Preparation of CeO₂ Loaded Chitosan-Based Nanofibrous Membranes for Antibacterial Applications

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Abstract CeO₂ nanospheres with different diameters were successfully prepared and embedded in one-dimensional chitosan/polyvinyl alcohol (CS/PVA) composite nanofibers by electrospinning technique for further antibacterial evaluation. The obtained composites exhibited different antibacterial activities to Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*). We propose that CeO₂-CS/PVA nanofibrous membranes with attractive antibacterial activities witness the practical use for tissue engineering such as wound dressing.

Keywords antibacterial, CeO₂ nanospheres, chitosan/polyvinyl alcohol fibers, electrospinning

Introduction

Chitosan (poly- β -1,4-glucosamine), obtained from the N-deacetylation of chitin, is one of the most abundant and widely used natural polysaccharides, which shows good biocompatibility, biodegradability and non-toxicity.^[1-4] In addition, chitosan also exhibits various attractive biological activities, such as antibacterial activity, antifungal activity and hemostasis activity, which not only caused chitosan to receive considerable attention but also attracted many researchers to focus on the antibacterial mechanism behind it.^[5] The most accepted mechanism is that the cationic amino group of chitosan can interact with the negatively charged bacterial membrane, which will influence the permeability of bacteria and lead to the leakage of components from bacteria. The antibacterial activity of chitosan is relatively low at physiological pH, so that the antibacterial agents are added to improve the antibacterial property, especially the antibacterial nanoparticles, such as Ag,^[6,7] CuO,^[8,9] ZnO,^[10,11] and TiO₂.^[12] In order to use chitosan in tissue engineering, researchers tried to combine chitosan with other bioactive polymers to improve its mechanical property and bioactivity, such as polyvinyl alcohol (PVA), collagen, gelatin, and sodium alginate, in which PVA is a spinning aid showing excellent biocompatibility and mechanical properties.^[13,14]

Although many studies on Ag, CuO, ZnO and TiO₂ antibacterial nanomaterials are in the literature, there is a lack of works introduce the antibacterial properties of cerium oxide (CeO₂) nanomaterials. Cerium is one of the most abundant elements of rare earth elements, and CeO₂ is the most important and representative rare earth metal oxide, which has been widely used in catalyst, sensor, biomedical and cosmetics fields.^[15-18] CeO₂ has unique electron structure and rich oxygen vacancies, and the most attraction point of CeO₂ is the different oxidation states switch between Ce³⁺ and Ce⁴⁺.^[19] The high oxygen storage capacity and excellent redox property endow CeO₂ with good antibacterial activities. Li. *et al.*^[20] modified the surface of degummed silk fiber with CeO₂ nanoparticles by dip-coating method, and the obtained modified silk showed the excellent UV-shielding ability and antibacterial activity. Gomez

et al.^[21] synthesized Ce³⁺ rich CeO₂ nanoparticles, which showed good antibacterial activities against both Gram-negative and Gram-positive bacteria due to the existing Ce³⁺ on surface and rich oxygen vacancies in CeO₂ nanoparticles. Although there are some reports about the antibacterial activity of CeO₂, researchers are mainly focused on the influence of CeO₂ to the antibacterial activity of Ag/CeO₂,^[22] ZnO/CeO₂^[23,24] and CuO/CeO₂^[25] hybrid nanomaterials. The investigation about the influence of particle size to the antibacterial activity of CeO₂ is relatively rare.

In this work, CeO₂ nanospheres embedded chitosan/PVA nanofibrous membranes (CeO₂-CS/PVA) were fabricated by electrospinning method, and the influence of CeO₂ diameters to the antibacterial activity of the electrospun nanofibrous membranes was also studied. Due to the attractive antibacterial property of the prepared CeO₂-CS/PVA nanofibrous membranes, they provide a great chance to be used in biomedical applications.

