Comparative Evaluation of Biological Performance, Biosecurity, and Availability of Cellulose-Based Absorbable Hemostats

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Abstract

Hemorrhage remains a leading cause of death after trauma, and developing a hemostat with excellent performance and good biosecurity is an extremely active area of research and commercial product development. Although oxidized regenerated cellulose (ORC) has been developed to address these problems, it is not always efficient and its biosecurity is not perfect. We aimed to refine ORC via a simple and mild neutralization method. The prepared neutralized oxidized regenerated cellulose (NORC) showed a superior gel property due to its chemical structure. The biological performance of both ORC and NORC was systematically evaluated; the results showed that ORC would induce erythema and edema in the irritation test, whereas NORC did not cause any adverse inflammation, indicating NORC had desirable biocompatibility. We further demonstrated that NORC confirmed to the toxicity requirements of International Organization for Standardization (ISO) standards; however, ORC showed an unacceptable cytotoxicity. The rabbit hepatic defect model stated that NORC exhibited better ability of hemostasis, which was attributed to its significant gel performance in physiological environment.

Introduction

Uncontrolled hemorrhage still remains the leading cause of preventable death after trauma in both the battlefield and civilian settings, accounting for approximately half of all deaths before a treatment facility is available.[1,2] Conventional materials and standard surgical techniques for bleeding control are insufficient or impractical in many clinical scenarios.[3-5] Therefore, a number of efficient hemostatic agents have been developed that can arrest bleeding and stabilize the casualty temporarily.[6-8] Additionally, some biological nanostructures such as phages have been regarded as the fascinating biomaterials and can be the promising candidates for the absorbable hemostats.[9,10]

Oxidized regenerated cellulose (ORC), among the topical hemostats, is of special interest because of its superior performance and several useful medical applications.[11] It can be obtained by the selectively oxidation to the primary hydroxyl groups in the anhydroglucose rings, producing the monocarboxyl cellulose.[12,13] Oxidized regenerated cellulose represents an important class of bioabsorbable polymers under physiological conditions,[14] being widely used as hemostat,[15-17] postsurgical adhesion prevention layer,[18] and as a macromolecular prodrug carrier.[19] Other applications of ORC to prepare

biomedical composites for hemostasis are also studied.[20,21] Especially, several successful clinic cases of ORC in controlling hemorrhage have been reported.[22,23] The mechanism of hemostatic action of ORC can be explained from 2 perspectives: physically and chemically. On the one hand, it can absorb blood and then swell slightly to form a plug at the injury site to seal off the damaged blood vessel; meanwhile, the swelling material generates pressure on the surrounding tissue contributing to hemostasis.[24] This initial adsorption process also assembles the fibrinogen, blood platelets, and cells. On the other hand, the low pH (approximately 3.1 with $16 \sim 24\%$ –COOH) of the carboxyl groups provides a primary local hemostatic action by stimulating the innate coagulation mechanism. It produces an acidic medium that converts hemoglobin to acid

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hematin, releasing Fe^{3+} in order to combine with –COOH and accelerate the clot formation.[25] Furthermore, the low pH also endows antibacterial activity to ORC.[26] Nevertheless, the previous study reveals that ORC exhibited strong cytotoxicity, indicating that it not only affects the survivability but also influence the proliferation ability of surrounding cells.[27] Intensive researches focused on modification of ORC have been carried out and obtained some achievements[28,29]; however, very few studies concentrate on its biosecurity.

In view of the dual challenges of hemorrhage and biological risk of biomedical materials, neutralized oxidized regenerated cellulose (NORC) is developed through a convenient neutralization route. The biological performance of the resulting material is systematically assessed by several qualitative and quantitative assays in vivo. And the biosecurity is identified both in vitro and in vivo. The hemostatic availability of the material has been also evaluated by the animal model.

Materials and Methods

Materials

Viscose filament yarn was obtained from the city of Xinxiang, Henan province, China. Nitrogen dioxide (NO_2) was purchased from Summit Specialty Gases Co, Ltd, Tianjin city, China. Carbon tetrachloride (CCl_4; AR) was received from Shuang Shuang Chemical Co, Ltd, Yantai, China. Sodium hydroxide (NaOH; AR) was supplied by Shuang Shuang Chemical Co, Ltd, Yantai, China. Ethanol absolute Analytical Reagent (AR) was purchased from Fu Yu Chemical Co, Ltd, Tianjin, China.

