

Mechanically Reinforced Chitosan/Cellulose Nanocrystals Composites with Good Transparency and Biocompatibility^{*}

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Abstract Chitosan/cellulose nanocrystals (CS/CNCs) composites were prepared with different contents of CNCs. Due to the homogeneous dispersion of CNCs and the strong interfacial interactions resulting from hydrogen bonding between CS chains and CNCs, the transparency of CS is well retained and the overall mechanical properties of CS are significantly improved. Furthermore, because both CS and CNCs are biocompatible materials, cell proliferation test shows that the obtained composites are noncytotoxic and can potentially meet safety requirements of biomedical applications. These advantages pave the way of potential applications of CS in the field of commercial plastics and encourage the use of CS as environment-friendly material and biomedical material.

Keywords: Chitosan; Cellulose nanocrystals; Reinforcement; Cytotoxicity.

INTRODUCTION

Chitosan (poly[β -(1-4)-2-amino-2-deoxy-d-glucopyranose]) (CS), the second most abundant natural polysaccharide after cellulose, is a non-toxic, biodegradable, biofunctional and biocompatible cationic polysaccharide produced by partial deacetylation of chitin^[1, 2]. It is a suitable material for food packing and biomedical applications, including drug delivery, tissue engineering, wound healing and various antimicrobial strategies^[3–6]. In recent years, CS has also been regarded as one of the best material sources for environmental protection^[7–9] and a candidate in response to environmental problem and energy issues^[10–12]. However, the relatively weak mechanical properties of this biopolymer (in comparison to petroleum-based polymers) restrict its usage^[8, 13]. As we know, addition of nanofillers into polymers is one of the most important techniques used to improve their properties. With this technique, significant improvement in mechanical, thermal and electrical properties has been reported. Therefore, in many reported studies, incorporation of nanofillers such as carbon nanotubes, clay, silica and graphene *etc.* has been adopted to improve the mechanical performances of CS^[13–15]. In most cases, however, these reported CS nanocomposites lost the transparency, biocompatibility and/or biodegradability of CS. Therefore, the preparation of mechanically reinforced CS nanocomposites with good transparency, biocompatibility and completely biodegradable properties is crucial to CS for its wide application.

Cellulose is the most abundant biopolymer and can be found in wood, cotton and hemp among other sources. Cellulose nanocrystals (CNCs), as a new kind of nanofiller, are highly crystalline rod-like nanomaterials isolated from cellulose fibers by acid hydrolysis^[16]. CNCs have many appealing intrinsic properties, such as

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nanoscale dimensions, high surface area, unique morphology, low density, high specific strength and Young's modulus, and very low coefficient of thermal expansion^[17–19]. Moreover, CNCs are biocompatible and biodegradable. Specially, there are abundant of hydroxyl groups on the surface of CNCs, which will be beneficial to the formation of hydrogen bonding between CNCs and polymer chains containing amidogen and/or hydroxyl, such as CS^[20]. Meanwhile, CNCs are also reported to be of great use in preparing transparent films, gels, as well as composites^[21–23], which will potentially retain the transparency of CS. Therefore, CNCs are theoretically ideal fillers to strengthen CS. However, only a few studies have been reported with this respect. In the study of Pereira *et al.*^[24], CS/CNCs nanocomposites were obtained through covalent linkage between methyl adipoyl chloride functionalized CNCs and natural CS. Obviously, this technique has a disadvantage of complexity and time consumption. In addition, due to the degradation of the CS chains during the expatiatory process and the formation of by-products during the covalent bonding, the mechanical increment of the prepared nanocomposites is still relatively low, the tensile strength of the CS/CNCs nanocomposites with 10 wt% CNCs is still lower than 60 MPa, and the obtained composites are difficult to be used in biomedical areas. In another report by Lacroix *et al.*^[25], the authors prepared CS/CNCs nanocomposites by a simple solution method. However, the CNCs used in their study are commercially supplied in dry redispersible powders. Thus, the dispersion of fillers in the CS matrix is not well and finally the 'nano-effect' of nanofillers cannot be fully brought into play. It was found that, even by incorporating 5 wt% CNCs, the tensile strength of CS was just increased by 26%, which is much lower than the theoretical value.