Experimental

Reagents

Cerium nitrate hexahydrate (Ce(NO₃)₃·6H₂O, 99.99%, Beijing HWRK Chem. Co., Ltd.), acetic acid (CH₃COOH, AR, Tianjin Concord Tech. Co., Ltd.) and ethylene glycol ((CH₂OH)₂, AR, Tianjin Concord Tech. Co., Ltd.) were used to synthesize CeO₂ nanoparticles. Chitosan (CS, deacetylation degree $\geq$ 95%, viscosity 100–200 mPa·s, 3A Chemicals Technology Co., Ltd.), poly (vinyl alcohol) (PVA, alcoholysis degree 87%–89%, viscosity 20–26 mPa·s, Tianjin Heowns Biochemical Technology Co., Ltd.), and dimethyl sulfoxide (DMSO, AR, Tianjin Concord Tech. Co., Ltd.) were used in the preparation of electrospun precursor solution. Glutaraldehyde (50% in water, ca. 5.6 mol/L, 3A Chemicals Technology Co., Ltd.) was used for the crosslinking of CS and PVA. Trypticase soy broth (TSB), 'LB nutrient agar and phosphate buffered solution powder (PBS) were obtained from Beijing Solarbio Science & Technology Co., Ltd. for antibacterial experiments. All chemicals were used as received without any further purification. Water used in relevant experiments was ultrapure water. *Staphylococcus aureus* (S.

aureus) and *Escherichia coli* (*E. coli*) were obtained from School of Chemical Engineering and Technology, Tianjin University (Tianjin, China), and stored at -20°C until used.

Material Synthesis

Preparation of the CeO_2 nanospheres with different sizes

CeO_2 nanospheres were synthesized by solvothermal method according to the reported method.^[26] Firstly, 2.0 g of $\text{Ce}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ was added in 2 mL of ultrapure water and dissolved completely. Then, 2 mL of acetic acid and 52 mL of ethylene glycol were added into above solution and stirred for 30 min to form a uniform solution. After that, the reagent was transferred into Teflon stainless autoclave and kept at 180°C for 200 min. While cooling to room temperature, the precipitation was separated by centrifugation and washed with ultrapure water and ethanol for several times, and calcined at 400°C for 4 h to obtain yellow powder (CeO_2). CeO_2 nanospheres with different particle sizes were prepared by adjusting the ratio between water and ethylene glycol. The diameters of CeO_2 spheres at 100, 200, 300 and 500 nm were marked as CeO_2 -100, CeO_2 -200, CeO_2 -300 and CeO_2 -500, respectively.

Preparation of CeO_2 embedded CS/PVA membranes (CeO_2 -CS/PVA)

CeO_2 nanospheres were dispersed in (CS/PVA)/($\text{H}_2\text{O}/\text{CH}_3\text{COOH}/\text{DMSO}$) solution uniformly, which will be used as the precursor solution of electrospinning to prepare the CeO_2 -CS/PVA nanofibrous membranes. In a typically experiment, 0.02 g of CeO_2 -100 was dispersed in 4 mL ultrapure water by ultrasonic dispersion to form a homogeneous solution. Then, 0.32 g PVA and 0.08 g CS were added into the mixture with 4 mL prepared $\text{CeO}_2/\text{H}_2\text{O}$ solution, 80 μL CH_3COOH and 1 mL DMSO, and stirred adequately by heat treatment until the solute dissolved completely. The precursor solution was added into syringes. The distance between the needle and the acceptor was 20 cm, the voltage was 16 kV, and the propulsion speed was 0.006 mL/min. Other samples with different CeO_2 particles sizes were prepared by the same method. In order to obtain a stable membrane in water, the CeO_2 -CS/PVA nanofibrous membranes were cross-linked by glutaraldehyde vapor after electrospinning and thoroughly washed with deionized water to remove the residual glutaraldehyde.

