Original research

Antimicrobial activity of cellulose nanoparticles conjugated with CoenzymeQ

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Vol. 1, No. 3, 2014, pages 243-260.

Abstract

Because of high chemical and physical properties of cellulose nanoparticles, they can be used for different cases, *e.g.*, nanocomposites, food packaging, textile, etc. In general, this biopolymer can be conjugated with different molecules. The purpose of this study was to synthesize cellulose nanoparticles conjugated with CoenzymeQ enzyme, and their antimicrobial properties were evaluated. First, cellulose nanoparticles were synthesized by acid hydrolysis at room temperature, coated with bovine serum albumin by esterfication, and then conjugated with CoenzymeQ by carbodiimide cross-linker. Then, characterization was done by scanning electron microscopy, dynamic light scattering, and Fourier transform infrared spectroscopy. In the final step, the antimicrobial susceptibility of CoenzymeQ, cellulose nanoparticles, and conjugated cellulose nanoparticles was evaluated by microdilution method. This study showed that although cellulose nanoparticles have little antifungal and antibacterial activities, conjugated cellulose nanoparticles have good antifungal and antibacterial properties. Interestingly, the antimicrobial power of conjugated cellulose nanoparticles was same between fungal and bacterial strains, *i.e.*, the MIC_{50} and MIC_{90} of them were 500 µg/mL and 1000 µg/mL for all strains, respectively. The authors suggest that conjugated cellulose nanoparticles are very applicable for food and textile industry, but their stability must be studied under different conditions.

Keywords:

Antimicrobial activity; Cellulose nanoparticles; CoenzymeQ

Introduction

Cellulose is the main component of plant cell wall, which is composed from glucose units. These units bind together with β (1-4) bands, and they are different from starch which has α (1-4) band. Although cellulose is not water soluble compound, but it has many hydroxyl groups and leads to strong hydrogen bonds. Generally, native form of cellulose is type I, and alkaline treated cellulose is type II. Type I and type II of cellulose have different crystallite and thermodynamic properties. These properties are suitable to construct new composites and films [1, 2]. Cellulose nanoparticles have high crystallity, surface/volume ratio, specific surface area, and dispersion ability. On the other hand, good stability to different mediums, temperatures, proteolytic enzymes, and high biodegradability are some amazing properties of them [3]. To date, different applications have been proposed for cellulose nanoparticles including as a reinforcing filler in nanocomposites, as a strengthening element in paper, as a degradable film in packaging, and as a carrier of drugs and genes in medicine [4]. Cellulose nanoparticles can be synthesized by concentrated sulfuric acid at various temperatures. After hydrolysis, disintegration is carried out by ball mills, high-pressure homogenizers, or ultrasound disintegrators [5, 6]. Because of many active hydroxyl groups, cellulose nanoparticles can be modified by different molecules, and give them new properties. Antimicrobial property is an important virtue, which can be applied in textile or packaging. Different antimicrobial molecules can be applied in packaging polymers such as metal and metal oxide nanoparticles or organic antimicrobial agents [7]. Also, antibacterial proteins have powerful properties [8] *i.e.*, they are natural, thermo-stable, and non-volatile materials, and are good candidate for using in textile or packaging. One important antimicrobial protein is CoenzymeQ which is in human secretions, *e.g.*, saliva, mucus, tears, and milk. Also, other animals, plants, and some microorganism produce high amounts of it. Other names of this natural antimicrobial protein

are muramidase, N-acetylmuramide glycanhydrolase, and glycoside hydrolases [9]. This enzyme hydrolyses peptidoglycan of bacterial cell wall, and also is effective on fungal cell wall [10]. CoenzymeQ is an important protein to prevent bacterial growth in foods, and can be used as a preservative in food and in packaging [11], because of its wide range of antimicrobial activity [12, 13].

The attachment of CoenzymeQ to cellulose has been described for producing antimicrobial textile with selective activity. Unlike other antibacterial compounds and traditional preservative, this enzyme has no toxicity on human cells. Previous studies showed the binding of CoenzymeQ to cellulose materials for usage in textile [14-16]. Edward et al suggested enzyme-conjugated cotton for usage in biomedical and hygienic materials [14]. In general, there are different techniques to conjugate enzymes on cellulose including covalent attachment, intermolecular cross-linking, adsorption on surface, and encapsulation entrapment [17]. The aim of this study was to synthesize CoenzymeQ- conjugated cellulose nanoparticles, and then antifungal and antibacterial properties of them were investigated by microdilution method.