Thus in this research, our purpose is to better evaluate the effect of CNCs incorporation on the mechanical, transparency and biocompatible properties of chitosan-based biodegradable films by preparing CS/CNCs nanocomposites with a homogeneous dispersion of fillers, and finally to help widen the application range of CS. Here, we use a simple solution casting method to prepare the nanocomposites and the CNCs suspension used in this study are prepared by sulfuric acid hydrolysis in our laboratory in order to make sure their good dispersion in the polymer matrix.

EXPERIMENTAL

Materials

Chitosan (CS) powders dissociated from crab shell, with a deacetylation degree of 86.98% and viscosity-average molecular weight of 300000 g/mol, are bought from Zhejiang Golden-Shell Biochemical Co. Ltd. (Yuhuan, China). Glacial acetic acid and sulfuric acid (98%) are purchased from Kelong Chemical reagent plant (Chengdu, China). Cellulose filter papers are purchased from DongYang Company (Fushun, China). Cellulose nanocrystals (CNCs) with a typical length of 100–300 nm and width of 20 nm are prepared by sulfuric acid hydrolysis according to the method described in our previous work^[20]. The morphology and chemical structures of CNCs and CS are shown in Figs. 1(a), 1(b) and 1(c).

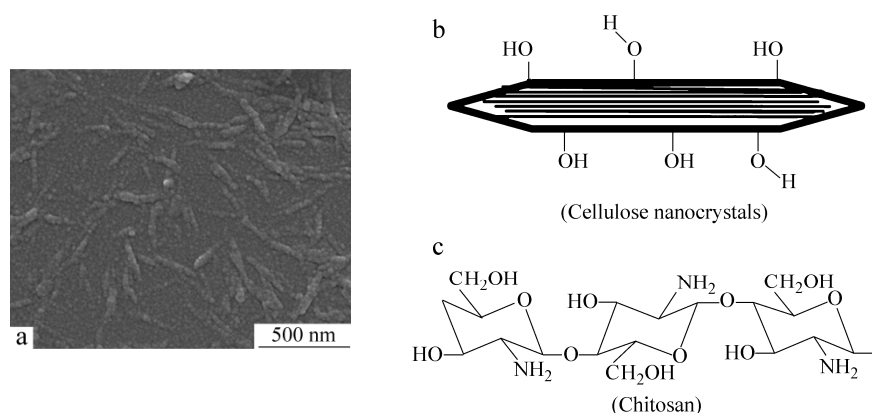


Fig. 1 (a) SEM micrograph of CNCs, chemical structures of (b) CNCs and (c) chitosan

Preparation of the CS/CNCs Composites

CS solution with the concentration of 2 wt% was prepared by dissolving CS in a 2% (V/V) aqueous acetic acid solution using a laboratory magnetic stirrer at 200 r/min for 2 h and filtered through a copper wire mesh to remove the impurity. The CNCs suspension was first diluted to 0.1% concentration by weight and ultrasonicated for 5 min. Then, the suspension was added into the CS solution dropwise, followed by stirring for 0.5 h. The obtained mixture was degassed for 3 h under vacuum. After that, the obtained CS/CNCs solutions were poured into hydrophobic glass plates, heated at 50 °C to remove the solvents. In this way, the CS/CNCs composites were produced. The obtained composites were cut into rectangular test samples using a razor blade and kept in an oven at 50 °C for 5 h to remove the remaining water before mechanical tests. The mean thickness of the composites was about 0.1 mm.

Characterization

The morphologies of cryogenically fractured surfaces of the composites were characterized by using an inspect field emission SEM (FEI Company, USA) with 5 kV accelerating voltage. Before SEM characterization, all the surfaces were coated with a thin layer of gold.

The Fourier transform infrared spectroscopy (FTIR) was used to characterize intermolecular interaction between the components in the prepared composites. The spectra were recorded with an FTIR spectrophotometer (American Perkin Elmer Co., USA) using KBr method. The spectra were recorded ranging from 4000 to 400 cm^{-1} .

The X-ray diffraction analysis (XRD) patterns of the composites were investigated using a Panalytical X'pert PRO WAXD diffractometer (the Netherlands) with Ni-filtered $\text{CuK}\alpha$ radiation at 40 kV and 40 mA in reflection mold. The angle range used was from 2° to 40° (2 θ).

The transparency of CS/CNCs composites were measured using a UV-Vis spectrometer at room temperature. The dimension of specimens was 25.00 mm in length, 10.00 mm in width and 0.1 mm in thickness.

