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PAPER

Synthesis of chitosan-Cu based bioactive material for coating catheters: *in vitro* cytotoxicity evaluation

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Abstract

Background: Catheters are polymeric materials frequently used in clinics and are associated with the risk of inflammation and coagulation. The development of bioactive catheter surfaces is worth applying because antibiotic resistance in bacterial infections is common. Copper (Cu) ion coordinated chitosan (Chitosan-Cu) coatings on medical catheters, and several studies have recently approved its application. *Objective*: It is crucial to investigate the possible cytotoxicity of Chitosan-Cu coatings on surrounding cells. *Methods*: The effect of the Chitosan-Cu complex coating, proven to have bioactive activities at different rates on L929 cells, was examined by the CCK-8 test kit. In 24 h, the cell viabilities of samples, with Chitosan: Cu ratios of 10:0, 10:1, 50:1, and 100:1, were measured as 105.14%, 89.90%, 91.91%, and 100.75%, respectively. In 72 h, they were measured at 119.45%, 109.33%, 110.24%, and 114.45%. The surface morphology of the coating was characterized by electron microscopy, and the entity of the Cu ions in the coating was characterized by x-ray photoelectron spectroscopy. *Conclusion*: Cytotoxicity assays showed that Cu, with a maximum concentration of 10% by volume, showed no toxic behavior.

1. Introduction

Biomedical materials are frequently used in clinics. Considering the different usage areas, most of these materials are mainly used in interaction with epithelial tissue, blood, and other body fluids. According to some usage areas, medical catheters among biomedical materials bring essential risks, especially the catheters used in the intravenous route. One of these risks is mostly inflammation, and the other is coagulation, depending on the application area [1–10]. Various drugs are given to the patient during application to prevent inflammation and blood clotting. Those drugs include blood anticoagulants, bactericidal and bacteriostatic antibiotics, and other anti-inflammatory drugs [7, 11–14]. There are recent works in biomedical materials research on inorganic and organic coatings changing the surface properties of the biomedical device to eliminate such complications. There are few alternatives to drug therapies and drug-eluting medical devices to solve inflammation complications [15, 16]. Recent studies show great interest in natural polymers, especially polysaccharides [17, 18]. Chitosan is a natural polysaccharide bringing sufficient antibacterial properties to the surface of biomedical materials [19–21]. Drug-loaded chitin/chitosan derivatives are often used as wound dressing or biomedical coating [22–25].

Copper (Cu) ions loaded bioactive coatings are predicted to create less drug resistance than drug-eluting coatings, prevent blood coagulation, and stimulate cell regeneration. The effectiveness of Cu ions containing chitosan films for antibacterial biomedical materials has been discovered [26, 27]. Moreover, Cu ion

Table 1. The designated Cu ion concentration of each prepared chitosan hydrogel.

Composition design Ch: Cu (v:v)	Chitosan solutions (ml)	$CuSO_4$ solutions (ml)
10:1	9	1
50:1	9.8	0.2
100:1	9.9	0.1
10:0	10	0

coordinated chitosan (Chitosan-Cu) complex coating on the medical catheters promises an alternative, especially where the drug resistance of the antibiotics is a problem [28–30]. It also brings anticoagulative properties [31]. In addition to the Cu-containing coatings on conventional catheters, rubber-based catheters containing Cu and their applications have recently attracted attention [32, 33]. Of course, besides the favorable results of this new application, its biosafety should also be questioned. It should also be taken into account that Cu ions, which primarily cause the death of bacteria, may cause cell death in the surrounding cells at the level where the application sensitivity is kept optimal. Cell death has not been considered until today in studies on the Cu ion-loaded coating and bulk material for catheter purposes.

