Stimuli-Responsive Nanocomposite: Potential Injectable Embolization Agent

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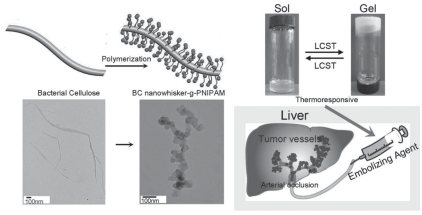
Liver cancer remains a significant medical problem and one promising therapeutic approach is to embolize the tumor. One emerging embolization strategy is to use thermoresponsive materials that can be injected but gel at the tumor site. It is now reported on thermoresponsive nanocomposites generated by grafting poly(*N*-isopropylacrylamide) chains on bacterial cellulose nanowhiskers. Chemical and physical evidences are provided for grafting and demonstrated a sol–gel transition when the temperature is increased above 34.3 °C. Cytotoxicity test in human umbilical vein endothelial cells indicates the excellent biocompatibility of these nanocomposites for use as embolic materials. These results suggest that the nanocomposites offer appropriate properties for embolization of hepatocellular carcinoma.



1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common form of cancer.[1] Transcatheter arterial embolization (TAE) is a treatment referring to "starving" the cells in liver tumors through arterial occlusion.[2] In TAE, the embolic materials must display properties of both low viscosity in the delivery process and high strength in the embolic process.[3] Lipiodol can reach the peripheral arteries rapidly, but it is easily eliminated by blood scouring. On the other hand, many synthetic materials [e.g., Gelfoam, Ivalon, poly(vinyl alcohol) (PVA) microparticles] and natural materials (blood clots) have been used for occlusion, but there are also some disadvantages in their clinical application.[3–5] Some polymer microspheres like PVA particles have a good permanent embolic effect in TAE therapy, but they have difficulty moving into the peripheral arteries because of their relatively large size.[6] Nanostructured materials, having a low viscosity in the delivery process, a high occlusion strength, and excellent biocompatibility are effective for this purpose. Zhao et al. reported that a temperature-sensitive poly(*N*-isopropylacrylamide-*co*-butyl methacrylate) nanosized gel could be used as embolic material. However, the strength and the biocompatibility of these materials still need improvement to be used as embolic agents.[3] Previously, our group reported on thermoresponsive BC whisker/poly(NIPAM-*co*-BMA) nanogel complexes as novel blood vessel embolic materials in the interventional therapy of liver tumors.[7]

Poly(*N*-isopropylacrylamide) (PNIPAM) is an important and extensively investigated thermoresponsive polymer, which has a lower critical solution temperature (LCST) around 32 °C in aqueous solution.[8] PNIPAM has been extensively used as a biomaterial in various applications such as responsive membranes,[9,10] drug delivery,[11] sensors,[12] and cell culture.[13] PNIPAM also shows potential for use as embolic material for TAE,[3] since it exhibits a change in solubility as the temperature increases from

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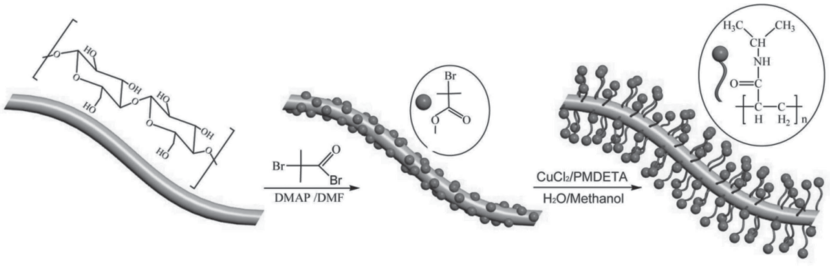
room temperature to body temperature (37 °C). However, its limited biocompatibility and poor mechanical properties greatly limit its application as embolic material.

Bacterial cellulose (BC) produced by *Acetobacter xylinum* is a form of the natural polymer cellulose,[14–17] whose network is composed of nanofibers with a diameter of about 30 nm.[18,19] BC nanowhiskers have excellent properties including high biocompatibility and a high crystallinity[20,21] that increases the modulus of whiskers to about 150 GPa.[22] BC nanowhiskers thus have a wide range of applications such as hydrogels for biomedical uses,[21] nanosilica composites,[23] coatings, and food additives.[24] Due to the high surface area of cellulose nanocrystals, more hydroxyl groups are available for modification,[14,18] they can be easily modified by a variety of chemical reactions leading to functionalizations including cationization, polymer grafting (grafting onto and grafting from), and other noncovalent and covalent modifications.[25]