The tensile strength, elongation and Young's modulus of the composites were measured on an Instron Universal Testing Machine 4302 (with a 500 N load cell and pneumatic side-action grips) at room temperature with gauge length of 20 mm and a tensile rate of 5 mm/min according to ASTM D882. The specimen dimension was 25.00 mm in length, 4.00 mm in width and 0.1 mm in thickness. Five parallel measurements were carried out for each sample.

Cell proliferation on the surface of CS/CNCs composites was assessed by colorimetric assay, which detected the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to formazan. Briefly, test samples with the dimensions of 10 mm × 10 mm × 0.1 mm (length × width × thickness) cut from the composites were sterilized by ethylene oxide vapor and immersed in RPMI 1640 medium with 10% FBS for 24 h. Then, the activity L929 fibroblasts were cultured on each test sample and determined after 1, 3 and 5 days. 20 mL/well of MTT solution (5 mg/mL in phosphate buffered saline (PBS)) was added in each well and the plate was incubated at 37 °C to allow the formation of formazan crystals. After 4 h, the dimethylsulfoxide (1.5 mL/well) was added to all wells after aspirating the culture medium and mixed thoroughly to dissolve the dark blue crystals at room temperature. Ten minutes later, the optical density (OD) at 492 nm was measured with a microplate reader (Model550, Bio Rad Corp. USA). All quantitative data were expressed as the means ± SD. Statistical analyses were performed using the statistic program SPSS 14.0. Probability values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Morphology of the CS/CNCs Composites

A series of SEM micrographs of the fractured surface of CS/CNCs composites are shown in Fig. 2. The SEM micrograph of pure CS is shown in Fig. 2(a) and the composites with 0.5 wt%, 3 wt% and 5 wt% CNCs are shown in Figs. 2(b), 2(c) and 2(d), respectively.

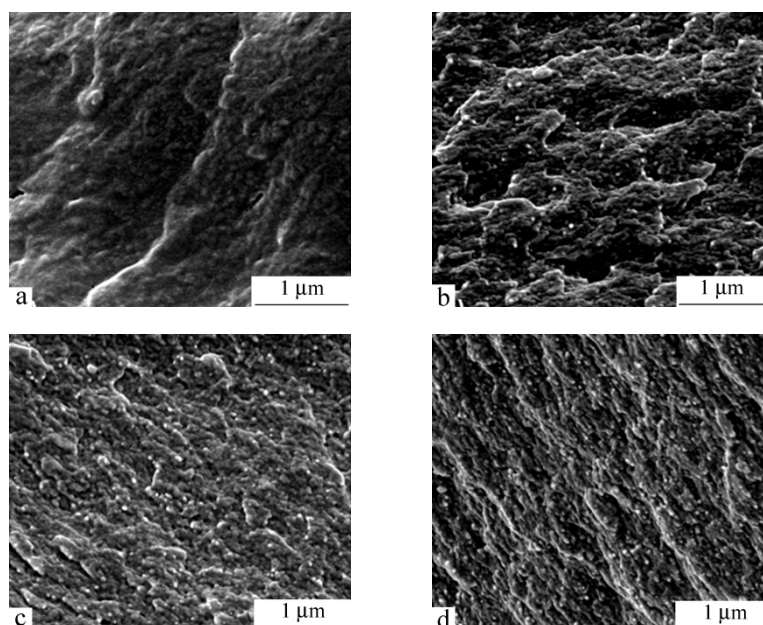


Fig. 2 SEM micrographs of pure CS (a) and the CS/CNCs composites with 0.5 wt% (b), 3 wt% (c) and 5 wt% CNCs (d)

Compared to the pure CS, some white dots appear on the fractured surface of the composites. With the increase of the content of CNCs, the quantity of white dots increases. The size of the dots is measured to be in the range of 20–30 nm by Image Pro Plus, which consists with the diameter of CNCs (as shown in Fig. 1). Therefore, it can be estimated that these white dots are the CNCs dispersed in the CS matrix^[26, 27]. The SEM micrographs of composites also indicate that CNCs are well dispersed in the matrix, because no large aggregates are observed in CS matrix even with 5 wt% of CNCs (Fig. 2d). Moreover, it is worth noting that, upon fracturing, most of the CNCs are broken rather than pulled out of the matrix. This indicates that there should be a strong interfacial adhesion between the fillers and the CS matrix. It is well known that the uniform dispersion of fillers and the strong interfacial adhesion can significantly affect the performances of polymer composites^[28]. Therefore, we characterize this interfacial interaction and then studied the mechanical performance, transparency and biological properties of the CS/CNCs composites in the following parts.

