Post-Print Properties of Nanocellulose/Cationic Polymer Composite Three-Dimensional Tissue Engineering Scaffolds

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Abstract

Nanocellulose was composited with high-cationicity acrylic and vinylamine polymers respectively to prepare three-dimensional porous tissue engineering scaffolds, the morphology and structure of which were observed via SEM, and the effects of the relative molecular mass and dosage of the high-cationicity acrylic and vinylamine polymers on the scaffold pore structure were investigated; image processing software was used to process the collected cross-sectional SEM images of the scaffolds, scaffold porosity data were obtained through statistical calculations, and a novel method for rapid determination of scaffold porosity based on SEM images was established; finally, the water retention value of the scaffolds was measured. The results showed that the porosity of the nanocellulose three-dimensional tissue engineering scaffolds prepared in this study was all greater than 90%. The porosity obtained by image processing was close to that measured by the liquid displacement method, with an error of less than 5%, indicating that the porosity determined by the image processing method is reliable. The nanocellulose three-dimensional tissue engineering scaffolds exhibited high water retention values (>200%). Adjusting the type and dosage of cationic polymers could regulate their porosity and water retention value, resulting in nanocellulose three-dimensional tissue engineering scaffolds suitable for tissue cell culture.

Full Text

Preamble

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Performance of 3D Tissue Engineering Scaffolds of Nanocellulose/High Cationic Polymers Composite

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Abstract
Three-dimensional (3D) tissue engineering scaffolds were prepared by compounding nanocellulose with polyacrylic cationic polymer and polyethylene amine cationic polymer respectively. The structural morphology of the scaffolds was characterized by scanning electron microscopy (SEM). The influence of the relative molecular mass and dosages of the polymers on the pore structure of the scaffolds was investigated, while a new method for fast measuring the porosity of the scaffolds was established based on SEM image processing. The water retention value (WRV) of the scaffolds was also measured. Results show that the porosity of all the nanocellulose 3D tissue engineering scaffolds is larger than 90%. The porosity value obtained by the new image processing method is close to that measured according to Archimedes principle with a difference less than 5%, which indicated that this method was reliable. All the 3D scaffolds have high WRV (>200%). Both the porosity and WRV of the 3D scaffolds can be adjusted by varying the species and dosage of polymers. Therewith the nanocellulose 3D tissue engineering scaffolds may optionally be prepared to meet the requirement for tissue and cell culture.

Keywords composites, nanocellulose, tissue engineering scaffold, image processing

Introduction

Tissue engineering, a frontier discipline of life sciences in the 21st century, applies fundamental principles of life science and engineering to develop biological substitutes that restore, maintain, or improve the function of damaged tissues or organs. This field has already seen clinical applications in bone, cartilage, nerve, blood vessel, skin, and gastrointestinal and urogenital tissues. Current tissue engineering has evolved to using three-dimensional scaffolds for cell culture and transplantation into the body to form new tissues. These scaffolds support cell proliferation, migration, growth, and differentiation while guiding the formation of desired tissues.

Nanocellulose derived from plant biomass is a rod-like fiber with diameter less than 100 nm and length ranging from tens to hundreds of nanometers, exhibiting excellent biocompatibility. Nanocellulose possesses unique properties in surface morphology and functional group interactions, making tissue engineering scaffolds based on this material exhibit high internal surface area to volume ratios that provide spatial support for favorable cell distribution. With a diameter

on the same order of magnitude as collagen fibers in the extracellular matrix, nanocellulose can be used to biomimetically construct the skeleton of extracellular matrix, showing promising prospects in biomedical applications.

Porous morphology, porosity, and water retention value are critical indicators for evaluating three-dimensional scaffolds used for cell culture in tissue engineering. Throughout the development of tissue engineering scaffolds, pore structure has been an important influencing factor. Pores must be interconnected to meet the requirements of cell growth and nutrient transport. Appropriate surface porosity can enhance mechanical connection between the scaffold and surrounding host tissue, providing necessary mechanical stability at critical interfaces. Therefore, rapid and accurate porosity measurement is of great significance. Current methods for porosity measurement mainly include liquid displacement, mercury intrusion porosimetry, and electron microscopy image analysis. Liquid displacement requires density measurement with cumbersome procedures and large errors, while mercury intrusion operates under high pressure that may compress and damage the sample structure, also introducing errors. Thus, establishing a rapid and simple porosity measurement method is extremely important. Tang et al. used SEM images to calculate the apparent porosity of soil particles and studied the influence of different thresholds and analysis region sizes on porosity. Zhou et al. proposed a characterization method for nanofiber membrane porosity based on digital image processing technology, with calculated porosity data closely matching density method results. Water retention is also a crucial parameter for tissue engineering scaffolds because physiological phenomena such as absorption of physiological fluids, transport of nutrients, and removal of metabolites occur within the scaffolds. Depan et al. studied the water retention performance of bone tissue engineering scaffolds and found that the scaffolds could retain more water than their own weight, which is beneficial for biomedical applications. Seniz Ucar et al. investigated the water retention performance of chitosan scaffolds prepared by wet spinning for biological delivery systems and found that these tissue engineering scaffolds could retain water equivalent to their own weight, which is essential for maintaining viscoelastic properties.