We anticipate that a combination of BC whiskers and PNIPAM prepared by the atom transfer radical polymerization (ATRP) could be beneficial to generate novel embolic materials. It should be recalled that ATRP is one of the reversible-deactivation radical polymerization (RDRP) methods, which can provide a controlled composition, architecture, and molecular weight distribution for polymers.[26,27] And the ATRP of the polar monomers such as (meth)acrylates and related block copolymers with a ppm amounts of Cu catalyst based on activators regenerated by electron transfer (ARGET) is called ARGET-ATRP.[28] Furthermore, with respect to the surface modification of cellulose and cellulose derivatives, ATRP is one of the most useful methods among all the RDRP technologies.[29–34] Malmström and co-workers[32] have reported that thermoresponsive PNIPAM and pH-responsive poly(4-vinylpyridine) (P4VP) were grafted on to a plant cellulose surface sequentially to form block copolymer chains of PNIPAM and P4VP. Esteves–Xin and co-workers[33] have used a surface-initiated ATRP approach to modify the hydrophilic surface of a cotton fabric with thermoresponsive PNIPAM. However, modified BC whiskers via ATRP have not been reported until now.

We now report on a novel type of embolic material obtained by ARGET-ATRP modification of BC nanowhiskers.[26,29–33,35,36] A catalyst system of Cu (II) and ascorbic acid (AsAc), known to provide good control over the polydispersity index (PDI) in the ATRP process, was used in this study.[37] The synthesis of BC nanowhisker-*g*-PNIPAM nanocomposites prepared via ATRP is described in Scheme 1. As embolic material, its nanometric dimensions make it easier to move into peripheral arteries than microparticles, and its transformation from a translucent fluid to a white shrunken gel at temperatures above the LCST is advantageous in the injection process of TAE.



Scheme 1. Grafting of PNIPAM brushes from BC nanowhiskers.

2. Experimental Section

2.1. Materials

The chemicals 2-bromoisobutyryl bromide (BriB; Aladdin), 4-dimethylaminopyridine (DMAP; Aladdin), *N,N*-dimethylformamide (DMF, waterless; Sinopharm), triethylamine (TEA; Sinopharm), methylene dichloride (Sinopharm), *N*-isopropylacryalamide (NIPAM; Wingch), copper(II) bromide (Sinopharm), *N,N,N′,N″,N‴*-pentamethyldiethylenetriamine (PMDETA; Aladdin), ascorbic acid (AsAc; Biosharp) and methyl alcohol (Sinopharm), and NIPAM were purified by *n*-hexane (Sinopharm). Milli-Q ultrapure water was used for all the experiments.

2.2. BC Nanowhiskers Preparation

Nanowhiskers from BC were obtained according to a method described by Jin and co-workers.[38] BC membranes were obtained from the fermentation of *A. xylinum* (*ATCC53582*). The composition of the culture medium was glucose 20 g, peptone 5 g, yeast extract 5 g, citric acid 1.5 g, disodium hydrogen phosphate dodecahydrate 6.8 g, and water 1 L, which was treated in a steam sterilizer for 20 min at 121 °C. The culture medium was inoculated with *A. xylinum* (*ATCC53582*) in a sterile environment and maintained in static cultivation for 8 d. The BC membranes produced were soaked in water for 3–4 d and then boiled in a 1 wt% NaOH solution for 30 min to remove the medium and bacteria. BC nanowhiskers were prepared by acid hydrolysis of the BC membranes. The membranes were processed in a high-shear homogenizer at 10 000 rpm for 15 min and hydrolyzed with 40 wt% sulfuric acid at 40 °C for 2 d, followed by dialysis (MWCO = 8000) with ultrapure water for 7 d. The resulting whiskers were stored at 4 °C until used.

2.3. Polymer Grafting

BC nanowhisker-*g*-PNIPAM synthesis was completed in two steps, which were the generation of the BC nanowhisker initiator