Fourier Transform Infrared Spectroscopy Analysis (FTIR)

FTIR experiments are carried out to investigate the interaction between CNCs and CS chains. Figure 3(a) shows the FTIR spectrum of neat CS and CS/CNCs composites with various amounts of CNCs. The enlarged spectrum of neat CS (Fig. 3b) exhibits the characteristic absorption bands at 3362 cm^{-1} and 3310 cm^{-1} . It is well known that the bands between 3200 and 3500 cm^{-1} are assigned to —OH and —NH_2 groups of CS. Compared with pure CS, the characteristic stretching bands corresponding to the —OH and —NH_2 groups of CS/CNCs composites shift to a lower wavenumber. For instance, the peak at 3362 cm^{-1} shifts to 3336 cm^{-1} after incorporating 5 wt% CNCs and the peak at 3310 cm^{-1} shifts to 3289 cm^{-1} under the same condition. All the red-shifts of the absorption bands indicate that hydrogen bonding, which is a strong interfacial interaction between fillers and polymer matrixes, formed between CS and CNCs^[29, 30].

X-ray Diffraction Analysis (XRD)

In order to better confirm the existence of interaction between CS and CNCs, XRD measurements of the composites are carried out. Figure 4 shows the XRD patterns of CNCs, CS and CS/CNCs composites with various amounts of CNCs. Pattern (b) shows characteristic peaks of CS at $2\theta = 12^\circ$ and 21° . The former peak is corresponded to the hydrated crystalline structure of CS, while the peak at 21° is due to the amorphous state of CS^[31, 32]. It is found that the characteristic peaks of virgin-CNCs at 14.7° , 16.6° and 22.7° observed in pattern (a)

disappeared in patterns (c) and (d). This suggests that CNCs are well surrounded and dispersed in the CS matrix, which is consistent with the SEM results shown in Fig. 2. On the other hand, it is worth noting that the incorporation of CNCs can also significantly affect the crystalline structure of CS. For example, after the addition of CNCs, CS/CNCs composites show a new characteristic peak around $2\theta = 16^\circ$ which is corresponded to the anhydrous crystalline structure of CS. This indicates that CNCs can induce the crystallization of CS. A similar phenomenon was also found in CS/graphite oxide nanocomposites. This can be explained by the hydrogen bonding which may result in a relatively ordered arrangement of the attached CS chains along the rigid template offered by CNCs (as shown in Fig. 5)^[33]. Thus, the XRD results further prove the existence of interaction resulting from hydrogen bonding. Besides, it could also be noticed that the diffraction peak of CNCs cannot be observed in the profiles of the composites. Considering that the sample preparation process could not destroy the crystalline structure of CNCs, this could be due to the relatively low content of CNCs and the low resolution of XRD used in this case.

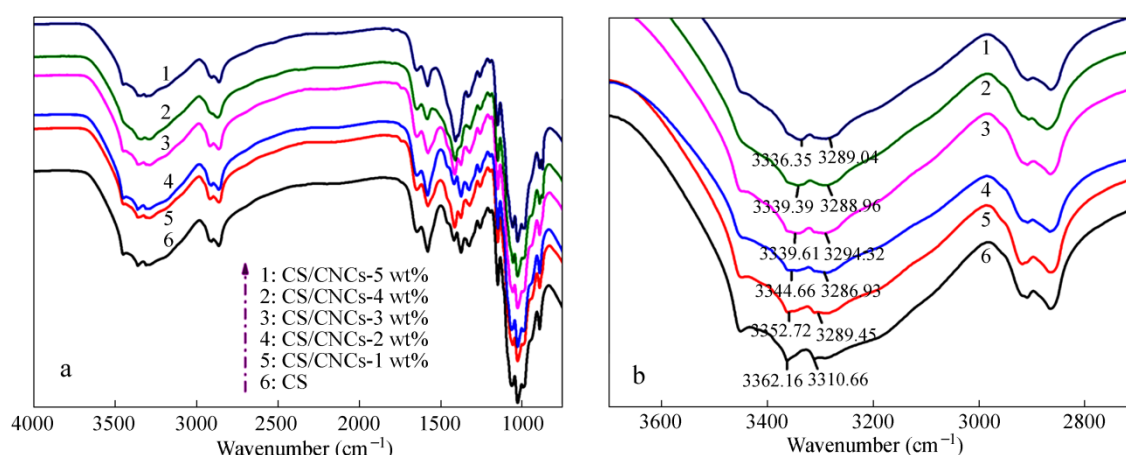


Fig. 3 (a) FTIR spectra of neat CS and CS/CNCs composites with various amounts of CNCs, (b) an enlargement of spectra in the wavenumber range between 2700 and 3700 cm^{-1} (CS/CNCs-5 wt% means CS/CNCs composite with 5 wt% CNCs.)