In this study, nanocellulose was used as raw material and compounded with high cationic degree polyacrylic and polyethylene amine polymers respectively to prepare nanocellulose three-dimensional tissue engineering scaffolds via freeze-drying. SEM was used to observe the morphology and structure, and the effects of polymer relative molecular mass and dosage on scaffold pore structure were investigated. To establish a rapid porosity measurement method, Matlab software image processing technology and statistical calculation functions were used to determine scaffold porosity and compare the results with liquid displacement measurements. The porosity and water retention value of scaffolds were regulated through control of polymer relative molecular mass, dosage, and scaffold forming conditions, aiming to prepare nanocellulose three-dimensional tissue engineering scaffolds with high porosity, high water retention value, certain mechanical strength, and suitability for tissue cell culture.

1 Experimental Methods

1.1 Materials

Bleached eucalyptus pulp with a beating degree of 25° SR and α-cellulose content of 86.8% was used. Polyacrylic cationic polymer with relative molecular weight of 6 million (designated as polymer A) and polyethylene amine cationic polymer with relative molecular weight of 3 million (designated as polymer B) were employed, both with cationic degree of 10.

1.2 Sample Preparation

Oxidized cellulose was prepared from bleached eucalyptus pulp using a neutral TEMPO-mediated oxidation system. The oxidized cellulose was dispersed in deionized water to prepare a 0.5% (mass fraction) suspension, which was then homogenized using a Nano De-BEE high-pressure homogenizer. The suspension was first homogenized 4 times at 30 Kpsi using a D8 nozzle, then 8 times at 40 Kpsi using a D5 nozzle to obtain nanocellulose aqueous dispersion, which was stored at 4°C.

Polymers A and B were dissolved in deionized water to prepare 0.05% (mass fraction) solutions, which were then mixed with 0.5% nanocellulose at different volume ratios. The mixtures were ultrasonicated for 80 s to ensure uniform dispersion, dropped into polytetrafluoroethylene templates (15 mm diameter, 7 mm depth), pre-frozen at -20°C for 12 h, and then freeze-dried for 15 h at -50°C to obtain three-dimensional porous tissue engineering scaffolds, which were stored in sealed bags.

1.3 Testing and Characterization

Nanocellulose aqueous dispersion was dropped onto mica sheets, vacuum-dried for 4 h, and observed using a Veeco Di Multimode SPM multi-mode scanning probe microscope in tapping mode. Scaffolds were sectioned with a sharp blade at room temperature while in polytetrafluoroethylene templates (15 mm diameter, 4 mm depth). The smooth sections were pasted onto metal sheets with conductive adhesive, sputter-coated with gold, and observed using a HITACHI S-4300 scanning electron microscope (SEM).

Porosity was measured using the liquid displacement method. At constant temperature of 30°C, a pycnometer was filled with anhydrous ethanol and weighed as W1. A sample of mass Ws was added to the pycnometer, and vacuum was applied cyclically until ethanol filled the sample pores. The pycnometer was refilled with anhydrous ethanol and weighed as W2. The ethanol-saturated sample was removed, and the remaining ethanol with pycnometer was weighed as W3. Four parallel samples were measured for each formulation and averaged. The scaffold porosity was calculated as:

$$\text{Porosity} = \frac{W2 - W3 - Ws}{W1 - W3} \times 100\%$$

Using Matlab software image processing functions, an appropriate threshold was selected to convert SEM images into binary black-and-white photos, where white represented pore walls and black represented pores. The resulting structure images most closely resembling the original structure were obtained. Based on Matlab' s mathematical operation functions, statistical calculations were performed according to the area values of different color regions in the binary photos to obtain scaffold porosity.

The water retention value (WRV) of scaffolds was measured according to the WRV determination method for fibers. A certain mass of sample was placed in a 200-mesh filter, soaked in water at 20°C for 10 min, then placed in a centrifuge tube and centrifuged at 4500 r/min for 10 min at 20°C. The wet weight W1 was measured after centrifugation, and the absolute dry weight W2 was measured after oven-drying for 4 h. Four parallel samples were measured for each formulation and averaged. The scaffold WRV was calculated as:

$$\text{WRV}(\%) = \frac{W1 - W2}{W2} \times 100\%$$

2 Results and Discussion

2.1 Morphology of Nanocellulose

[Figure 1: see original paper] shows the AFM height image of nanocellulose. The nanocellulose exhibits short rod-like structural characteristics with some ellipsoidal particles. The aspect ratio is approximately 10, with most fiber lengths around 100 nm and a few reaching 200 nm, while all fiber widths are less than 10 nm. Due to the enormous specific surface area effect, nanocellulose tends to aggregate and intertwine to form dense structures.