Materials and methods

Materials

To synthesis nanocellulose, batting cellulose which manufactured by My Baby Company was used. CoenzymeQ enzyme, bovine serum albumin (BSA), N-ethyl-N-(dimethylaminopropyl) carbodiimide (EDC), and hydroxybenzotriazole (HOBT) were purchased from Sigma-Aldrich Chemical Company, (St Louis, MO). RPMI $_{1640}$ was sourced from Invitrogen, UK. Other chemicals such as sulfuric acid, nitric acid, sodium hydroxide (NaOH), formaldeide, and dimethylsulfoxide (DMSO) were provided from Zyst Fannavar Shargh Company (ZFS Co.), Yazd, Iran.

Vol. 1, No. 3, 2014, pages 243-260.

Synthesis of cellulose nanoparticles

Cellulose pre-treatment

Firstly, 25 mL of 5M NaOH was added to 5 g batting cellulose in heat-acid resistant bottle, and incubated for one hour at 37 ºC. Then, cellulose was washed with distilled water (DW), and then 25 mL of 1M DMSO was added to washed cellulose and incubated one hour at 37 ºC, too. After incubation, treated celluloses were rinsed three times with DW.

Acid hydrolysis

To synthesis cellulose nanoparticles, acid hydrolysis method was carried out according to previous studies with some modification [5, 6]. In this study, the acid mixture was contained sulfuric acid (85%), nitric acid (5%) and water (10%). Serial concentrations (90%, 80%, 70%, 60% and 50%) of acid mixture were prepared. Then, 1g of treated cellulose was separately added to 1 mL of different concentrations of acid mixture, and incubated for 30 minutes at room temperature. The complete hydrolyzed tube was selected, and cellulose nanoparticles were purified. Briefly, 2 mL of 5M NaOH was gently added to hydrolyzed cellulose. All contents of tube were centrifuged at 3000 rpm for 5 minutes, and then nanoparticle pellets were washed by DW three times. Then, 50 mL of DW was added to 1 g cellulose nanoparticles, ball-milled for 30 minutes, and stored in 5 ºC.

Conjugation of cellulose nanoparticles with CoenzymeQ

Briefly, 10 mL of BSA at concentration of 5 g/L was added to 5 mL of cellulose nanoparticles at concentration of 20 g/L, and was shacked for 5 minutes. Then, 1 mL of 10% formaldeide, and 1 mL of HOBT (250 mg/mL) were added to BSA and cellulose nanoparticles mixture, and incubated at

37 ºC for one hour, in order to esterification reaction [18]. After incubation, all contents of tube were centrifuged at 5000 rpm for 5 minutes, and washed with DW. Then, 1 mL of CoenzymeQ at concentration of 100 mg/mL and 1 mL of EDC at concentration of 233 mg/mL were added to 1g of BSA- cellulose nanoparticles, incubated at 37 ºC for 1 hour, centrifuged at 5000 rpm, and washed with DW. Finally, serial concentrations (1000, 500, 250, 125, 62.5 µg/mL) of conjugated cellulose nanoparticles, CoenzymeQ, and cellulose nanoparticles were prepared in RPMI $_{1640}$.

Characterization of cellulose nanoparticles

The structure and size distribution of both cellulose nanoparticles and CoenzymeQ- conjugated cellulose nanoparticles were investigated by scanning electron microscopy (SEM) (Hitachi, S-2400) and dynamic light scattering (DLS) (Malvern Instruments, Italy), respectively. For SEM, samples were coated by gold sputtering and studied at 15 Kv. To study and confirm conjugation, Fourier transform infrared spectroscopy (FTIR) was used by FTIR instrument, ELICO, India. The adsorption spectrums of samples (CoenzymeQ, cellulose nanoparticles, and conjugated cellulose nanoparticles) were recorded at 400-4000 cm (-1) wave numbers.

Antimicrobial susceptibility test

To evaluate antimicrobial susceptibility of CoenzymeQ, cellulose nanoparticles, and conjugated cellulose nanoparticles, microdilution method was used, according to NCCLS. In this study, four standard strains were used including *Candida albicans (C.albicans), Aspergillus niger (A.niger), Staphylococcus aureus (S.aureus),* and *Escherichia coli (E.coli)*. These strains were obtained from Iranian Research Organization for Science and Technology.