There are many methods to measure the *in-vitro* cytotoxicity of biomedical devices and materials [34, 35]. They can be categorized into three groups, the first direct-contact method, where the biomedical devices or materials are contacted directly by a solid or liquid medium or separated with a selective film: the latter is the indirect contact method, where cell cultures are exposed step-by-step with leachates of biomaterials in biological medium [34]. Another category can be summarized by analytical ways where ion tolerance of cell cultures is investigated with an assay. Later degradation or diffusion study of biomedical devices or biomaterials is held separately to investigate the contaminant concentration over time. Later those numerical results can be interpolated/extrapolated or logically compared with min/max values to examine the toxicology of each material [36]. While indirect methods give high-throughput results, direct contact methods better reflect clinical use, especially for polymeric materials [35]. Cell Counting Kit-8's (CCK-8) counting assay is a quantitative method to measure cell proliferation and viability [37, 38].

Based on all this, a short cytotoxicity study was carried out by considering the effect of Cu-loaded catheter material on cell death. The cytotoxicity of chitosan and Chitosan-Cu coated silicon rubber samples were investigated through the CCK-8, according to the ISO 10993–5 standards, by a direct-contact method. They were compared with the bare silicon rubber samples chosen as a control group. The surface morphology and elemental composition of the coating is investigated by a scanning electron microscope (SEM), and the electron configuration of the Cu ion in the chitosan layer is revealed with x-ray photoelectron spectroscopy (XPS).

2. Materials and methods

Chitosan (Molecular weight: 50–800Da) was purchased from Beijing Biotechnology Co. Ltd, and Copper Sulfate ($CuSO_4{\cdot}5H_2O$, 99%) was purchased from Yongda Chemical Reagent Co. Ltd. Other chemicals (AR grade) were used as received without further purification. The medical-grade silicone rubber disk. Chitosan and $CuSO_4$ solutions were prepared the same way as published in previous works and mixed with various volume ratios of Cu and Chitosan solutions [29, 31]. 9:1, 9.8:0.2, and 9.9:0.1 volume ratios of two solutions were combined to obtain the 10:1, the 50:1, and the 100:1, Chitosan:Cu ratio.

L929 murine fibroblasts cell lines were obtained from Mingjin Biology Co. Ltd. The developed bacterial culture for cytotoxicity assay, L929 murine fibroblasts cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10 vol% fetal bovine serum (FBS) and 1 vol% penicillin-streptomycin solution. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Chemical Technology (Shanghai) Co. Ltd.

2.1. Material Preparation

Disc samples (Ø = 10 mm) were cut from a medical-grade silicone rubber mat. The disk samples were washed and sterilized for 15 min with 75% ethanol solution under ultrasonic waves and then dried at room temperature. $CuSO_4$ dissolved Chitosan and Acetic acid solutions with various Cu content were sprayed on the dried surfaces by an atomizer (nozzles size is 35 mm, 1 atm pressure). Various (table 1) Chitosan-Cu complex hydrogel-coated samples were dried at room temperature for 24 h and in an oven at 60 °C for 8 h to establish uniform Chitosan-Cu complex films on the substrate.

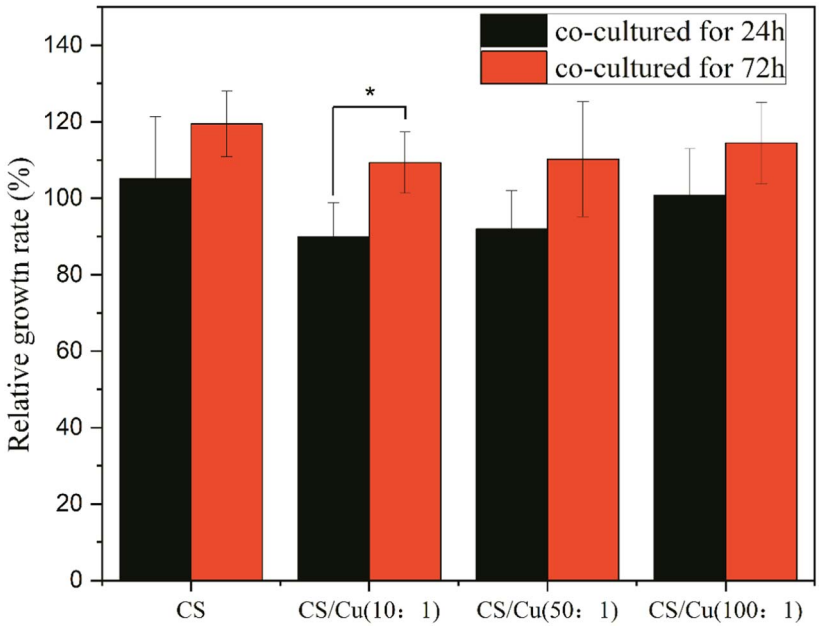

Figure 1. Relative growth rate of L929 cells co-cultured for 24 and 72 h with different chitosan films with various Cu content.