Material characterization

The structure of CeO_2 nanospheres with different sizes were observed by transmission electron microscopy (TEM, JEOL JEM-2800, Japan) and high-resolution TEM (HRTEM) with an acceleration voltage of 200 kV and the morphology of CeO_2 -CS/PVA nanofibrous membranes were obtained from field emission scanning electron microscope (FE-SEM, JEOL JSM-7800F, Japan) with an acceleration voltage of 15 kV. The functional group present in the samples were recorded by fourier-transform infrared (FTIR, Bruker-Tensor 37, Germany) using KBr disks. The crystalline phases of the CeO_2 embedded in CeO_2 -CS/PVA electrospun nanofibrous membranes was performed on X-ray diffractometer (XRD, Rigaku Smart Lab 3 kW, Japan) using Cu-K α radiation, and the scan area was from 10° to 80° with a speed of $10^{\circ}/\text{min}$.

Antibacterial activity of CeO_2 -CS/PVA membrane

The antibacterial activities of different CeO_2 -CS/PVA nanofibrous membrane were tested against both *E. coli* and *S. aureus* using the co-culture method.^[27] In detail, 0.02 g of CeO_2 -CS/PVA nanofibrous membrane was cross-linked using glutaraldehyde and removed residual glutaraldehyde by exchanging with ultrapure water for several times. After this, the

materials were sterilized with alcohol and replaced with PBS for four times to obtain sterile membranes. Then, the membranes were added into 24-well culture plate with 990 μL of TSB and 10 μL of bacteria solution. In addition, the bacterial solution without any membrane was set as the blank group. The plate was incubated in a reciprocal shaker with a shaking speed of 160 rpm at 37°C for 12 h. After incubation, the optical density (OD) value of bacterial suspension which diluted 20 times in each well was measured using a Multiskan spectrum reader (Thermofisher Varioskan LUX, Finland) at wavelength of 600 nm. All the samples were measured in triplicate.

The progress of material synthesis and the antibacterial experiments are shown in Scheme 1.

Scheme 1 Schematic illustration of the synthesis of CeO_2 -CS/PVA nanofibrous membrane and subsequent application in antibacterial experiment

Results and Discussion

The morphology and structural properties of CeO_2 and CeO_2 -CS/PVA were thoroughly researched by FE-SEM (Figure 1) and TEM (Figure 2). Electrospun nanofibrous membranes were comprised of countless fibers, and the surface of fibers was smooth. Figures 1a and 1b were the fibers of CS/PVA with different magnification, and Figures 1c and 1d were the fibers of the CeO_2 -200 embedded CS/PVA. All these figures demonstrated that the obtained fibers still maintained the original one-dimensional morphology just with the CeO_2 nanospheres being wrapped inside the fibers, when CeO_2 nanospheres were added into precursor solution.

Figure 1 SEM images of CS/PVA nanofibrous membranes (a, b) and CeO_2 -200-CS/PVA (c, d) nanofibrous membranes with different magnifications.

CeO_2 nanospheres with different diameters were further examined by TEM, and the results were shown in Figure 2. CeO_2 nanospheres with four different sizes were prepared to investigate the influence of size of nanospheres on antibacterial activities. Figures 2a—2d were corresponded to the CeO_2 nanospheres with diameters at about 100, 200, 300 and 500

nm. The pictures showed that CeO₂ nanospheres distributed uniformly, especially when the diameter of nanospheres was smaller. The bigger CeO₂ nanospheres with 300 and 500 nm diameters were not so uniform (Figures 2c and 2d). HRTEM images (Figure 2e and 2f) clearly displayed that the CeO₂ nanospheres were assembled by many small CeO₂ nanoparticles, for which the diameter was about 5 nm. All in all, CeO₂ nanospheres were successfully inserted into nanofibrous membranes by electrospinning method, which will be used in the following antibacterial experiments.

Figure 2 TEM images of CeO₂ with different size: CeO₂-100 (a), CeO₂-200 (b), CeO₂-300 (c), CeO₂-500 (d) and partial enlarged detail of CeO₂-100 (e, f).