Preparation of ORC

The detailed preparation process was carried out as follows. Prior to oxidation, the viscose filament yarns (regenerated cellulose [RC]) were knitted with a knitting machine to obtain single-layer weft knitting rectangular fabrics. The oxidant solution was prepared by dissolving NO_2 into CCl_4 to prepare the 20 wt% NO_2/CCl_4 solution, and then the RC-knitting fabrics were immersed into a round-bottomed flask containing the mentioned NO_2/CCl_4 oxidant solution to achieve a concentration of 25 mg/mL (RC-knitting fabrics/oxidant solution). The round-bottomed flask was sealed, and the oxidation reaction was performed at 19.5°C for 40 hours with constantly vigorously stirring. After the reaction, the product was washed thrice with CCl_4 and then rinsed 5 times with 50% alcohol aqueous solution. Finally, ORC fabrics were frozen in a −80°C freezer for 4 hours followed by freeze-drying at −50°C in vacuum for 48 hours.[30-34]

Fabrication of NORC

Firstly, 12.0 g of NaOH was dispersed into 150 mL ethanol absolute in an Erlenmeyer flask, then the mixture was stirred vigorously overnight to form a homogenous NaOH/ethanol solution at a concentration of 2.0 mol/mL. Next step, 6.0 g of ORC fabrics were immersed into the NaOH/ethanol solution.

Then the flask was sealed with a rubber stopper and shook it vigorously by a reciprocating shaker at room temperature for 24 hours. After the neutralization reaction, NORC has been synthetized. The obtained NORC fabrics were washed 5 times with 50% alcohol aqueous solution. After that, the NORC fabrics were frozen in a −80°C freezer for 4 hours, then the fabrics were frozen-dried at −50°C in vacuum for 48 hours.

Determination of Carboxyl Content

Carboxyl content was measured according to United States Pharmacopoeia (USP23-NF18). Briefly, approximately 0.5 g of sample was accurately weighted and suspended in 50 mL of a 2% (wt/wt) fresh calcium acetate solution for 15 hours. The mixture was titrated with 0.1 M NaOH, and the volume of NaOH solution consumed was corrected by the blank. The content of −COOH in the sample was calculated by the following equation:

$$-COOH(\%) = \frac{N \times V \times MW_{COOH}}{m} \times 100, \quad (1)$$

where N is the normality of NaOH solution, V is the consumed volume of NaOH which was corrected by the blank, MW_{COOH} is the molecular weight of –COOH, and m is the mass of sample.

Structural Analysis

Fourier Transform Infrared Spectroscopy (FTIR) was obtained as potassium bromide (KBr) pellets,[35] with a Nicolet-Nexus670 spectrophotometer.

Biological Evaluation

All animal handling strictly followed the research protocol approved by National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. The biological evaluation was performed according to the ISO 10993 principles. Prior to the test, the extract of the sample was prepared, using 0.9% sodium chloride (SC) solution and cotton seed oil (CSO) as extraction media. Briefly, the sample was immersed into SC (or CSO) at a surface area to volume ratio of 9:1, followed by standing at 37°C for 72 hours. The liquid that resulted from the extraction served as extract. Pure SC and CSO extraction media were prepared similarly to serve as the reagent control.

Pyrogenicity test. The extract was prewarmed to 37°C, and the temperature of each rabbit was measured 30 minutes ahead of injection and recorded as the baseline temperature. Each rabbit received a single intravenous injection of the extract via the marginal ear vein at 10 mL/Kg of body weight. Rectal temperature was measured and recorded at 30-minute intervals between 1 hour and 3 hours after injection. The maximum temperature rise for each rabbit was determined.[36]