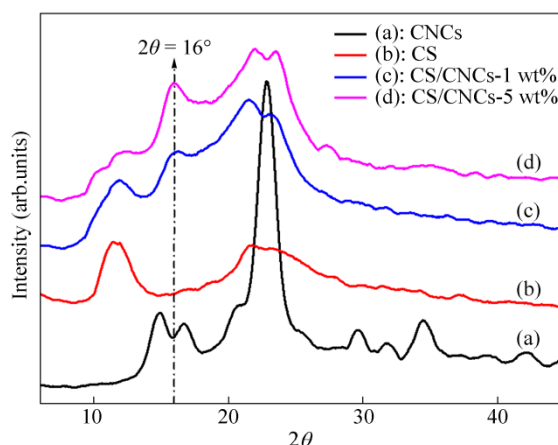


Fig. 4 XRD patterns of neat CS, CNCs and CS/CNCs composites with various amounts of CNCs (CS/CNCs-1 wt% means CS/CNCs composite with 1 wt% CNCs.)

On the basis of above characterizations, we schematically propose the possible microstructure of the obtained CS/CNCs composites (Fig. 5). As shown in Fig. 5, CNCs are homogeneously distributed in the CS matrix. Due to the large amounts of hydroxyl groups both on the surface of CNCs and CS chains, hydrogen

bonding is easily formed on the interfaces of CS and CNCs, and consequently improves the interfacial interactions significantly. Besides, the formation of hydrogen bonding between CNCs and CS chains also result in the anhydrous crystalline structure in the composites. Since uniform dispersion of fillers and strong interfacial interactions between polymers and fillers can usually affect the material performances of composites, we study the transparency, mechanical properties and cytotoxicity of the obtained CS/CNCs composites in the next part.

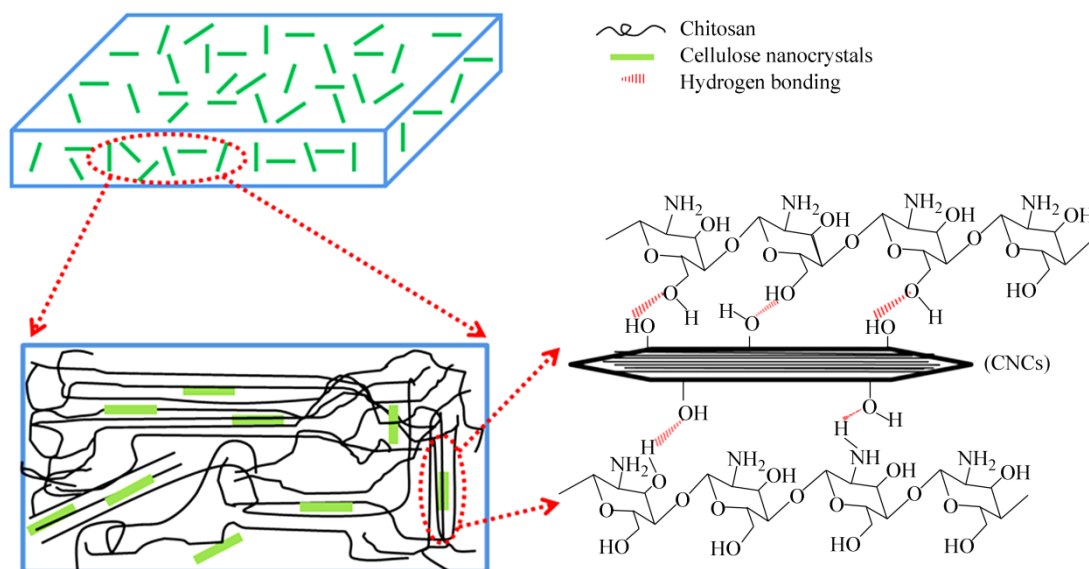


Fig. 5 Sketch of the possible microstructure of the obtained CS/CNCs composites

Transparency of the CS/CNCs Composites

The light transmittances of samples are investigated by UV-Vis spectrometer at room temperature. Figure 6(a) shows the light transmittances curves of pure CS and CS/CNCs composites with different CNCs contents as a function of wavelength. Figures 6(b), 6(c), 6(d) and 6(e) show the digital images of CS, CS/CNCs-1 wt%, CS/CNCs-3 wt% and CS/CNCs-5 wt% composites, respectively.