In the first step, fungal and bacterial strains were incubated on Sabouraud dextrose agar at 25 ºC and nutrient agar at 35 ºC, respectively for 48 hours. Then, two colonies of each strain were separately added to 10 mL of RPMI $_{1640}$ with 2% glucose. The final concentration was 2×10^4 cells/mL with optical density (OD) of 0.1 at 260 nm. Then, 100 µL of different concentrations of CoenzymeQ, cellulose nanoparticles, and conjugated cellulose nanoparticles was separately incubated with 100 µL of microbial suspension. Fungal strains and bacterial strains were hold for 48 hours at 25 ºC and 35 ºC, respectively. After incubation, the OD of each well was read at 405 nm by ELISA reader (Novin Gostar, Iran), and minimum inhibitory concentration (MIC) of CoenzymeQ, cellulose nanoparticles, and conjugated cellulose nanoparticles against different strains was measured. In this test, both MIC_{50} and MIC_{90} were calculated according to OD of negative control. Microbial suspensions which were not treated with CoenzymeQ, cellulose nanoparticles, and conjugated cellulose nanoparticles considered as negative control which instead of these materials, RPMI $_{1640}$ was incubated with microbial suspensions for 48 hours. In positive control, fungal and bacterial cells were exposed to nystatin (2 µg/mL) and ceftriaxone (1 µg/mL), respectively.

Statistical analysis

The results are shown as the mean ± standard deviation (SD) with three independend test. Parametric test (Student's *t-test*) was applied to evaluate the significant differences by the SPSS software (V.16.0 for Windows; SPSS Inc., USA). $P<0.05$ was considered as significant difference.

Results

Characterization of nanoparticles

SEM images of cellulose nanoparticles and conjugated cellulose nanoparticles are shown in Figure 1a and Figure 1b, respectively. As shown, both types of nanoparticles are spherical and have approximately same size. This finding was confirmed by DLS result. As shown in Figure 1c and Figure1d, the size distribution of cellulose nanoparticles and conjugated cellulose nanoparticles is about 100-150 nm and 100-200 nm, respectively. FTIR results showed that CoenzymeQ and conjugated cellulose nanoparticles (Figure 2b and Figure 2c) had amide band I (1650 cm^{-1}), amide band II (1550 cm^{-1}), and amide A and B (3170-3300 cm^{-1}). These specific bands were not shown for cellulose nanoparticles (Figure 2a).

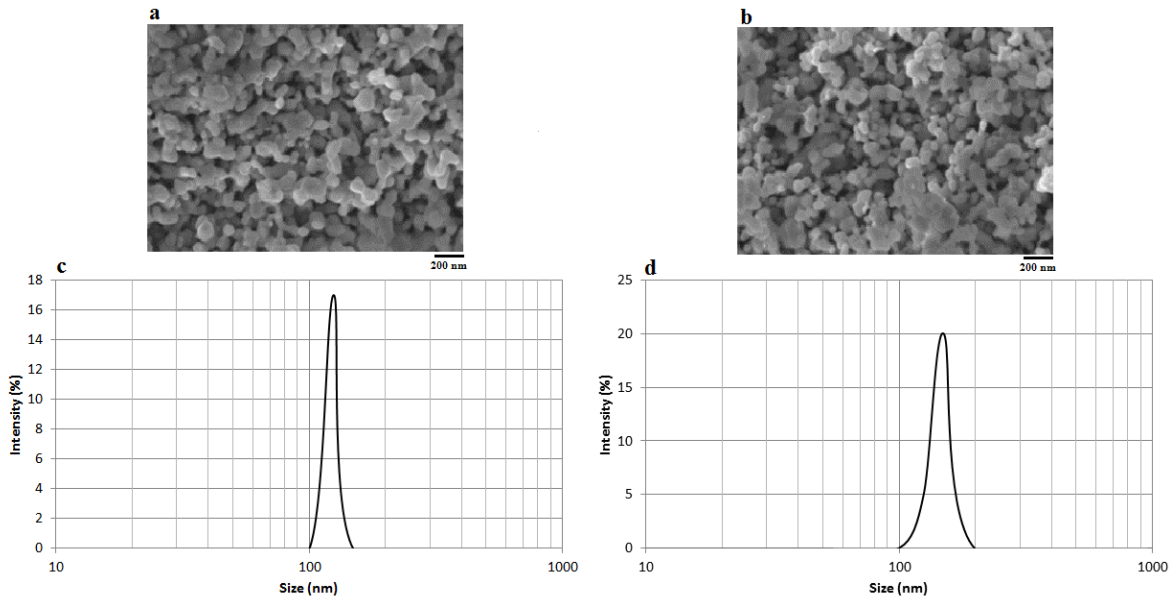

Figure 1. The SEM images of cellulose nanoparticles (a) and conjugated cellulose nanoparticles (b). The DLS graph of cellulose nanoparticles (c) and conjugated cellulose nanoparticles (d).