Irritation test. Each rabbit was closely clipped free of fur from the back and both sides of spinal column to yield a sufficient

injection area on the day before the test. Just before injection, the clipped area was wiped with 70% alcohol and allowed to dry. Then a 0.2-mL dose of the extract (or reagent control) was injected intracutaneously into 5 separate sites. Tissue reaction for each injection site at each time interval was observed, and the result was recorded. If no rabbit showed either erythema or edema, the sample met the requirement for irritation. If the animal showed very slight erythema or edema, the irritation symptom grade was classified as the first. If the animal showed well-defined negative response, the grade was belonged to the second. If the animal showed moderate anaphylaxis, its irritation belonged to grade 3, and the symptom of severe adverse effect was classified as grade 4.[37]

Skin sensitization test. Three rows of intradermal injection sites were chosen (2 sites per row, injection site [a], [b], and [c]) and given to each Albino guinea pig within an approximate 2 cm × 4 cm boundary of the fur-clipped area. Then, the test animal was injected (a) 0.1 mL of 50: 50 (vol/vol) mixture of Freund's complete adjuvant (FCA) and the extraction media, (b) 0.1 mL of test extract, and (c) 0.1 mL of 50:50 (vol/vol) mixture of the concentration used at sites (a) and (b), whereas the control animal was injected (a) 0.1 mL of 50: 50 (vol/vol) mixture of FCA and the extraction media, (b) 0.1 mL of extraction media, and (c) 0.1 mL of 50:50 (vol/vol) mixture of the concentration used at sites (a) and (b).

Seven days later, the same area was clipped free of fur and treated with 10% sodium lauryl sulfate suspension in petrolatum. The suspension was massaged into the skin over the injection site to provoke a mild acute inflammation, and the area was left uncovered. After 24 hours, a gauze patch (20 mm × 40 mm) saturated with fresh extract (or reagent control) was topically applied to the previous injection sites. The gauze was secured with an occlusive dressing and removed after 48 hours.

Two weeks later, the gauze soaked in the solution at the concentration of site (c) was applied to cover the left upper flank of each animal and it was removed after 24 hours. The appearance of the injection skin site was observed 24 hours and 48 hours after the removal of the gauze.[38]

Biosecurity Evaluation

Acute systemic toxicity test. Prior to dosing, the mice were identified and weighted. Twenty mice were divided into 4 groups. Each group was injected with the fresh extract (or the corresponding reagent control) at a dose of 50 mL/Kg by the intraperitoneal route. Mice were observed for adverse reactions immediately after injection, 4 hours, 24 hours, 48 hours, and 72 hours, respectively. The body weights were recorded. In acute systemic toxicity test, if the mice showed slight but noticeable symptoms of hypokinesia, dyspnea, or abdominal irritation, the sample presented slight acute systemic toxicity. If the mice exhibited definite evidence of toxicity, and the weight usually dropped to between 15 and 17 g, the material denoted moderate toxicity response. And, if the mice showed prostration, cyanosis, tremors, or severe symptoms of

abdominal irritation, diarrhea, ptosis, or dyspnea and if the mice showed extreme weight loss less than 15 g, the test sample would be recorded as marked toxicity. The test material would be identified to be expired and deadly systemic toxicity if the animals died.[39]

Cytotoxicity test. The test was carried out as the followed procedures. L929 Mouse fibroblast cells were prepared at 37°C in sealed flasks containing Grand Island Biological Company (GIBCO) minimum essential medium (MEM), supplemented with 10% calf and 1% L-glutamine. For this study, 2 plates (Φ = 100 mm) were seeded in 10 mL suspension of 3.0×10^5 cells per milliliter and incubated at 37°C for 24 hours in order to obtain a confluent monolayer of cells. Equal mounts of double strength MEM and 3% agar were combined to form an MEM-agar mixture. Minimum essential medium–agar mixture (10 mL) was then placed in the cell culture plates containing the confluent monolayer of cells, and it was allowed to solidify over the cells to form the agar overlay. After that, freshly prepared neutral red vital stain (10 mL) was added gently to cover the entire solidified agar surface and the strong light was shielded for 15 minutes. Then, 2 replicate test samples, 1 negative control (high-density polyethylene sheet) and 1 positive control (an organotin-stabilized polyvinylchloride sheet), were applied symmetrically to the surface of the agar with the edge of the samples approximately 15 mm from the edge of the plate. And then, the plates were incubated at 37°C under 5% carbon dioxide in air for 24 hours.[40]