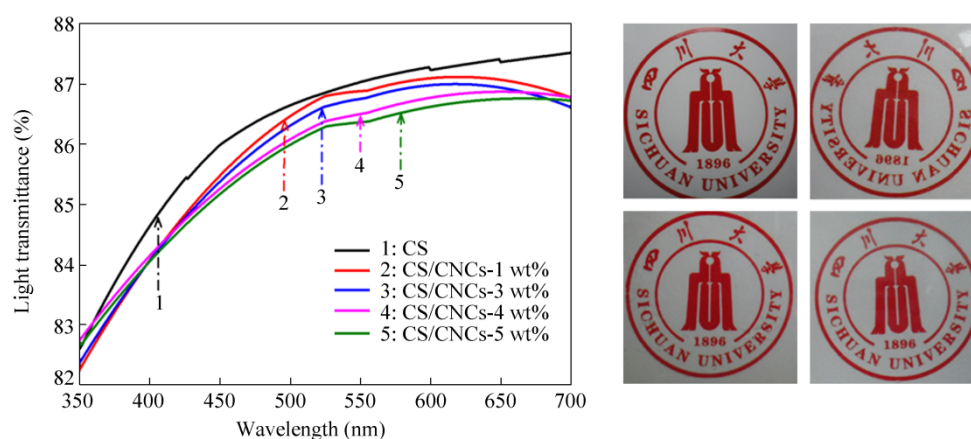


Fig. 6 (a) UV-Vis spectrometer analysis of the CS/CNCs composites films and the corresponding digital photos of the films: (b) CS, (c) CS/CNCs-1 wt%, (d) CS/CNCs-3 wt% and (e) CS/CNCs-5 wt% (CS/CNCs-1 wt% means CS/CNCs composite with 1 wt% CNCs.)

As shown in Fig. 6(a), the light transmittance of pure CS is larger than 84% and the light transmittance of all composites is also above 84% within the wavelength range of visible light (approximately 400–700 nm). Moreover, even when the content of CNCs is as high as 5 wt%, there is almost no change in transparency (as shown in Figs. 6b and 6e). These results indicate that the addition of CNCs does not affect the light transmittance of CS and all the obtained composites show high light transmittances. On the one hand, as shown in Fig. 1(a), the length of CNCs is less than 300 nm, which is much smaller than the wavelength of visible light (400–800 nm). On the other hand, CNCs are uniformly dispersed in matrix and no large aggregates are observed in the matrix even with a CNCs content of 5 wt% (as shown in Fig. 2). Therefore, the visible light can go through the composite films without any barriers, and consequently the composites show good transparency.

Mechanical Properties of the CS/CNCs Composites

The mechanical behaviors of the samples with various contents of CNCs were investigated by tensile tests at room temperature. Figure 7(a) shows the stress-strain curves of the CS/CNCs composites with different contents of CNCs. Young's modulus is measured from the initial slopes in the elastic region and shown in Fig. 7(b).