and the NIPAM "grafting from" process using the modified BC nanowhiskers by ATRP. A 1 g sample of BC nanowhiskers was subjected to solvent exchange from water to dry DMF by centrifugation (1000 rpm for 10 min), mixed with 1 g of DMAP, 100 mL of dry DMF and 30 mL of dry TEA, and stirred well while adding 5 mL of BriB dropwise under nitrogen atmosphere, followed by storage at room temperature for 24 h. The resulting initiator-BC nanowhiskers were purified by centrifugation in dry DMF once, methylene chloride twice, and ultrapure water twice. In the second step, a whisker dispersion was prepared by mixing 0.1 g of BC nanowhisker initiator, 20 mL of H_2O, and 20 mL of methyl alcohol. A NiPAM solution was prepared by mixing 6.3 g (55.7 mmol) of NiPAM, 266 μL (1.08 mmol) of PMDETA, 16 mL of Cu^{2+} (0.04 mmol mL^{-1} in water, 0.64 mmol), 50 mL of methyl alcohol, and 50 mL of H_2O under nitrogen atmosphere, and injecting 16 mL of ascorbic acid (AsAc) (0.04 mmol mL^{-1} in water) 5 min later. Both solutions were combined using a double-ended canula, followed by ATRP under nitrogen atmosphere at room temperature for 24 h. The products were centrifuged twice at 25 °C, dialyzed (MWCO = 8000) against ultrapure water for 1 week, and then recovered by lyophilization.

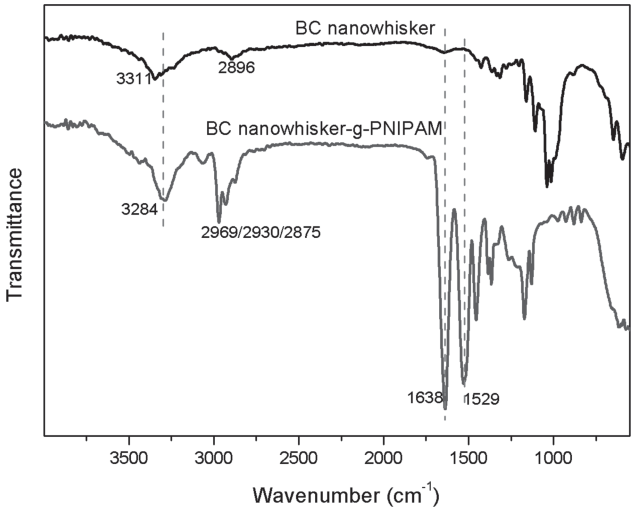


Figure 1. ATR FTIR spectra for purified BC nanowhisker-*g*-PNIPAM and unmodified BC whiskers.

2.4. Characterization Techniques

Fourier transform infrared (FTIR) spectra were recorded on a VERTEX 70 instrument with an ATR cell. All spectra were collected with a scanning range from 4000 to 600 cm^{-1} after 64 continuous scans. Differential scanning calorimetry (DSC) was performed on a Perkin Elmer Jade DSC from 10 to 50 °C with a heating rate of 5 °C min^{-1}. Molecular weights (M_w) and molecular weight distributions of the polymers were obtained by gel permeation chromatography (GPC; Agilent 1100) equipped with refractive index (RI) detection and using PL gel column (10 μm; 10^4 Å). The eluent was THF at room temperature at a flow rate of 1 mL min^{-1}, and the polymer for calibration was polystyrene M_w ranging from oligomers to millions. The samples were prepared by cleaving the grafted polymer with 2% NaOH solution for 48 h while stirring, and dialysis in ultrapure water for 1 week (MWCO = 3000).

Transmission electron microscope (TEM) imaging was performed on a JEM-1230 instrument (Japan) operating at an accelerating voltage of 200 kV. The dry samples were diluted with ultrapure water to 0.1% concentration, and one drop was placed on a carbon film support grid. After staining with 1% phosphotungstic acid, the samples were dried at either 25 or 37 °C for 2 h.

2.5. Cell Cultures and Cell Viability Assays

Human umbilical vein endothelial cells (HUVECs) were grown in Dulbecco's modified Eagle medium (DMEM; GIBCO), supplemented with 10% bovine calf serum (GIBCO) and 1% antibiotics. The cells were cultured in a humidified incubator (5% CO_2, 37 °C) and were subcultured every 2 d. The cells were seeded in 96-well plates at a density of 3000–5000 cells per well and were incubated at 37 °C in a 5% CO_2/95% air humidified incubator. Cell viability was measured using the Cell Counting Kit-8 (CCK-8; WST-8) assay (DOJINDO, Japan) by taking media from the following wells: the experimental group (containing different concentrations of nanocomposite), the control group (media incubated with normal cells), and the blank group (media without any cells). Before measuring the absorbance (A), 10 μL of CCK-8 was added to every well and the samples were incubated for 1 h at 37 °C before reading their optical density at 450 nm in a multimode microplate reader. The cell viability was calculated using the equation below:

$$\text{Cell viability}(\%) = (A_{\text{experimental group}} - A_{\text{blank group}})/(A_{\text{control group}} - A_{\text{blank group}}) \times 100\%$$