Hemostatic Availability

Hemolysis test. The pure SC was used as the negative control, and distilled water served as the positive control. Firstly, diluted anticoagulated rabbit blood was prepared as follow. Fresh rabbit blood (4 mL) was mixed with 0.2 mL of 20 g/L potassium oxalate in a tube, and then 5 mL of SC was added. Three replicates of the blood were prepared per extract and control, and each tube was added 10 mL extract (or control) to start the test. All tubes were incubated at 37°C for 30 minutes, followed by the addition of 0.2 mL fresh diluted anticoagulated rabbit blood, and the tube was shaken gently and maintained at 37°C for 60 minutes. After that, the tubes were centrifuged for 5 minutes at 800g, and the $D_{545\ nm}$ of the supernatant was determined spectrophotometrically. The extent of hemolysis was expressed as a percentage and calculated according to the formula:

$$\frac{D_\mathrm{t} - D_\mathrm{nc}}{D_\mathrm{pc} - D_\mathrm{nc}} \times 100\% \qquad (2)$$

where D_t is the absorbance of the test extract, D_nc showed the absorbance of the negative control, and D_pc represented the absorbance of the positive control.[41]

Animal injury experiments. Rabbits were placed on their backs and anesthetized, and the tissues including skin and overlaying muscles were transected with scalpel to expose the liver. The injury was induced by scooping out the tissue in the middle of
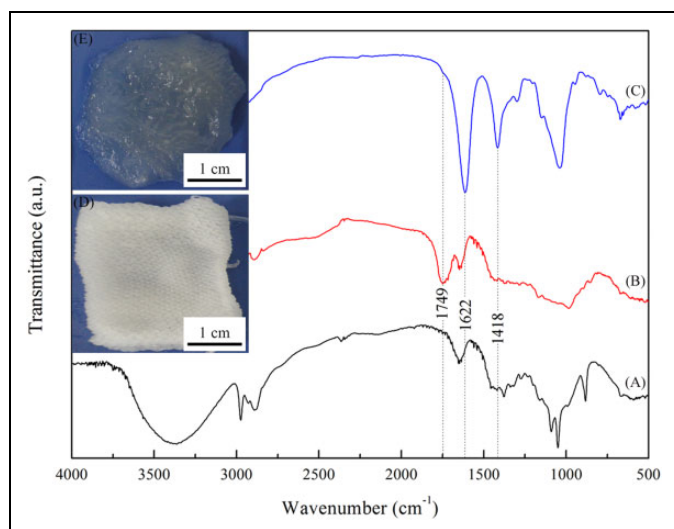
Figure 1. FTIR spectra of: (A) RC, (B) ORC, (C) NORC, and photographs of (D) ORC saturated with water and (E) NORC saturated with water. NORC indicates neutralized oxidized regenerated cellulose; ORC, oxidized regenerated cellulose; RC, regenerated cellulose.

the lobe of liver. The wound measured approximately 0.5 cm × 0.5 cm × 0.3 cm. As the injury started to bleed, the blood was absorbed with conventional sterile gauze immediately. And then a layer of sample was applied to the site of injury with manual compression. The hemorrhage situation was monitored, and the hemostatic time was also recorded (n = 5).[42]

Results and Discussion

Structure and Gel Performance of ORC and NORC

Figure 1 showed the FTIR spectra and photographs of the RC, ORC, and NORC.

In Figure 1, the absorption peak around 3471 cm^{-1} was attributed to the stretching vibration of O–H, and the twin peaks at 2974 and 2891 cm^{-1}, respectively, corresponded to the asymmetrical and symmetrical stretching vibration of –CH_2.[43] The peak at 1653 cm^{-1} was assigned to the bending vibration of absorbed H_2O,[44] while the absorption at 1049 cm^{-1} was due to the stretching vibration of C–O–C pyranose ring in cellulose.[45] By comparison, double peaks around 2974 and 2891 cm^{-1} for both ORC and NORC were dramatically attenuated in intensity.

The FTIR spectrum of NORC (Figure 1C) showed the decrease in the signal at 1749 cm^{-1} and a corresponding increase of asymmetrical and symmetrical stretching vibration absorption signal of –COO^- at 1622 and 1418 cm^{-1} with respect to the reduction of –COOH in NORC, indicating that ORC has been neutralized by NaOH as proposed.