2.2. Cytotoxicity assay by direct contact

Control group and samples coated with only Chitosan and Chitosan-Cu by 10:1, 50:1, and 100:1 Chitosan:Cu ratios were put into 48-well plates with 3 × 104 cells in each well. Cultured L929 cells were detached from the culture flask by adding 0.25% trypsin-EDTA solution into a 48-well plate medium. Bacterial culture media are incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 24 and 72 h.

Bacterial culture media were removed after incubating at 37 °C for 24 or 72 h; then 225 μl of the medium and 25 μl of the CCK-8's *2-(2-methoxy-4-nitrophenyl)—3-(4-nitrophenyl)-5-(2,4- disulfophenyl)-2H-tetrazolium, monosodium salt* (WTS-8) solution was added into the wells. After incubation for 2 h, the obtained WTS-8-formazan crystals' optical density (OD) was measured by a microplate reader (FilterMax F5, Molecular Devices) with a filter with an absorbance wavelength is 450 nm. The results were expressed as percentages relative to the control experiment. Moreover, the cell viability was calculated using the following Formula (1):

$$Cell\ viability = (OD_{material} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\% \quad (1)$$

OD_{blank} represents the optical density of the well without cell, neither medium nor WTS-8, and $OD_{control}$ represents the optical density of the well with bacterial medium after adding WTS-8 without the Chitosan/Cu coating.

2.3. Surface characterization of Chitosan-Cu film-coated silicon rubber

The surface morphology of the 10:1 Chitosan-Cu film coated on the silicone rubber disc was obtained by secondary electron imaging (FE-SEM, XL 30 FESEM FEG, FEI Company, Oregon State) after a short time carbon sputtered to the surface of each specimen to bring electron conductivity to the polymer. Quantitative analysis of the surface made by Energy Dispersive Spectroscopy (Oxford Instruments) The electron configuration of Cu ions in chitosan hydrogel is investigated with x-ray photoelectrons. (ESCALAB250, Thermo VG).

3. Results

The CCK-8 assay has been used to evaluate the cytotoxicity of the chitosan and Chitosan-Cu coated samples for 24 and 72 h. The results indicate that the amount of L929 murine fibroblast cells on each substrate increased with culture time (figure 1). In 24 h, the cell viabilities of samples, with Chitosan:Cu ratios are 10:1, 50:1, and 100:1, were measured as 105.14% (±16.23), 89.90% (±8.89), 91.91% (±10.11), and 100.75% (±12.21), respectively (figure 1). There was no significant difference in the relative growth rate among the four groups. In 72 h, the cell viabilities of samples with Chitosan:Cu ratios are 10:1, 50:1, and 100:1 were measured at 119.45% (±8.57), 109.33% (±8.02), 110.24% (±15.07), and 114.45% (±10.59), respectively. There was no significant difference in

