS}/\text{Fe}_3\text{O}_4$ particles with this method.

The chemical structure of all cross-linked nanofibers, $\text{CS}/\text{Fe}_3\text{O}_4/\text{PVA}$, $\text{CS}/\text{GA}/\text{Fe}_3\text{O}_4/\text{PVA}$, and $\text{CS}/\text{TPP}/\text{Fe}_3\text{O}_4/\text{PVA}$ prepared by electrospinning from PVA aqueous solution was investigated using SEM and FTIR.

In sample of $\text{CS}/\text{Fe}_3\text{O}_4/\text{PVA}$ which the magnetic nanoparticles were added directly to the solution, distribution of Fe_3O_4 were not uniform and aggregations were formed (Fig. 4). The size of nanofibers was found to be 300 nm, and at some points, irregular shape nanofibers were formed.

SEM image of sample $\text{CS}/\text{GA}/\text{Fe}_3\text{O}_4/\text{PVA}$ showed a complex network of tissue nanofibers. The length of each fiber was decreased, and terminal sides of nanofibers were attached to each other resulting in a complex network (Fig. 5a). This is due to the presence of GA as a strong cross-linker which has probably cross-linked CS/CS nanofibers producing a network scaffold. It is assumed that the release of functional molecules from these fibers takes place slower due to the network.

The magnetic nanoparticles were attached strongly to the nanofibers which had deformed the fibers in some point. Fibers with the average size of 150 nm and in some parts spindle shape were detected in this sample.

Electrospinning of $\text{CS}/\text{TPP}/\text{Fe}_3\text{O}_4/\text{PVA}$ resulted in complete smooth and homogenous nanofibers where the magnetic

nanoparticles were distributed uniformly without aggregation in the scaffold (Fig. 5b). The average size of nanofibers was 150 nm, and nanoparticles with smaller size compared to the other samples were detected. Applying cross-linkers enhanced the dispersion of magnetic NPs in terms of size and uniform dispersion.

The best electrospun nanofibers were obtained in sample of $\text{CS}/\text{TPP}/\text{Fe}_3\text{O}_4/\text{PVA}$ in terms of a uniform combination of nanofibers and nanoparticles. It was concluded that TPP acts as PVA in enhancing electrospinning ability of chitosan. Due to the properties of TPP in forming a linkage with functional molecules, these nanofibers are a suitable candidate for loading drugs.

The presence of PVA, CS, GA, TPP and Fe_3O_4 in the composite fibers was confirmed by FTIR (Fig. 6).

For pure PVA, absorption bands that are around 2940, 1660, 1430, and 1090 cm^{-1} correspond to the C-H, C=O, CH_2 and C-N groups, respectively (Fig. 6a) (38,39). In the case of Fe_3O_4 , the absorption at 585 cm^{-1} is assigned to Fe-O vibrations (40,41). The FTIR spectra of each set of fibers show a combination of the characteristic bands of PVA, CS, and Fe_3O_4 , confirming that PVA, CS, and Fe_3O_4 are all present in the composite materials. The cross-linking and activation with glutaraldehyde were confirmed by FTIR spectra.

Fig. 4. SEM image of electrospun $\text{Fe}_3\text{O}_4/\text{CS}/\text{PVA}$ nanofibers

Fig. 5. SEM image of electrospun **a** $\text{Fe}_3\text{O}_4/\text{CS}/\text{GA}$: PVA nanofibers and **b** $\text{Fe}_3\text{O}_4/\text{CS}/\text{TPP}$: PVA nanofibers

The peak at 1412 cm^{-1} represents the C-N bond results with a cross-linking reaction (Fig. 6b). The aldehyde groups of glutaraldehyde molecules react with the amino group of chitosan through schiff base linkage. Other peaks at 1646 and 1054 cm^{-1} may attributed to C=N and polysaccharide structure. For the electrospun sample of CS- Fe_3O_4 -PVA cross-linked by tripolyphosphate (CS/TPP/ Fe_3O_4 /PVA), absorption bands at around 1230 and 1140 cm^{-1} correspond to the P=O, group (Fig. 6c). The cross-linking with

tripolyphosphate was confirmed by FTIR spectra. The peak at 1644 cm^{-1} represents the cross-linking of tripolyphosphate molecule with the amino group of chitosan through C=N (42–46). Cross-linking of nanofiber with glutaraldehyde is associated with the absorption peak near 1090 cm^{-1} . This indicates the formation of acetal ring with increasing PVA–glutaraldehyde cross-linking reaction time. The IR spectra also show a broad O–H stretching and an alkyl C–H stretching vibration absorption around 3300 and 2900 cm^{-1} is related to cross-link of PVA nanofiber with glutaraldehyde. Moreover, the PVA O–H stretching band to higher wave number can be attributed to the hydrogen bonding among O–H groups of PVA cross-link with glutaraldehyde.