To quantitatively analyze the neutralization degree, the carboxyl content of the 2 materials was determined. The –COOH in ORC was 18.77%, while it significantly reduced to only 1.27% in NORC. The molar ratio of –COOH to NaOH was 1:12 in the experiment; however, the degree of the neutralization was not carried out completely so that –COOH still remained in NORC. This could be explained by the different reaction zones in ORC, including surface and core, and the reactivity of –COOH in the 2 zones was significantly different. The neutralization rate was rapid in the initial stage happened on the fiber surface, since NaOH accessed easily to –COOH because of no stereo-hindrance and the large amount of –COOH. As –COOH in core was fewer and penetration of NaOH became difficult, the reaction rate decreased.

Furthermore, from the photographs of the materials saturated with water, we could see that ORC-knitted fabric swelled inconspicuously and still maintained its original morphology. However, due to the change happened to the molecular structure, NORC-knitted fabric could rapidly convert into a transparent and sticky gel.

Biological Performance

The biological evaluation was a key point for the development and application of biomaterials and biomedical devices.[46]

Firstly, the samples were evaluated in the rabbit for material-mediated pyrogenicity. In this study, any temperature decrease was considered as zero rises. If no animal showed an individual temperature rise of 0.5°C or more above the baseline, it indicated that the sample met the requirement for the absence of pyrogens. If any rabbit showed an individual rise of 0.5°C or more, a retest was conducted using 5 other rabbits. If not more than 3 of the 8 rabbits showed individual rise of 0.5°C or more, and the sum of the individual maximum temperature rises did not exceed 3.3°C, the sample was nonpyrogenic.[47] The results of pyrogenicity test for ORC and NORC were listed in Table 1.

As shown in the data of pyrogenicity test, the maximum temperature rises as compared to baseline in the 3 ORC groups were 0.4°C, 0.4°C, and 0.3°C, respectively, and the maximum temperature rises in the NORC groups were 0.4°C, 0.2°C, and 0.4°C. In other words, all temperature rises were less than 0.5°C, and the total rises were within acceptable limits, suggesting that no evidence of material-mediated pyrogenicity was observed. Therefore, both ORC and NORC were judged as nonpyrogenic.

Secondly, the intracutaneous reactivity test was conducted to assess the potential of the samples to induce irritation.[48] For each sample and reagent control, the irritation grades obtained at each time interval were added together and then divided by the total number of observations. The grades of the samples were compared with the corresponding reagent control, and the final data of the irritation test were presented in Table 2.

From the irritation test result, the difference between the mean grades of sample and the CSO control was 0.0 in ORC group, this meant no evidence of significant irritation from the CSO extracts of ORC. However, a visible symptom of significant irritation from the SC extracts of ORC could be seen, that is, the grades of erythema were 1.0, 1.0, and 1.0, and the edema grades were 1.6, 2.3, and 2.3. According to the requirements,

Table 1. The Results of Pyrogenicity Test.

| Sample | Weight (kg) | Dose (mL) | Baseline (°C) | Hours After Injection (hours) | | | | | | Max Rise (°C) |
| | | | | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | |
				Temperature (°C)						
ORC	2.0	20	38.6	39.0	38.7	38.8	38.6	38.5	38.7	0.4
	1.9	19	38.8	39.2	39.0	38.8	38.8	38.9	38.7	0.4
	1.9	19	39.1	39.4	39.2	38.9	39.0	39.0	38.9	0.3
NORC	2.5	25	38.6	38.8	38.9	39.0	39.0	39.0	38.9	0.4
	2.6	26	39.0	39.1	39.2	39.2	39.2	39.0	39.2	0.2
	2.3	23	38.7	38.8	39.1	39.1	39.1	39.1	39.1	0.4

Abbreviations: NORC, neutralized oxidized regenerated cellulose; ORC, oxidized regenerated cellulose.