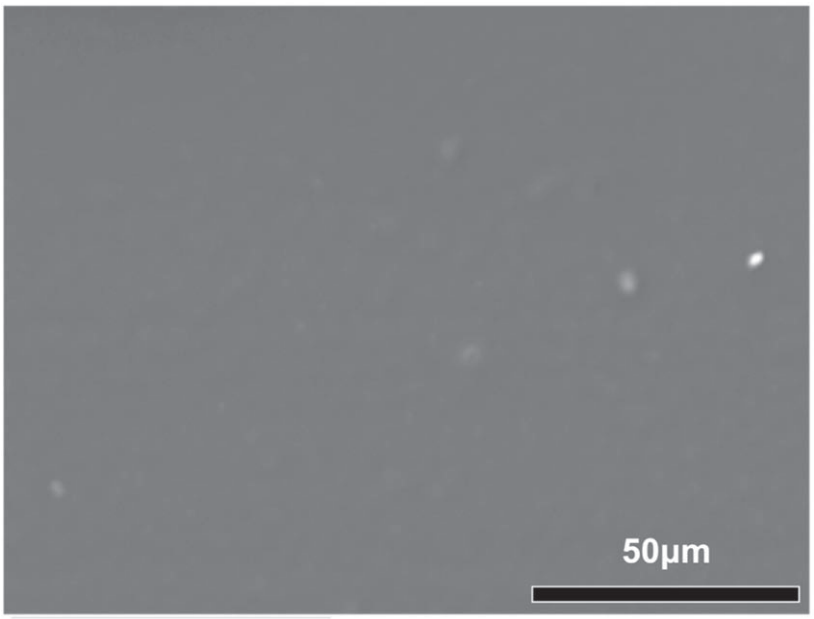

Figure 2. Surface morphology of Chitosan-Cu film on silicon rubber disk made by 10:1 Chitosan:Cu coating.

Table 2. Elemental composition of Chitosan-Cu film on silicon rubber disk made by 10:1 Chitosan:Cu coating.

Elements	Atomic percent (%)
C	63.63
O	29.17
Si	6.56
Cu	0.64
Total	100

the relative viability among the four groups. Whereas the cell proliferation measures of 10:1 (Chitosan:Cu) samples were significantly different at 72 h compared to 24 h $(P < 0.05)$.

The surface morphology of the coating with the highest Cu content was visualized by secondary electrons and is shown in figure 2. Although arbitrary discontinuities are observed on the surface, it is seen that the surface is completely covered with polymer with some defects. A quantitative surface analysis was made by reflected x-ray from the surface, and its elemental composition is listed in table 2. In addition, the presence and electronic structure of Cu ions on the surface were investigated by XPS and are shown in figure 3.

Cu 2p peaks of each composition and the control groups have been compared in figure 4. And the full spectrum of each sample is shown in figure 5.

4. Discussions

Previous studies demonstrate that Copper grafted Chitosan coating has anticoagulative, antibacterial, and antiproliferative properties. 10:1 Chitosan:Cu ratio in chitosan coating decreases the platelet adhesion by 45% in 30 min and 77% in 180 min. Furthermore, postpone blood clotting time by 8 min (where the optical density was 0.1) [31]. 10:1 Chitosan:Cu ratio in chitosan coating decrease also decreases the bacterial concentration of the surface when applied on the catheter surface also eliminates the risk of inflammation at peri-implant tissue [29]. However, toxicity might be caused by excessive copper ion exposure [39–41]. Despite the multi-functionality associated with the copper ions, they thus should be considered for long-lasting medical applications. By the summary of previous works, the average daily intake of copper is 2–5 mg in healthy adults; approximately 40% of ingested copper is absorbed from the intestine to the blood and is mainly transported into the liver. 10 mg of copper is in the blood of healthy adults. Approximately 90% of serum copper is ceruloplasmin-bound copper