Fig. 6. FTIR spectra of **a** $\text{Fe}_3\text{O}_4/\text{CS}/\text{PVA}$, **b** $\text{Fe}_3\text{O}_4/\text{CS}/\text{GA}/\text{PVA}$ and **c** $\text{Fe}_3\text{O}_4/\text{CS}/\text{TPP}/\text{PVA}$, (chitosan/PVA, 30:70)

Fig. 7. *In vitro* release profile of BSA loaded Fe_3O_4 containing PVA/CS nanofibers (weight ratio of PVA/CS: 70/30) with different cross-linkers at 37°C and pH 7.4

To investigate the *in vitro* drug release behavior of the cross-linked PVA/CS hybrid nanofibers, BSA as a model of protein drugs was loaded into the fibers with an encapsulation efficiency of 100%. The non-crosslinked nanofibers released BSA molecules within 3 h (Fig. 7). Consequently, the purpose of sustaining and controlled drug release could not be achieved by these nanofibers. Contrast to this cross-linked, TPP nanofibers released 60% of BSA in 30 h. GA cross-linked nanofibers decreased the rate of release to a slower and low release to 30% after 30 h. This is attributed to the complex network of cross-linked GA nanofibers which has largely slowed the release rate.

There are few methods for measuring the porosity such as conventional methods using apparent density and bulk density, image analysis by FESEM and transmission electron microscopy (TEM), mercury porosimeter, and Brunauer–Emmett–Teller (BET). However till date, an accurate estimation of porosity in these grades of materials (nanofiber mat) has been a difficult task. The misleading results could be obtained via mercury porosimetry due to the mechanical deformation of the nanofibers and BET measurements lead to only specific surface area value. Also simple experiment based on liquid absorption can be inaccurate due to swelling of nanofibers in the liquid.

The FESEM result indicated GA cross-linked nanofibers have smaller pore compared to TPP nanofibers which is due to cross-linked CS-CS fibers. This will lead to a lower porosity. Due to the fact that release through diffusion is lower in these fibers, proteins were released in low concentrations in GA cross-linked nanofibers.

The release burst in the initial stage of experiments could be explained as follows: the swelling of the soluble polymers in physiological condition (pH=7.4) was inevitable although part of the NH_2 on CS chains and BSA was cross-linked together. Accordingly, BSA molecules near the surface of the hybrid fibers were diffused into the medium quickly. After that, the drug was released gradually deep into the fibers (47).

CONCLUSION

The magnetic nanoparticles (Fe_3O_4) were effectively included in cross-linked electrospun chitosan nanofibers using different cross-linkers. These nanocomposites were used for BSA loading and release applications. The composite fibers which contained Fe_3O_4 nanoparticles were dispersed relatively uniform. Sodium tripolyphosphate (TPP) and glutaraldehyde (GA) as cross-linkers had effects on the size of the obtained composites. The characterization results suggest that Fe_3O_4 nanoparticles with average size of 45 nm were successfully bound on the surface of chitosan. Bovine serum albumin (BSA) was used as a model protein which was encapsulated to form CS/BSA/TPP/ Fe_3O_4 and CS/BSA/GA/ Fe_3O_4 nanoparticles. These nanocomposites were electrospun with poly vinyl alcohol (PVA) to form cross-linked nanofibers. The composite structure was confirmed by characterization techniques, field emission scanning electron microscopy (FESEM), and Fourier transform infrared spectroscopy (FTIR). FTIR data show that these two polymers have intermolecular interaction. The best sample was considered to be TPP cross-linked nanofibers in terms of uniform and homogenous CS/PVA nanofibers with fine distribution of Fe_3O_4 nanoparticles. A controlled slow

release rate with higher amount of BSA release to 60% after 30 h was obtained. These electrospun magnetic chitosan nanocomposite fibrous networks could be used for efficient controlled release of proteins due to their magnetic properties. Hence, these magnetic electrospun chitosan nanofiber composites have great potential for biomedical applications.

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