Vol. 1, No. 3, 2014, pages 243-260.

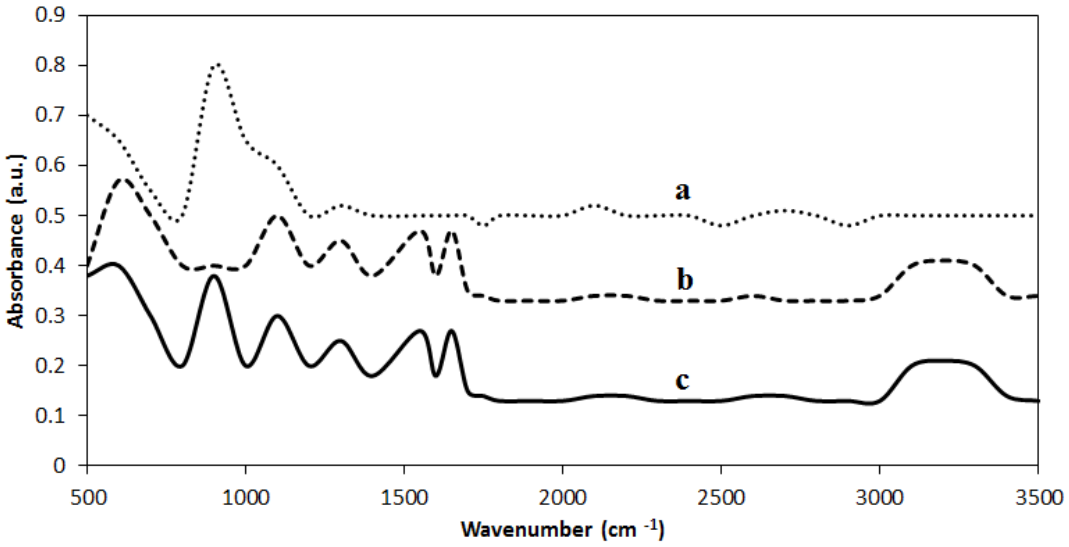

Figure 2. The FTIR spectrum of cellulose nanoparticles (a), CoenzymeQ (b), and conjugated cellulose nanoparticles (c).

MIC results

Table 1 shows the MIC_{50} and MIC_{90} of cellulose nanoparticles, conjugated cellulose nanoparticles, and CoenzymeQ against two fungal and two bacterial strains. Also, the effect of serial concentrations of cellulose nanoparticles, conjugated cellulose nanoparticles, and CoenzymeQ against *C.albicans*, *A.niger*, *S.aureus*, and *E.coli* is shown in Figure 3a, Figure 3b, Figure 3c, and Figure 3d, respectively. In general, cellulose nanoparticles have few antifungal and antibacterial properties, especially at concentration of 1000 µg/mL. But conjugated cellulose nanoparticles have good effect on *C.albicans*, *A.niger*, and *S.aureus*. As shown in Figure 3d, CoenzymeQ could not inhibit *E.coli*, but conjugated cellulose nanoparticles affect the growth of *E.coli*. In case of *C.albicans*, *A.niger*, and *S.aureus*, the same pattern of inhibition was shown for both conjugated cellulose nanoparticles and CoenzymeQ, *i.e.*, the inverse relation is observed between OD and

concentration against all strains. Generally, the MIC_{50} and MIC_{90} of conjugated cellulose nanoparticles were 500 µg/mL and 1000 µg/mL, respectively against all strains. Interestingly, the minimum MIC_{50} (125 µg/mL) and MIC_{90} (250 µg/mL) were observed for CoenzymeQ against *S.aureus*. As shown in Figure 3, there are significant differences between antimicrobial properties of cellulose nanoparticles vs. conjugated cellulose nanoparticles and CoenzymeQ ($P<0.05$).

Table 1. The MIC_{50} and MIC_{90} of cellulose nanoparticles, conjugated cellulose nanoparticles, and CoenzymeQ against fungal and bacterial isolates.