The XRD patterns of CeO₂-100, CS/PVA fibers and CeO₂-100-CS/PVA nanofibrous membranes were shown in Figure 3. For CeO₂ nanospheres, all the locations of diffraction peaks were consistent with the fluorite structure of CeO₂, suggesting a pure phase acquired (JCPDS 34-0394).^[28] After embedding CeO₂ nanospheres in CS/PVA nanofibers, the compound still exhibited corresponding diffraction patterns with CeO₂ compared to CS/PVA fibers. However, the intensity of peaks is weaker, which may be due to the low content of CeO₂ in the composite nanofibers. In addition, the broad diffraction peak at about 20° was attributed to glass slide. The results of XRD proved that CeO₂ nanospheres have been integrated into CS/PVA nanofibrous membranes.

Figure 3 XRD patterns of CeO₂-100, CS/PVA nanofibrous membranes and CeO₂-100-CS/PVA nanofibrous membranes.

The FTIR spectra of PVA, CS and CeO₂-100-CS/PVA membrane were shown in Figure 4. The FTIR spectrum of PVA shows a broader peak in the range of 3500–3200 cm⁻¹, which is caused by the stretching of OH. Beyond that, there is a peak at 1733 cm⁻¹^[29] assigned to the stretching vibration of the carbonyl due to the partially hydrolysis from the acetate group. In addition, the peaks located at 2924, 2850 and 1092 cm⁻¹ are attributed to C—H and C—O stretching vibration, respectively.

In the spectrum of CS, a peak observed at 1080 cm⁻¹ is ascribed to the stretching vibration of C—O—C bond, and the absorption at 1259 cm⁻¹^[30] is the amide III vibration of the combination of N—H deformation and C—N stretching vibration. The peak at 1383 cm⁻¹ is belonged to CH₂ deformation, the weak bands at 2860 and 2922 cm⁻¹ are assigned to the stretching vibration of C—H. The band at 1600 cm⁻¹ is the NH₂ group. In the FTIR of CeO₂-CS/PVA membrane, the characteristic absorption peaks of PVA and CS are included, which indicated that the addition of CeO₂ has no influence to the structure of CS/PVA nanofibers. These results confirm the successful recombination of CeO₂ nanospheres and CS/PVA nanofibrous membranes and the embedding of CeO₂ has no influence to the structure of membranes.

Figure 4 FTIR spectra of PVA, CS and CeO₂-100-CS/PVA nanofibrous membranes.

As the electrospinning nanofibrous membranes have hydrogel-like properties after being cross-linked by glutaraldehyde, it is necessary to test the swelling ability of samples in aqueous solution. The swelling ratios of CS/PVA nanofibrous membranes and CeO₂-CS/PVA nanofibrous membranes were displayed in Figure 5. It indicated that embedding different sizes of CeO₂ nanospheres into CS/PVA membranes can induce the increase of swelling ratios compared to CS/PVA nanofibrous membranes, which may be due to the disruption of the structure of the membranes by the addition water absorption. As the diameter of CeO₂ nanospheres increased from 100 to 500 nm, the swelling ratios of the membranes decreased gradually. For the same embedded weight, the numbers of embedded CeO₂-100 was much higher than CeO₂-500, resulting in more defects through the fibers and higher water absorption.

Figure 5 The equilibrium swelling ratios of the membranes after crosslinking by glutaraldehyde.