Table 2. The Results of Irritation Test.

| Category | Time (hours) | Grade | | | | | |
| | | ORC | | NORC | | Control | |
		Erythema	Edema	Erythema	Edema	Erythema	Edema
SC group	24	1.0	1.6	0.0	0.0	0.0	0.0
	48	1.0	2.3	0.0	0.0	0.0	0.0
	72	1.0	2.3	0.0	0.0	0.0	0.0
CSO group	24	0.0	0.0	0.0	0.0	0.0	0.0
	48	0.0	0.0	0.0	0.0	0.0	0.0
	72	0.0	0.0	0.0	0.0	0.0	0.0

Abbreviations: CSO, cotton seed oil; NORC, neutralized oxidized regenerated cellulose; ORC, oxidized regenerated cellulose; SC, sodium chloride.

the sample would be standard qualified if the difference between the mean grades of sample and the control was not more than 1.0. But the differences between the mean grades of SC extract of ORC and control were approximately 1.5, so it could be concluded that ORC did not meet the requirement. However, under the same conditions, the differences between NORC and control were all 0.0, indicating that NORC would not induce any significant stimulation to the animals.

Based on the data and analysis above, it could be inferred the irritation response was closely related to the pH of samples. Oxidized regenerated cellulose contained 18.77% −COOH and its pH was about 3.5, and this strong acidity could cause skin stimulation manifested by intradermal erythema and edema. However, the −COOH in NORC significantly decreased to 1.27% so that its pH increased to approximately 6.0, such weak acidity would not result in any inflammatory response.

Finally, the skin-sensitizing potentials of both ORC and NORC were evaluated. The skin-sensitizing reactions for erythema were described and scored in accordance with the criteria as below: if no visible change occurred to the animals, the sample would get 0 point. If the skin site showed discrete or patchy erythema, the material would gain 1 point. If the injection site appeared moderate and confluent erythema, the sample would obtain 2 points, and the materials would be scored 3 points if the erythema was severe.[49] The results of skin sensitization test were shown in Table 3.

Table 3. The Results of Skin Sensitization Test.

| Hours Following Gauze Removal (hours) | Score | | | |
| | 24 | | 48 | |
Extraction media	SC	CSO	SC	CSO
ORC	0	0	0	0
NORC	0	0	0	0
Control	0	0	0	0

Abbreviations: CSO, cotton seed oil; NORC, neutralized oxidized regenerated cellulose; ORC, oxidized regenerated cellulose; SC, sodium chloride.

All animals appeared clinically normal throughout skin sensitization test. Whether extracted in SC or CSO, the skin sensitization scores of both ORC and NORC were determined as 0 point. It showed that ORC and NORC would not lead to the skin sensitization.

Biosecurity Evaluation

All observations of the animals during the acute systemic toxicity test were listed in Table 4.

The negative and positive controls performed as anticipated, and all animals appeared clinically normal throughout the experiment. Under the conditions of this study, the weights

Table 4. The Results of Acute Systemic Toxicity Test.

Category	No.	ORC			NORC			Control		
		Time (hours)			Time (hours)			Time (hours)		
		24	72		24	72		24	72	
		Weight (g)		$\frac{Dead}{Total}$	Weight (g)		$\frac{Dead}{Total}$	Weight (g)		$\frac{Dead}{Total}$
SC group	1	19.3	23.5	0/5	19.6	24.5	0/5	19.3	24.6	0/5
	2	19.6	24.9		19.6	23.9		19.0	23.5	
	3	18.9	23.4		19.2	23.7		19.4	23.8	
	4	19.2	24.7		19.3	24.1		19.5	24.3	
	5	19.1	23.2		19.1	23.2		19.7	24.5	
CSO group	1	19.7	24.4	0/5	19.3	22.7	0/5	19.1	23.1	0/5
	2	19.3	23.8		19.0	23.3		19.2	23.9	
	3	19.5	23.8		19.7	22.9		19.2	22.8	
	4	19.6	22.7		19.7	23.2		19.6	23.7	
	5	19.0	23.4		19.5	22.9		19.5	23.4	

Abbreviations: CSO, cotton seed oil; NORC, neutralized oxidized regenerated cellulose; ORC, oxidized regenerated cellulose; SC, sodium chloride.

Figure 2. The photograph of hemostatic experiment of ORC. ORC indicates oxidized regenerated cellulose.

of all animals have increased within acceptable limits,[39] and there was no mortality or other evidence of acute systemic toxicity from the extract of the 2 test samples. In summary, both ORC and NORC would not cause any acute systemic toxicity.