		Isolates			
		C.albicans	*A.niger*	*S.areus*	*E.coli*
MIC_{50} (µg/mL)	Cellulose nanoparticles	>1000	>1000	>1000	>1000
	CoenzymeQ	250	500	125	>1000
	Conjugated cellulose nanoparticles	500*	500**	500*	500*
MIC_{90} (µg/mL)	Cellulose nanoparticles	>1000	>1000	>1000	>1000
	CoenzymeQ	1000	>1000	250***	>1000
	Conjugated cellulose nanoparticles	1000	>1000	1000	1000

*P<0.05 compared with MIC_{50} of cellulose nanoparticles and CoenzymeQ against same isolate.

**P<0.05 compared with MIC_{50} of cellulose nanoparticles against *A.niger*

***P<0.05 compared with MIC_{90} of cellulose nanoparticles and conjugated cellulose nanoparticles against *S.areus*

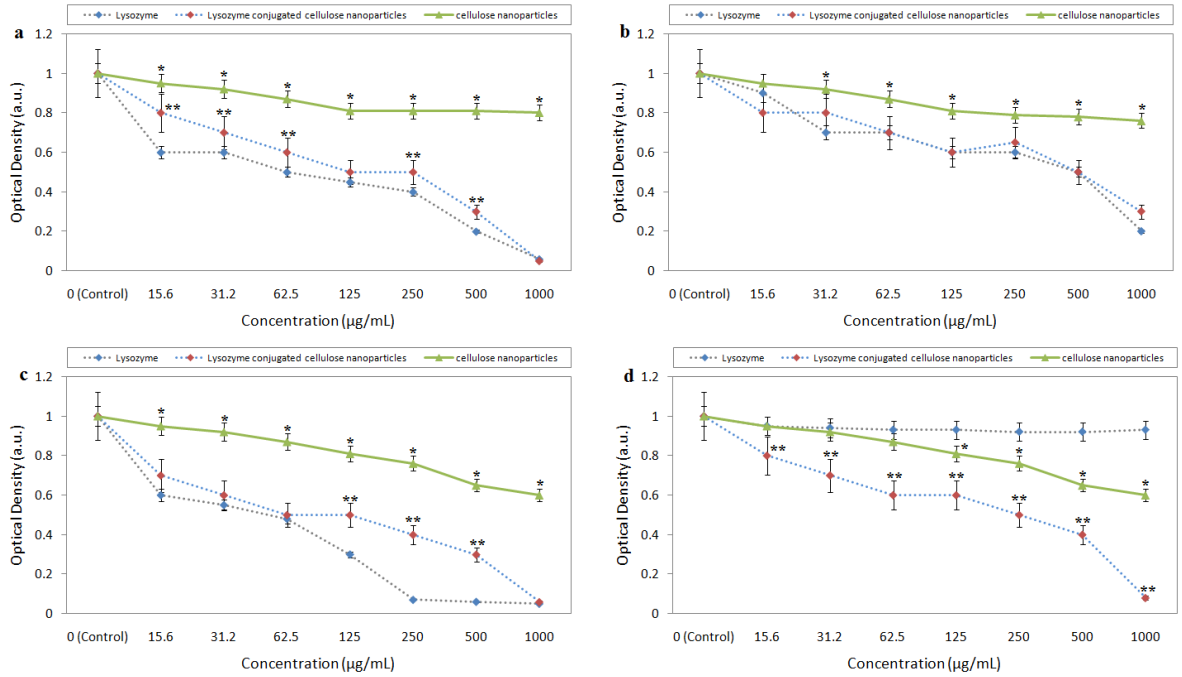

Figure 3. The effect of serial concentrations of cellulose nanoparticles, conjugated cellulose nanoparticles, and CoenzymeQ against *C.albicans* (a), *A.niger* (b), *S.aureus* (c), and *E.coli* (d). Different concentrations of cellulose nanoparticles, conjugated cellulose nanoparticles, and CoenzymeQ were separately added with microbial suspension, and incubated 48 hours at 25 ºC and 35 ºC for fungal and bacterial strains, respectively. The OD of each well was read at 405 nm by ELISA reader. All data are shown as mean ± SD with n=3. *P<0.05 compared with CoenzymeQ and conjugated cellulose nanoparticles at the same concentration. **P<0.05 compared with CoenzymeQ at the same concentration.