The antibacterial activity of CS/PVA nanofibrous membranes and CeO₂-CS/PVA nanofibrous membranes were studied extensively to explore their utility as a potential antibacterial material. The antimicrobial properties of the membranes against *S. aureus* and *E. coli* were tested by the method of co-culture. *S. aureus* and *E. coli* were incubated with different membranes in TSB at 37 °C for 12 h, and the relevant results are shown in Figure 6a. In this part, the bacterial

suspensions without any antibacterial material were marked as blank and the bacterial suspensions with CS/PVA nanofibrous membranes were marked as control. As shown in Figure 6a, the suspensions with CeO₂-CS/PVA nanofibrous membranes were much clearer than the blank and control groups, and the most transparent bacterial suspensions for *S. aureus* and *E. coli* were the suspensions with CeO₂-500-CS/PVA nanofibrous membranes and CeO₂-100-CS/PVA nanofibrous membranes, respectively. More visualized antimicrobial results were obtained by subsequent spread plate method. Figure 6b shows the results of the agar plates, which were coated with the diluted co-cultured antibacterial suspensions and incubated at 37 °C constant temperature for 12 h, and the results were the same as the turbidity of the suspensions mentioned above. To quantitatively investigate the antibacterial activity of the different antibacterial materials, the absorbance of suspensions after co-culture was detected at 600 nm by the Multiskan spectrum reader. From Figure 6c, we can know that the best antibacterial materials for *S. aureus* and *E. coli* were CeO₂-500-CS/PVA nanofibrous membranes and CeO₂-100-CS/PVA nanofibrous membranes, respectively. Although all CeO₂-CS/PVA nanofibrous membranes have antibacterial activity for two typical bacteria, the antibacterial activities of CeO₂-CS/PVA nanofibrous membranes embedded with different size of CeO₂ nanospheres diverse as the bacteria types change, which may be due to the different cell membrane structure between them.

Figure 6 The results of antibacterial activity assay. The photograph of the bacterial growth inhibition results after the co-culture for 12 h (a), the photograph of spread plates that the suspension and agar were incubated at 37 °C for 12 h (b), antibacterial effects of PVA/CS and CeO₂-PVA/CS nanofibrous membranes against to *S. aureus* and *E. coli* (c).

The co-cultured materials were dehydrated gradiently and characterized by SEM. The results are shown in Figure 7. The figures show that after the co-culture of bacteria, the fibers still retain their original morphology, and CeO₂ nanospheres do not fall off. Therefore, the composite materials we prepared have certain stability.

Conclusions

In summary, we synthesized CeO₂ nanospheres embedded CS/PVA nanofibrous membranes (CeO₂-CS/PVA) which show attractive antibacterial activity to both *S. aureus* and *E. coli*. The CeO₂ nanospheres with different sizes distribute uniformly in

Figure 7 SEM images of CeO₂-100-CS/PVA nanofibrous membranes (a, b) after the co-culture.

fibers and show diverse inhibition actions to the growth of different kinds of bacteria. The CS/PVA nanofibrous membranes embedded with CeO₂-500 showed the best antibacterial activity to *S. aureus*, while for *E. coli* the CeO₂-100 embedded CS/PVA nanofibrous membranes revealed the best antibacterial activity, which may be due to the different structure of the bacteria's cell membranes. The addition of CeO₂ nanospheres enhanced the antibacterial activity of CS/PVA nanofibrous membranes because of the special properties of CeO₂, which can produce reactive oxygen species to kill the bacteria. Electrospinning the CeO₂ nanosphere into membranes is beneficial for the attachment of bacteria on the surface of membranes and is also convenient for the post-treatment during antibacterial experiments. Overall, the CeO₂-CS/PVA nanofibrous membranes represent a class of CeO₂-based antibacterial materials, for which the antibacterial activity is prompted compared to pure CS/PVA nanofibrous membranes, and shows a great potential application in wound healing in the future.

Acknowledgement

The financial support from the China National Funds for Excellent Young Scientists (Nos. 21522106 and 21971117), the National Key R&D Program of China (No. 2017YFA0208000), 111 Project (No. B18030) from China, and the Fundamental Research Funds for the Central Universities, Nankai University (No. 023-63191410) is gratefully acknowledged.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Received August 1, 2019

Accepted September 21, 2019