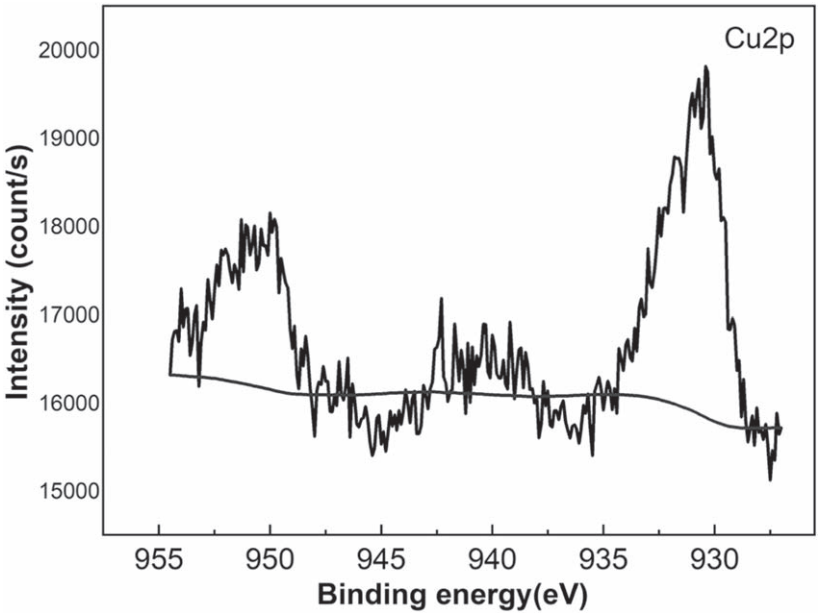

Figure 3. Binding energies of Cu-ion on the outmost surface of Chitosan-Cu film on silicon rubber disk made by 10:1 Chitosan:Cu coating.

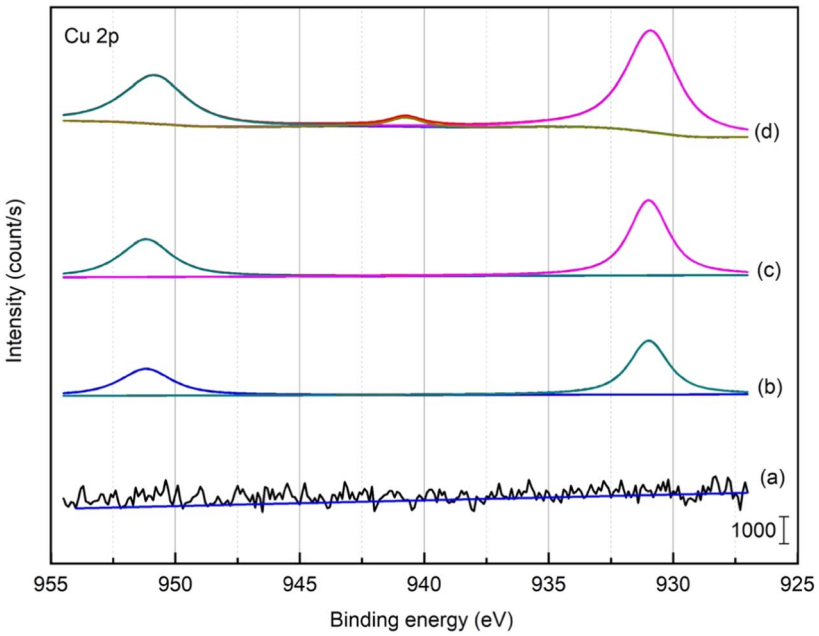

Figure 4. Binding energies of Cu ion on the outmost surface of Chitosan-Cu film on silicon rubber disk made by (a) 10:0, (b) 100:1, (c) 50:1, and (d) 10:1 Chitosan:Cu coating.

(holoceruloplasmin), while around 10% is albumin or amino acid-bound copper (free copper), which may be involved in copper transport into various tissues. Almost all the copper is excreted through the intestine in a healthy adult [40].

Besides the copper-modified chitosan coating on conventional catheters, copper-loaded rubber materials have been studied recently. MTT Cell viability assay of L929 cells contact with carboxymethyl chitosan modified CuO particles modified natural latex results shows 80% viability after 24 h [42].

Previous studies demonstrate that Cu-grafted Chitosan coating has anticoagulative, antibacterial, and antiproliferative properties. 10:1 Chitosan:Cu ratio in chitosan coating decreases the platelet adhesion by 45% in 30 min and 77% in 180 min. Furthermore, postpone blood clotting time by 8 min (where the optical density was 0.1) [31]. 10:1 Chitosan:Cu ratio in chitosan coating decrease also decreases the bacterial concentration of the