3. Results and Discussion

The Fourier transform infrared spectroscopy (ATR FTIR) spectrum obtained for the purified product (Figure 1) indicates a successful "grafting from" polymerization. In the polymerization process, the ratio of monomer to anhydroglucose units [NiPAAm]/[AGU] is 30:1.[26,31] The amide and N–H functionalities of PNIPAM are clearly seen at about 1640 and 1530 cm^{-1}, respectively. The signal intensity for the hydroxyl groups at about 3390 cm^{-1} remains nearly unchanged, because a large number of sugar units inside the BC nanowhiskers remain unaffected after the chemical modification. GPC analysis of the purified PNIPAM chains obtained after cleavage from the surface of the whiskers yielded a PDI of 1.85 [M_w (weight-average molecular weight) ≈ 1600], which is higher than typical for these types of reactions. The ester bonding between PNIPAM chains and cellulose nanowhiskers was cleaved by the saponification (2% NaOH). Structure of the cleaved polymer chains from the whiskers was analyzed by ^1H NMR (Figure S1, Supporting Information), which showed

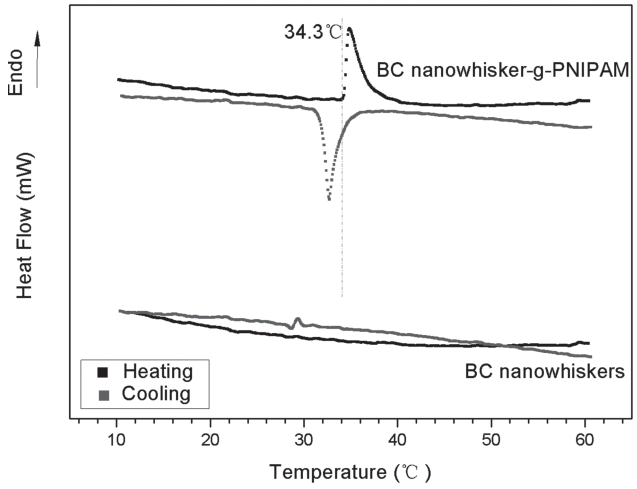


Figure 2. DSC thermograms for BC nanowhisker-*g*-PNIPAM and unmodified BC nanowhiskers.

that the ester in the side chain of PNIPAM remained intact after hydrolysis.[31]

DSC data for the unmodified BC nanowhiskers and for BC nanowhisker-*g*-PNIPAM are shown in Figure 2. The transition temperature of the nanocomposite was determined from the onset temperature in the DSC curves during heating.[39] The sol–gel phase transition temperature for BC nanowhisker-*g*-PNIPAM is about 34.3 °C. The chemical and physical evidences provided in Figure 1 indicate that PNIPAM was successfully grafted from the cellulose nanowhiskers and this polymer confers temperature-responsive properties to the nanocomposite.

The structures of BC nanowhiskers and the BC nanowhisker-*g*-PNIPAM nanocomposites are compared in Figure 3. BC nanowhiskers obtained from the hydrolysis of BC membranes have a diameter from 10 to 60 nm, and a length ranging from 200 to 800 nm, as seen in the TEM image shown in Figure 3a. Comparison of Figure 3b,c, showing the BC nanowhisker-*g*-PNIPAM nanocomposites at 25 and 60 °C, respectively, shows that the formation of globular cluster structures is obvious at 60 °C. It is clear that the BC nanowhiskers are embedded in the middle of PNIPAM globular clusters. Figure 3c provides TEM images for the nanocomposite at three different tilt angles of −30°, 0°, and 30°. This set of images further confirms that the BC nanowhiskers act as a backbone embedded in the middle of the nanocomposites (indicated by the black and white arrows). The globular structures are generated by collapsed PNIPAM chains grafted from the hydroxyl groups on the surface of the BC nanowhiskers, which are responsible for the thermoresponsive properties. At high polymer brush grafting densities, neighboring globular clusters can connect with each other during the thermally induced collapse process. The PNIPAM chains connecting multiple BC nanowhiskers could become part of the network when the temperature reaches 37 °C. The vial inversion method was used to study the sol–gel phase transition behavior (Figure 3d) of the grafted nanowhiskers at different concentrations. Above 5 wt%, the polymer could form a stable stagnant gel from a flowing sol at 37 °C. The morphologies shown in Figure 2 and the observation of sol–gel phase transitions confirm that the nanocomposites have excellent properties for applications as embolic materials.