2.2 Effect of Polymer Dosage on Scaffold Morphology

To investigate the influence of polymer dosage on scaffold morphology, two different types of cationic polymers with different main chain structures and relative molecular masses were added to the scaffolds. Polymer A is a polyacrylic cationic polymer with relative molecular mass of 6 million, while polymer B is a polyethylene amine cationic polymer with relative molecular mass of 3 million. Both polymers contain amino-based cationic groups.

[Figure 2: see original paper] presents SEM images of scaffolds with different amounts of cationic polymer A, where Figure 2a shows the pure nanocellulose scaffold without cationic polymer. [Figure 3: see original paper] shows SEM images of scaffolds with different amounts of polymer B. Figure 2a indicates

that the pure nanocellulose scaffold formed closed-pore structures on the surface with poor pore connectivity, which is unfavorable for physiological nutrient transport and metabolic waste removal, and thus detrimental to cell growth and development. Therefore, this pure nanocellulose scaffold does not meet tissue engineering requirements.

From the surface morphology images in Figure 2, nanocellulose scaffolds prepared with polymer A formed porous network structures containing both large pores of 40-300 m diameter and micropores of several micrometers. The pore morphology and quantity were related to the dosage of polymer A. As the dosage of polymer A increased, the number of surface pores decreased, closed-pore phenomena increased, and pore compactness improved. At a polymer A mass fraction of 0.79%, the surface pores were most regular and orderly, with the least closed-pore formation.

From the cross-sectional images in Figure 2, nanocellulose and polymer formed interconnected porous structures within the scaffolds, with observable fiber network structures that are highly beneficial for cell growth. As polymer A dosage increased, the number of internal micropores decreased and pore arrangement regularity deteriorated.

From the surface morphology in Figure 3, nanocellulose scaffolds prepared with polymer B also exhibited porous network structures, but with smaller pore sizes —larger pores of 50-200 m and micropores of several micrometers. Similar to polymer A, closed-pore phenomena occurred to varying degrees on the surface of nanocellulose scaffolds with polymer B, being most severe at a polymer B mass fraction of 7.41%.

The cross-sectional images in Figure 3 indicate that nanocellulose scaffolds with polymer B formed vertically interconnected channels with good pore connectivity. The scaffold morphology varied significantly with polymer B mass fraction. At mass fractions of 0.79% and 7.41%, more micropores and severe closed-pore phenomena were observed, while at 1.57% and 3.85%, fewer closed pores, more large pores, better pore size distribution uniformity, and better internal pore connectivity were achieved. The vertically interconnected porous structures formed in the scaffold architecture are favorable for cell adhesion, growth, and nutrient transport.

2.3 Porosity Measurement

2.3.1 Liquid Displacement Method Porosity was first measured using the liquid displacement method. [Figure 4: see original paper] shows the relationship between porosity and polymer dosage for scaffolds prepared with cationic polymers A and B. For polymer A, porosity generally decreased with increasing mass fraction, dropping from 97.42% to 93.70% as the mass fraction increased from 0.79% to 7.41%, reaching the minimum at 7.41%. For polymer B, the porosity variation differed. As polymer B mass fraction increased from 0.79% to 1.57%, porosity increased from 93.96% to 96.76%, but further increasing

polymer B dosage caused porosity to decrease from 96.76% to 90.77% as mass fraction rose from 1.57% to 7.41%.

Comparing the porosity of scaffolds prepared with both cationic polymers in Figure 4, the minimum porosity occurred at 7.41% mass fraction for both. This may be because higher cationic polymer mass fraction creates more binding sites with fibers, resulting in denser nanocellulose composite scaffold structures and thus lower porosity. Regardless of the mixing ratio, all scaffolds prepared with both polymers exhibited porosity exceeding 90%. Since scaffold porosity should preferably be greater than 80% for tissue engineering applications, these polymer-modified scaffolds meet the requirements for tissue cell growth and proliferation.

2.3.2 SEM Image Processing Method Matlab software was used to convert SEM images into binary black-and-white photos. The cross-sectional images from Figures 1 and 2 were selected for processing, with results shown in [Figure 5: see original paper]. Statistical calculations based on area values of different color regions in the binary photos yielded porosity values. During statistical calculation, images in Figure 5 were divided into pores and pore walls based on black and white colors, as illustrated in [Figure 6: see original paper], where white represents pore walls and black represents pores. Porosity values were obtained based on the area occupied by both parts.