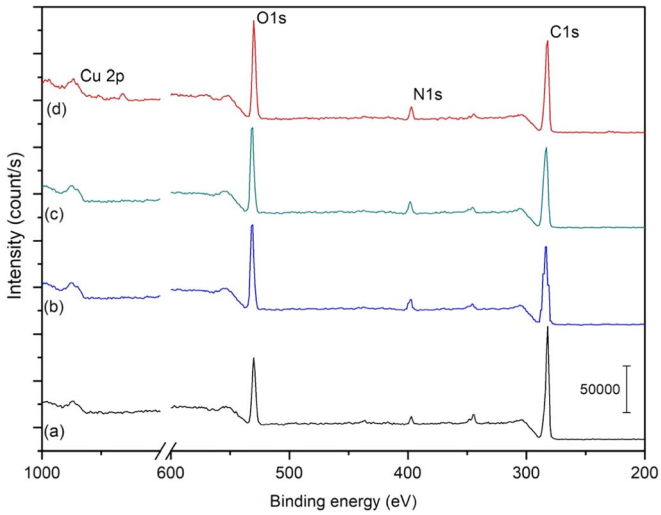

Figure 5. The full spectrum of the outmost surface of Chitosan-Cu film on silicon rubber disk made by (a) 10:0, (b) 100:1, (c) 50:1, and (d) 10:1 Chitosan:Cu coating.

surface when applied on the catheter surface also eliminates the risk of inflammation at peri-implant tissue [29]. However, toxicity might be caused by excessive Cu ion exposure [39–41]. Despite the multi-functionality associated with Cu ions, they thus should be considered for long-lasting medical applications. By the summary of previous works, the average daily intake of Cu is 2–5 mg in healthy adults; approximately 40% of ingested Cu is absorbed from the intestine to the blood and is mainly transported into the liver. 10 mg of Cu is in the blood of healthy adults. Approximately 90% of serum Cu is ceruloplasmin-bound Cu (holoceruloplasmin), while around 10% is albumin or amino acid-bound Cu, which may be involved in Cu transport into various tissues. Almost all the Cu is excreted through the intestine in a healthy adult [40].

Besides the Cu-modified chitosan coating on conventional catheters, Cu-loaded rubber materials have been studied recently. MTT Cell viability assay of L929 cells contact with carboxymethyl chitosan modified CuO particles modified natural latex results shows 80% viability after 24 h [42]. Despite chitosan coating on natural rubber increasing the biosafety of the natural rubber, still, the viability of cells is less than Cu-modified chitosan coating on silicon rubber. There are also recent works on Silver (Ag) modified chitosan coatings for natural latex; despite its bioactive properties being well studied, the biosafety of Ag-modified chitosan coatings is unknown [33].

As seen in the compared Cu 2p peak results of the 10:1 (CS:Cu) sample to 100:1 and 50:1 (CS:Cu) samples, the peak of $CuSO_4$ copper is observed. This means this film also contains copper, which is not fully chelated with chitosan.

5. Conclusion

Cu-loaded Chitosan film is an anticoagulative, antiproliferative, antibacterial coating for medical catheters. The CCK8 assay method was used to evaluate the cytotoxicity of the coating with different ratios of Cu ions after co-culturing for 24 and 72 h. The obtained results showed that all the groups' relative growth rate was above 100%, indicating that the cytotoxicity rating was Level 0. Thus, all the Chitosan-Cu coatings prepared in this study were safe. Furthermore, cytotoxicity assays prove that Cu with a maximum concentration of 10% by volume shows no toxic behavior. It is also safe because of cell viability to use in further research.

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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Author contributions

D E Erisen performed a literature search, part of the experiments, conceptualization, and manuscript writing. G S Gu performed the literature search, part of the experiments, data analysis, and visualization. S.S. Chen contributed to experimental design, resources, and project administration. M G Shen, B C Zhang contributed to experimental design. K Yang contributed to the supervision and funding acquisition. All authors have approved the final version of the manuscript. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethical compliance

There is no experiment involved with human tissues or animal subjects in this study. Only L929 cells have been used for in-vitro experiments to achieve cytotoxicity results without damaging any animal/human subject due to the 1964 Helsinki Declaration.

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