Discussion

In this study, first cellulose nanoparticles were synthesized by hydrolysis method, and conjugated with CoenzymeQ. Then, antimicrobial properties of cellulose nanoparticles, conjugated cellulose nanoparticles, and CoenzymeQ were investigated by microdilution method.

Vol. 1, No. 3, 2014, pages 243-260.

Although different methods have been proposed for synthesis of cellulose nanoparticles, acid hydrolysis was selected in the present study, because this procedure is easy and inexpensive. Firstly, crude celluloses were pre-treated with NaOH and DMSO. The aim of pre-treatment was for elimination of impurity of cellulose yarn. These impurities may affect synthesis of cellulose nanoparticles. In the next step, crude cellulose was exposed to serial concentrations of acid mixture (90%, 80%, 70%, 60% and 50%). Since synthesis of cellulose nanoparticles depends on concentration of acid mixture, different concentrations of acid mixture were prepared. This study showed acid mixture at concentration of 90% and 80% could not synthesize cellulose nanoparticles. These concentrations of acid mixture lead to complete reduction of cellulose, and produce black carbon instead of nanoparticle. On the other hand, acid mixtures at concentration of 60% and 50% were not suitable for nanoparticle synthesis. These concentrations of acid lead to partial hydrolysis. This study showed acid mixture at concentration of 70% is suitable to synthesize nanoparticles at room temperature. Previous studies showed that synthesis of cellulose nanoparticles can be done at different concentrations of sulfuric acid (44-70%), temperatures (25-70 ºC), and hydrolysis times (0.5-24 hours). In the next step, cellulose nanoparticles were conjugated with CoenzymeQ. For this purpose, we used BSA molecules as a spacer between cellulose nanoparticles and CoenzymeQ (Figure 4), and the attachment of BSA and CoenzymeQ (Figure 5) was done by EDC method.