The statistical results are shown in [Figure 7: see original paper]. For nanocellulose scaffolds with polymer A, porosity decreased with increasing polymer A mass fraction, but the overall decrease was small. When polymer A mass fraction increased from 0.79% to 1.57%, porosity decreased noticeably from 94.98% to 93.43%. With further increases in polymer A mass fraction, porosity changed insignificantly, reaching 93.70% at 7.41% mass fraction. For scaffolds prepared with polymer B and nanocellulose, porosity variation with polymer dosage showed no regular pattern. Within the 0.79%-7.41% mass fraction range, scaffold porosity ranged between 90.67%-94.93%, with the maximum porosity of 94.93% occurring at 1.57% mass fraction.

2.3.3 Comparison of Methods To explore the feasibility of image processing technology for porosity measurement, porosity values obtained by liquid displacement and image processing methods were compared and analyzed for scaffolds prepared with both polymers A and B, as shown in [Figure 8: see original paper], [Figure 9: see original paper], , and .

[Figure 8: see original paper] and show that the porosity of nanocellulose/polymer A composite scaffolds measured by both methods had some deviation but not substantial, with an average absolute error of 1.88% and average relative error of 2.02%. The smallest error occurred at 1.57% polymer A mass fraction, while the largest error occurred at 7.41%, with absolute error of 3.33% and relative error of 3.68%, where image processing results were higher than liquid displacement results. However, all deviations were less than

5%, indicating that image processing characterization of nanocellulose/cationic polymer A composite scaffold porosity is relatively accurate.

Data in [Figure 9: see original paper] and show that porosity values of nanocellulose/polymer B composite scaffolds measured by both methods were also very close, with consistent variation patterns relative to polymer B mass fraction. The maximum absolute error was 1.83%, maximum relative error was 1.89%, average absolute error was 1.50%, and average relative error was 1.6%. Compared with polymer A composite scaffolds, image processing evaluation of nanocellulose/cationic polymer B composite scaffold porosity was more accurate.

The errors between image processing and liquid displacement methods may arise from: (1) SEM images themselves may have errors due to refraction and reflection during electron microscopy scanning of scaffold surfaces, causing partial image distortion; (2) subjective human factors in threshold selection during SEM image processing. Despite these errors, porosity values measured by both methods were very close, with maximum absolute error of 3.33% and maximum relative error of 3.68%. Particularly, image processing technology offers greater convenience and speed. Therefore, using image processing technology to rapidly determine scaffold porosity is feasible and reliable.

2.4 Water Retention Value of Scaffolds

The water retention values of scaffolds prepared with both polymers were measured, with results shown in [Figure 10: see original paper]. The pure nanocellulose scaffold without high cationic degree polymer had a WRV of 1575%, reaching the maximum value. As polymer A mass fraction increased, scaffold WRV decreased. At 0.79% polymer A dosage, WRV was 654.10%, decreasing from 649.12% to 349.1% as polymer A dosage increased from 1.57% to 7.41%. This trend is consistent with porosity measured by liquid displacement, indicating that higher porosity leads to higher WRV.

As shown in [Figure 10: see original paper], scaffolds with polymer B also exhibited decreasing WRV with increasing polymer dosage, dropping from 790.78% to 239.44% as mass fraction increased from 0.79% to 7.41%. This trend differs from porosity variation measured by both methods, suggesting that WRV is not only related to porosity but also affected by other factors. Both polymers used in this study had cationic degree of 10 with amino-based cationic groups. Polymer A contains anionic carboxyl groups that may repel nanocellulose with carboxyl groups, resulting in higher porosity. Meanwhile, carboxyl groups may have stronger water adsorption capacity, leading to increased WRV. The higher relative molecular mass of polymer A also contributed to higher WRV. Under the experimental conditions of this study, nanocellulose scaffolds with both polymers exhibited high WRV values exceeding 200%, which is beneficial for scaffold swelling and cell growth.

3 Conclusion

Three-dimensional porous tissue engineering scaffolds were successfully prepared by mixing nanocellulose with polyacrylic cationic polymer A and polyethylene amine cationic polymer B at different ratios using freeze-drying methods. Porosity was measured using both liquid displacement and image processing methods, and the reliability of the image processing method was evaluated through absolute and relative error analysis, demonstrating its feasibility. The nanocellulose composite scaffolds prepared with both polymers exhibited porosity greater than 90% and WRV greater than 200%, making them suitable for tissue cell growth and proliferation. By adjusting the polymer type and dosage, scaffolds with regulated porosity and water retention properties can be obtained for specific tissue engineering applications.

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Note: Figure translations are in progress. See original paper for figures.