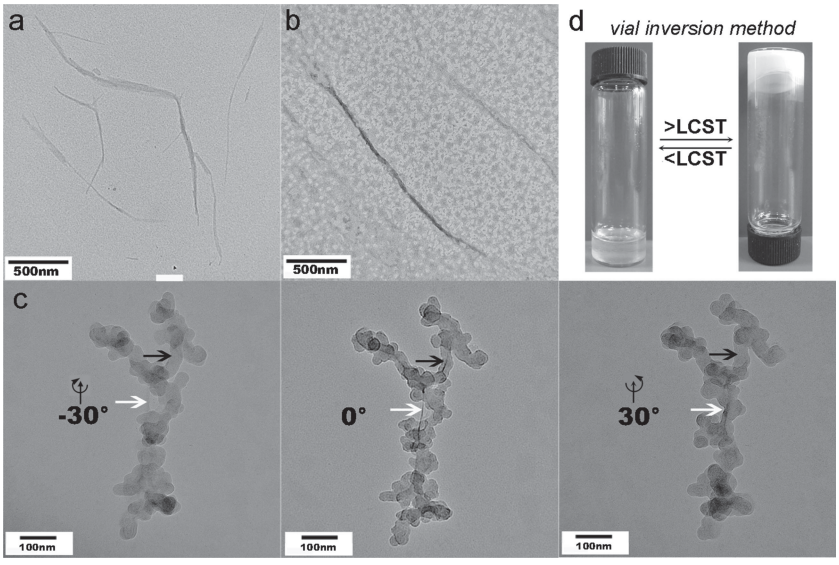


Figure 3. TEM images for a) unmodified BC nanowhiskers at 25 °C, b) BC nanowhisker-*g*-PNIPAM at 25 °C, c) BC nanowhisker-*g*-PNIPAM at 60 °C (globular structures) at different tilt angles, and d) the sol–gel phase transition behavior of the nanocomposites.

HUVECs were used to investigate the biocompatibility of the nanocomposites synthesized. PNIPAM is known to display poor biocompatibility because of its high cytotoxicity.[40] To achieve better visualization and understanding of the effects on HUVECs, CCK-8 experiments were used to show the effect of the nanocomposites for HUVECs. As shown in Figure 4, the cell viabilities determined at different polymer concentrations were above 85% under all test conditions (culture times of 24 and 48 h, Figure 4). And the viability rate of the test group cultured 48 h had a small increase, even higher than the control group. It might be that gelation network structure of the nanocomposites decreased the level of oxygen, resulting in the advancements of expression of hemeoxygenase and cytoplasmic free Ca^{2+}, and promoted the proliferation of

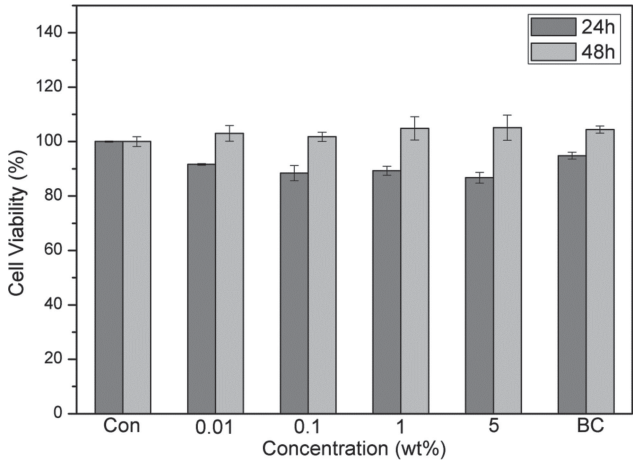


Figure 4. CCK-8 measurement of HUVEC viability after 24 and 48 h of culture.

HUVEC.[41] Moreover, cell viability with the unmodified BC nanowhiskers was higher than 95%, confirming the high biocompatibility of this material. CCK-8 experiments indicated that the nanocomposites were not cytotoxic. All the above experiments therefore indicated that the nanocomposites are suitable for biomaterial applications.

4. Conclusions

In conclusion, compared with unmodified BC nanowhiskers, collapsed structures generated by the PNIPAM chains on the surface of BC nanowhiskers were observed. The LCST of the nanocomposites is 34.3 °C; they could therefore display a phase transition from a flowing sol to stable stagnant gel as the temperature increases from room temperature to body temperature. Cell culture experiments with the resulting nanocomposites demonstrated their high biocompatibility. The nanometric size of these materials, their easy injection, and high biocompatibility demonstrate the excellent potential of the nanocomposites as biomedical materials for TAE.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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