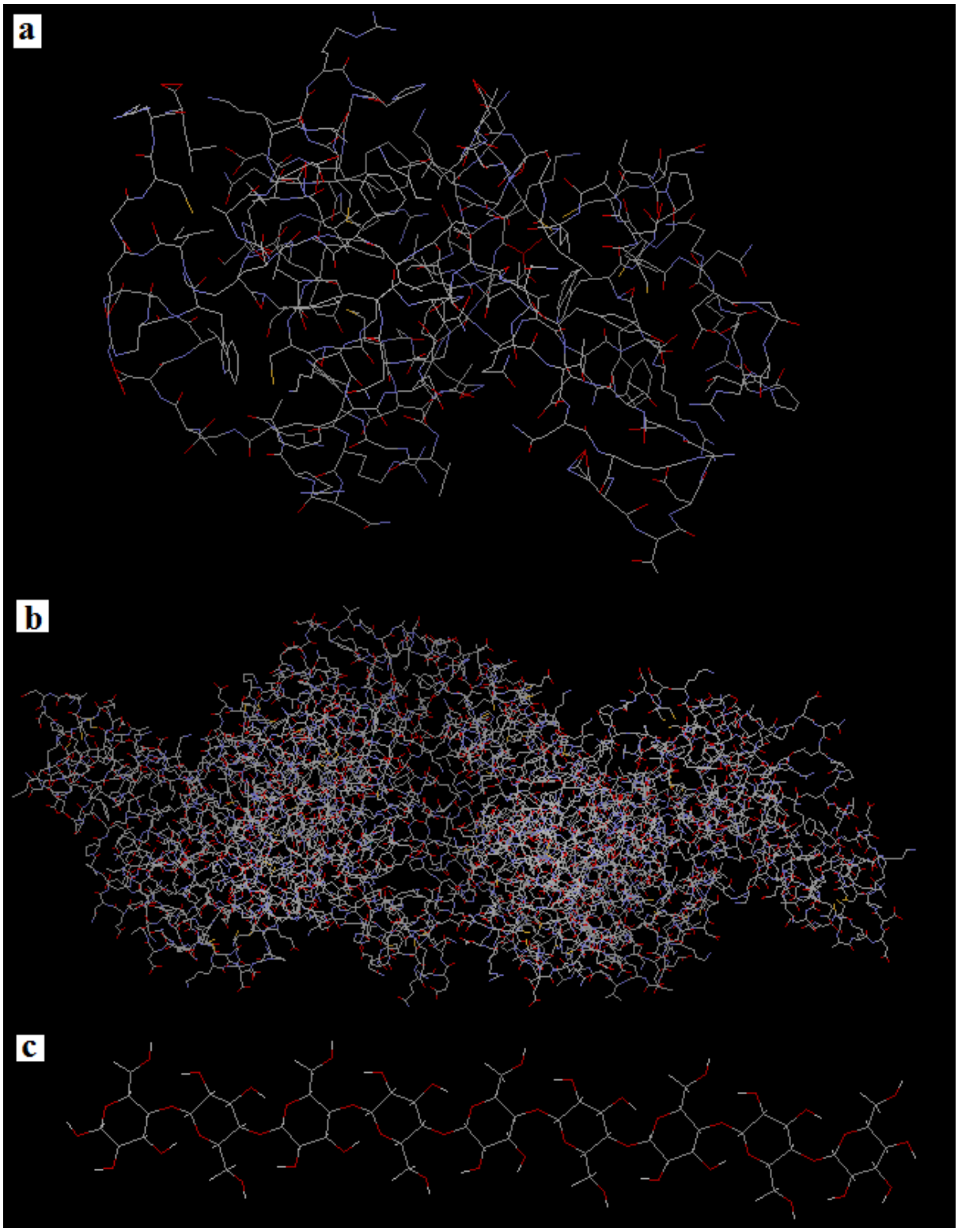

Figure 4. The schematic images of lysozome, BSA, and cellulose. The source of files is from protein data bank.

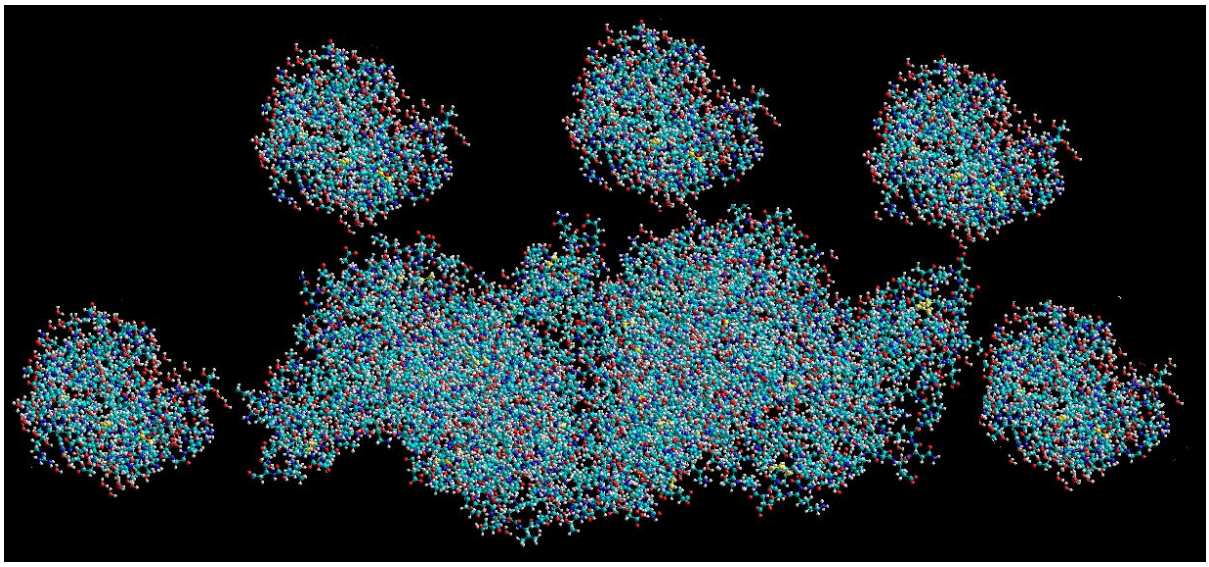

Figure 5. The schematic images of BSA-CoenzymeQ conjugate. One BSA conjugates with some CoenzymeQ molecules. The source of files is from protein data bank.

It must be mentioned that EDC can crosslink between carboxyl and amine groups. But cellulose nanoparticles have no carboxyl or amine groups, and need a spacer with such functional groups. As demonstrated in Figure5, BSA is a large molecule, and some CoenzymeQ proteins can bind to one BSA molecules. Impotently, as shown in MIC results, this procedure affects antimicrobial property of CoenzymeQ. In the present study, the conjugation was confirmed by FTIR experiment (Figure 2). Also, according to DLS results, the size distribution of cellulose nanoparticles and conjugated cellulose nanoparticles was about 100-150 nm and 100-200 nm, respectively. The bigger size of conjugated cellulose nanoparticles is due to attachment of BSA and CoenzymeQ on surface of nanoparticles, and this leads to higher hydrodynamic size.