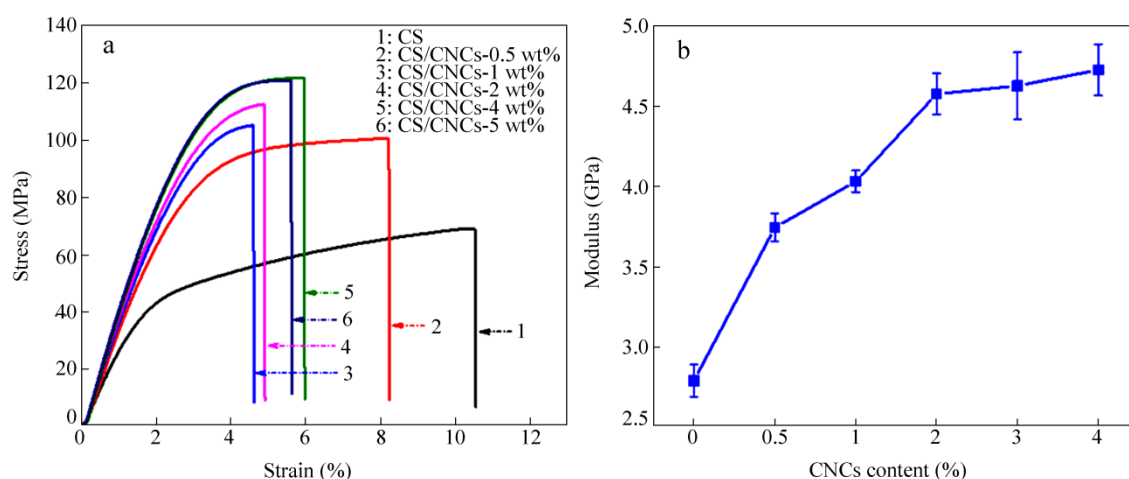


Fig. 7 (a) Typical stress-strain curve for CS/CNCs with different CNCs weight contents at room temperature and (b) the Young's modulus as function of CNCs content at room temperature (CS/CNCs-0.5 wt% means CS/CNCs composite with 0.5 wt% CNCs.)

Clearly, pure CS shows features with low tensile strength and Young's modulus. Such poor mechanical behaviors restrict the practical application of pure CS. However, the addition of CNCs leads to a significant improvement in the tensile strength and Young's modulus. For example, with the incorporation of 4 wt% CNCs, the tensile strength and Young's modulus of CS increase by 76% from 69.07 MPa to 121.82 MPa and by 65% from 2.79 GPa to 4.6 GPa, respectively. Even with only 0.5 wt% of CNCs, the tensile strength still increases by 45%. These results indicate a great reinforcement effect resulting from the nano-scale dispersed CNCs within the CS matrix. Unfortunately, it is found that it is difficult to obtain a higher mechanical strength by adding more than 4 wt% CNCs. When 5 wt% CNCs are incorporated, the Young's modulus increases slightly and the tensile strength keeps stable. This is because more water would be introduced into the mixed system of CS/acetic acid/CNCs suspension with increasing the addition of CNCs suspension, which consequently deteriorated the homogeneity of the CS/acetic acid/CNCs system. Thus, 4 wt% CNCs is considered to be the best ratio for reinforcing CS in our study.

Cytotoxicity of the CS/CNCs Composites

Since both CS and CNCs are biocompatible materials, the cytotoxicity of the CS/CNCs composites are evaluated by culturing the L929 cells on the composite films over a period of 1, 3 or 5 days at 37 °C. Cell viability based upon intact metabolic activity is monitored using an MTT assay, which was usually applied to screen the

polymer cytotoxicity for its reliability and sensitivity^[34]. As shown in Fig. 8, compared to the blank sample (cells cultured with PBS), no significant reduction in viability is found in the CS/CNCs composites. Statistical analysis results show no significant difference between the blank and the composites, which indicates that the obtained CS/CNCs composites are noncytotoxic and can meet safety requirements of biomedical applications.

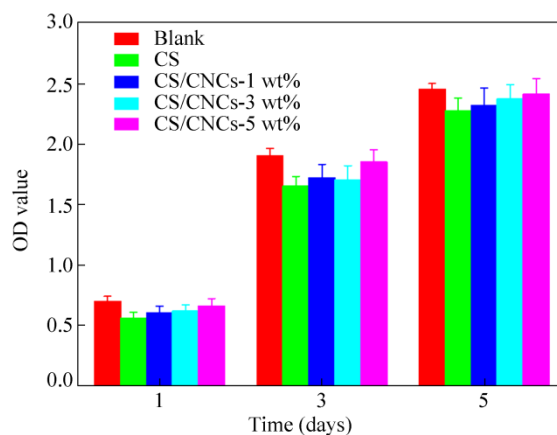


Fig. 8 Proliferation of L929 cells cultured on the composites

CONCLUSIONS

In this study, it is demonstrated that CS composites with significantly improved tensile strength and Young's modulus can be realized by incorporating a very small amount of biodegradable CNCs. This significant improvement can be ascribed to the strong interfacial interactions resulting from the hydrogen bonding between CS chains and CNCs. Due to the homogeneous dispersion of fillers, the transparency of CS is well retained. Furthermore, we prove the noncytotoxicity of the obtained CS/CNCs composites by MTT tests. In summary, these advantages clearly pave the way of potential applications of CS in the field of commercial plastics and encourage the use of CS as environment-friendly material and biomedical material.

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