To further study the potential for cytotoxicity of both ORC and NORC, the mouse fibroblast cells were used to evaluate the grade of cytotoxicity. Scoring for cytotoxicity was based on the following criteria. No detectable zone around or under specimen meant the absence of toxicity. Some malformed or degenerated cells under specimen represented slight cytotoxicity. The material was mildly cytotoxic when the reactivity zone could be detected only in the area under specimen. The cytotoxicity was moderate if the reactivity zone extended specimen up to 1.0 cm, and it was severely cytotoxic when the extending of reactivity zone was further than 1.0 cm beyond specimen.[40]

In our experiment, all cells of the negative control grew well and no abnormal reactivity was found, while most of the cells contacted with the positive control became round, showing cytotoxic reactivity of cell lysis and membrane rupture, and the extending of reactivity zone was further than 1.0 cm beyond the specimen. In the case of ORC, most of the cells became round and degenerated, the membrane ruptured and the reactivity zone extended specimen up to 1.0 cm, which indicated the cytotoxicity of ORC was moderate. Nevertheless, some cells were also degenerated and lysed in NORC group, but the symptoms could be detected only under the specimen, revealing that NORC exhibited mild cytotoxicity. This was also likely contributed to −COOH in the 2 materials, which would change the acidity of the living condition for the cells, so that the cells showed some cytotoxic symptoms. However, the amount of −COOH in NORC was much fewer than that in ORC, so the cytotoxic response of NORC was significantly slighter compared to that of ORC, that was to say, NORC
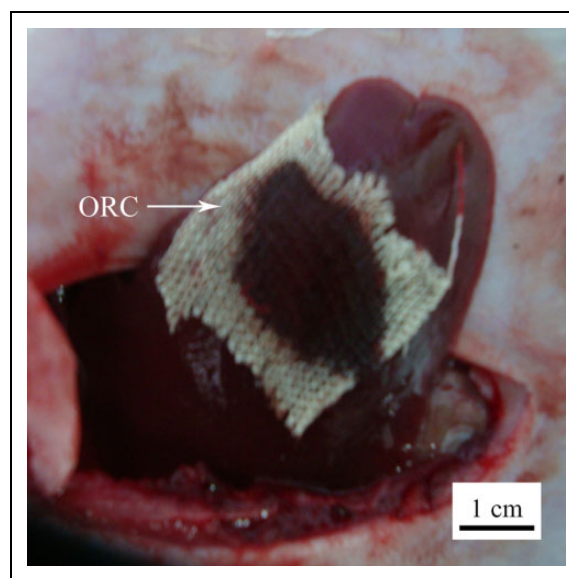
would be safer for using as a biomedical material in the human body.

Hemostatic Availability

The hemostatic performance of ORC and NORC has been comparatively evaluated by the jury model. Figure 2 illustrated the hemostatic experiment of ORC. When ORC-knitted fabric was applied onto the wound, it quickly absorbed the blood, subsequent with the color change of the blood from bright red to dark red. The hemorrhage could be controlled within 290.4 ± 13.4 seconds.

Through the hemolysis test, it has been demonstrated that ORC would cause hemolysis of red blood cell, and the hemolytic ratio was 56.9%. That is to say, ORC produced an acidic medium that converted hemoglobin to acid hematin, giving rise to black color. In addition, −COOH was able to capture the Fe^{3+} in hematin, eventually forming a dark red clotting to seal off the injury. So it could be concluded that the hemostatic availability of ORC was determined by −COOH, which was accorded with the previous studies.[24,25] Neutralized oxidized regenerated cellulose also underwent the same color change when the blood penetrated onto it. It should be noted that NORC became gel rapidly once it was soaked with blood and adhered to the tissue, as shown in Figure 3. The hemostatic time in NORC group was 231.8 ± 14.8 seconds, significantly shorter than ORC groups.