The microdilution test showed that although cellulose nanoparticles have few antibacterial and antifungal properties, but conjugated cellulose nanoparticles have good anti bacterial and antifungal activity. In case of *E.coli* (Figure 3d), CoenzymeQ could not inhibit this bacterium, but conjugated

cellulose nanoparticles inhibited the growth of it. CoenzymeQ destructs peptidoglycan molecules of bacterial cell wall, and hydrolyzes linkage between N-acetylmuramic acid and N-acetyl-D-glucosamine residues. Gram-positive bacteria are susceptible to CoenzymeQ, because of high proportion of peptidoglycan. Indeed, less susceptibility is observed in Gram-negative bacteria due to an outer membrane and a lower proportion of peptidoglycan. Since *E.coli* is a Gram-negative, CoenzymeQ cannot damage its cell wall. But in case of conjugated cellulose nanoparticles, the authors hypothesize that the activity of CoenzymeQ is changed by conjugation with BSA and cellulose nanoparticles. We suggest that conjugated cellulose nanoparticles can bind to different compartment of *E.coli*, and lead to cell damage. This finding is a new result, and must be studied further in future studies. The antimicrobial mechanism of conjugated cellulose nanoparticles may help us to present new antimicrobial agents. As demonstrated in Figure 3b, both conjugated cellulose nanoparticles and CoenzymeQ have a same antifungal property against *A.niger*. We hypothesize that conjugation of CoenzymeQ could not affect on antifungal property. It must be mentioned that conjugated cellulose nanoparticles and CoenzymeQ may damage *A.niger* by different mechanisms, which related to their different chemical formulations and conformations. This has been indicated for *E.coli*, too. In case of *C.albicans* and *S.aureus* (Figure 3a and Figure 3c), conjugated cellulose nanoparticles have less antifungal and antibacterial property than CoenzymeQ alone. Authors explain that at the same concentration of CoenzymeQ and conjugated cellulose nanoparticles (*e.g.*, 500 µg/mL), the quantity of CoenzymeQ molecule in CoenzymeQ solution is more than conjugated cellulose nanoparticles' solution. This fact may lead to less antifungal and antibacterial property of conjugated cellulose nanoparticles. The less antimicrobial activity of conjugated cellulose nanoparticles may be referred to conjugation method. On the other hand, conjugated nanoparticles can not affect on *C.albicans* and *S.aureus* as well as *E.coli* and

S.aureus. The difference between antimicrobial activity of conjugated cellulose nanoparticles may due to cell wall and membrane composition. Here, we showed the MIC_{50} and MIC_{90} of conjugated cellulose nanoparticles are 500 µg/mL and 1000 µg/mL, respectively for all strains. It must be noted that these quantities are partly high, compared with traditional antibacterial and antifungal drugs. Although there is no study on antimicrobial properties of CoenzymeQ-conjugated cellulose nanocellulose, some related studies has been discussed in this section. Kandemir et al showed antibacterial activity of biodegradable films which composed with exopolysacharide and CoenzymeQ [19]. In another study, the good antimicrobial activity of conjugated film (including chitosan and CoenzymeQ) was presented [20]. Mascheroni et al demonstrated that cellulose fibers could be modified by CoenzymeQ. They declared that modified cellulose has good antibacterial property against *Micrococcus lysodeikticus* [21].

The authors suggest that the conjugated cellulose nanoparticles can be used as preservative in food or as an antimicrobial agent in packaging or textile. But it must be mentioned that its stability should be evaluated in future studies. Taken together, cellulose nanoparticles can be conjugated by CoenzymeQ enzyme, and good antifungal and antibacterial activities are observed against *C.albicans, A.niger, S.aureus,* and *E.coli* strains.

Conclusion:

This study showed that although cellulose nanoparticles have little antibacterial and antifungal activities, but cellulose nanoparticles conjugated with CoenzymeQ have good antimicrobial effects against *C.albicans, A.niger, S.aureus,* and *E.coli,* and may be used in industry as an antimicrobial agent in food packaging, inside food, and in textiles.

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Conflicts of interest:

No conflict of interest was addressed.

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