Carboxyl content of NORC was much less than ORC, which was supposed to lead NORC to lose the major hemostatic efficiency provided by −COOH. And it was exactly so, the hemolysis ratio of NORC did significantly decrease to 8.4%. However, the injury model showed that NORC exhibited a more superior hemostatic performance than ORC. It could be inferred that this abnormal phenomenon was contributed to the
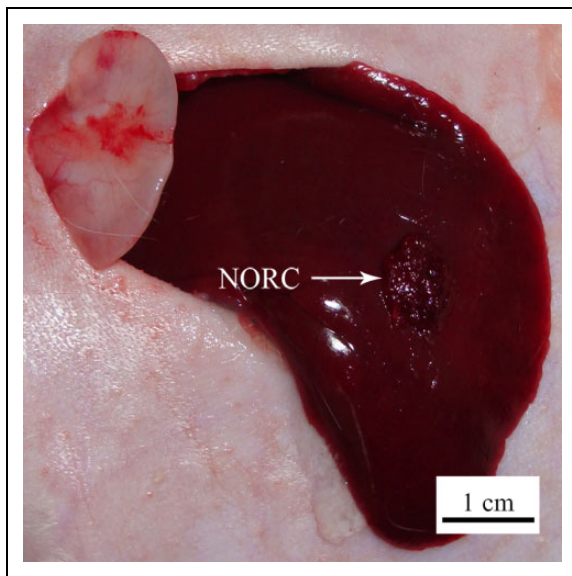
Figure 3. The photograph of hemostatic assay of NORC. NORC indicates neutralized oxidized regenerated cellulose.

significant gel property of NORC. On the one hand, it was able to concentrate the blood to aggregate more red blood cells and blood platelets, facilitating hemostasis of body's self-healing mechanism. On the other hand, also the most important, NORC could rapidly transform into a sticky gel when it absorbed the blood and subsequently plugged up the damaged blood vessel to arrest the bleeding. Besides, the residual –COOH could also contribute to the hemostasis. Thus, integrating the above 3 aspects of effectiveness, the hemostatic performance of NORC did become more effective.

In summary, an ideal hemostatic agent should have the capacity to stop the hemorrhage within minutes, should be simple to apply with minimal training, and should sustain hemostasis to allow for transport to critical care centers.[50] Furthermore, it needed to ensure the safety and biocompatibility of the materials, such as noncytotoxicity and nonimmunogenicity.[51] The approved absorbable hemostatic agents to date mainly included gelatin,[52] collagen,[53] fibrin glues, chitosan bandages, and zeolite powders, ORC. Although many of these have shown efficacy in animal studies and humans, there were several disadvantages to these dressings. For example, since most of these products were prepared from pooled blood of multiple donors, they presented an associated risk of negative immune responses.[50] Zeolite powders were difficult to apply in windy environments and may cause severe burns. Chitosan bandages were arguably the most promising of the hemostatic dressings mentioned here and have been adopted by the US Department of Defense. However, the mechanism of action of the chitosan bandage was not entirely understood, and the bandage appeared to be most suitable for noncomplex wounds.[3] Our cellulose-based hemostatic material, whose raw was extracted from natural plants, could avoid the risk of negative immune responses. We have also demonstrated that the NORC has good biocompatibility and

acceptable biosecurity. Furthermore, NORC showed effective hemostatic ability and it was flexible for complex wounds; in the meantime, its hemostatic mechanism was relatively clear. Moreover, if ORC was implanted into the human body, it would damage nervous system due to its high acidity. However, pH of NORC was close to neutral, which was a potential alternative as a hemostat for hemorrhage in human head. Also, this moderate acidity allowed NORC to be used together with some acid-sensitive drugs to further promote the hemostatic availability, such as thrombin.

Conclusion

In this study, NORC has been prepared through an extremely facile and efficient neutralization method, and the resulting structure rendered NORC special and desirable gel property. The biological evaluation, including pyrogenicity test, irritation test, and skin sensitization test, suggested that NORC presented better biological performance than ORC. In addition, NORC did not exhibit any acute systemic toxicity and had acceptable cytotoxicity according to ISO standards. All these results suggested that NORC showed indeed excellent biocompatibility and good biosecurity. We further demonstrated that the hemostatic mechanism of NORC partially depended on the residual –COOH which was able to destroy the red blood cells and combine with Fe^{3+}, and it was worth noting that the remarkable gel performance imparted NORC with superior hemostatic ability. Therefore, NORC was a promising candidate for potential application in surgery and emergency for hemorrhage control.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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