Investigation of Genipin-crosslinked Hydroxybutyl Chitosan Polymeric Stent for Cardiovascular Diseases

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A limitation of the use of polymers as stent matrices is their inherent weak mechanical strength. To reinforce the mechanical strength of the hydroxybutyl chitosan (HBC) stent, a genipin-crosslinked hydroxybutyl chitosan polymeric stent was developed. The inner structure of the HBC-genipin stent (HBC-G) was long narrow strip structure, superior to the porous structure of its counterpart, the chitosan-genipin stent (CS-G). With the increase of the crosslinking time, the crosslinking degree increased whereas the self-expansion ratio decreased. The amount of BSA absorption in the HBC-G was 48.9 ± 4.2 μg/mL, about 62.3% of that in the CS-G (78.5 ± 5.6 μg/mL). The hemolysis rate of HBC-G was under 5% and lower than the CS-G, and, which was in line with the national standard. The viability of the human umbilical vein endothelial cells (HUVECs) was above 80% at any concentration of the HBC-G, demonstrating that the stent possesses good blood compatibility and cytocompatibility to HUVECs. The results of degradation test in vitro indicated that the degradation rate of stent was associated with the crosslinking degree. HBC stent with 3 hours crosslinking time could be more easily degraded. These results provided evidence that HBC-G is exciting and promising for an alternative cardiovascular stent. Journal of Nature and Science, 1(5):e100, 2015

Hydroxybutyl chitosan | Genipin | Cardiovascular stent | Polymeric stent

1 Introduction
Cardiovascular diseases (CVDs) are the leading cause of death globally [1, 2], including disorders can affect the heart and the blood vessels, leading to myocardial infarction and peripheral artery disease. Percutaneous transluminal coronary angioplasty (PTCA), based on stent implantation, has been an important procedure in the treatment of cardiovascular diseases, particularly coronary artery disease (CAD) [3]. The first generation stent is called bare-metal stent (BMS), which is a small, tubular, wire-mesh device pre-loaded in a collapsed form onto a catheter balloon. It threads to the narrowed section of the artery and expands within the vessel. But this metal foreign object could lead immunological response with an upregulation of inflammatory mediators, ultimately cause neointimal hyperplasia. The proliferation of vascular smooth muscle cells could cause a re-narrowing of the vessel, which tends to threaten patient’s life and, what’s worse, to result in his death [4]. To avoid these problems, the drug-eluting stent (DES) was developed by coating polymer with special drugs on surface of stent for prevention of vascular restenosis [5]. DES has dramatically reduced restenosis rates, whereas the use of DES has been associated with an increased risk of late stent thrombosis and accelerated neointimal atherosclerosis, resulting in the failure of stent [6-8]. There are urgent needs for developing an alternative stent, which is able to avoid the cardiovascular disease effectively, as well as finally disappear from the blood vessel after treatment. For this purpose, the biodegradable polymeric stent is considered as a promise choice because of its favourable plasticity, biocompatibility, and biodegradability [9]. Chen et al. developed a genipin-crosslinked chitosan polymeric stent and it exhibited improved mechanical property, favourable cytocompatibility and feasibility in animal study. After loading with sirolimus, this stent coated with heparin allowed the release of drug in a sustained manner and the released sirolimus remained its activity in inhibiting smooth muscle cell proliferation [10]. Yang and his co-workers successfully synthesized PEG-PCL copolymer stent with excellent shape-memory effect functionality. The shape-memory behavior and degradation rate could be modulated by adjusting the weight ratio of PEG/PCL. The stent can also recover from a linear configuration to a spiral shape with a transition temperature around body temperature. The stent could also sustainably release the anticoagulant drug (curcumin) over 14 days and the antiproliferation drug (mitomycin C) over 70 days [11].

Chitosan (CS) is a nature polymer with good biocompatibility, biodegradability, low toxicity [12]. By conjugating the hydroxybutyl groups to the hydroxyl and amino groups of chitosan molecular skeleton, hydroxybutyl chitosan (HBC) with temperature-sensitivity can be synthesized. Compared to CS, HBC has good water solubility and the dramatic physical responses against the change of external temperature. Meanwhile, HBC has no toxicity to smooth muscle cells [13], human mesenchymal stem cells (MSCs) and intervertebral disk cells, suggesting that it has potential for application of biomedical fields [14-16]. In our previous study HBC could transform from solution to hydrogel when the environment temperature is higher than its phase inversion temperature (PIT), while the mechanical strength is limited for using as cardiovascular stent. Genipin, a kind of crosslinker, could react with the amine group on HBC molecular skeleton to form dark blue pigments [10, 17, 18]. After crosslinking reaction, the biomaterial’s mechanical and biological property of HBC may change and it is a good process to improve the HBC’s mechanical property.

In this study, genipin-crosslinked HBC polymeric stent was successfully synthesized. The characteristics of the stent were investigated in vitro including the crosslinking degree, protein absorption, blood compatibility, cytocompatibility and degradability in vitro. The objective of this study was to evaluate the potential of the genipin-crosslinked HBC stent as a stent platform.

2 Materials and methods
2.1 Materials
Chitosan (Molecular Weight, MW: 1154 KDa, Degree of Deacetylation, DD: 85%) was purchased from Laizhou Haili Biological Product Co. Ltd. Hydroxybutyl chitosan was synthesized refer to Wang’s method [14]. Genipin was provided by Shanghai Yuanye Biological Technology Co, Ltd. All other chemicals and reagents were of analytical grade and provided by Qingdao Huasheng Biological Technology Co. Ltd.

2.2 Preparation and characteristics of test stents
HBC solution (4.0 wt.%) was prepared at 4 °C. Then the temperature was elevated to 25 °C (the PIT of the HBC is 20 °C), HBC solution transferred to gel state and dried for 3 d at

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25 °C to form film. Then, the film was cut into pieces (length: 60 mm, width: 3 mm) and fixed the pieces onto the mandrel in spiral shape and immersed in a 0.5 % genipin solution (dissolved in 0.02M PBS) at 37 °C for specific time period (Fig. 1). After fixation, the stents were rinsed several times with ethanol to remove the residual crosslinking agent. Then the HBC-genipin stent (HBC-G) was taken down from the mandrel and lyophilized for 48 h. The chitosan-genipin stent (CS-G) was made by the same method. The lyophilized HBC-G and CS-G were put into the liquid nitrogen and the cross-sectional morphologies were captured with a scanning electron microscopy (SEM) (KYKY-2800B, Scientific Instrument Co., Ltd., China) after being sputter coated with platinum-gold.

The crosslinking degree of test stents was determined by the ninhydrin assay. The self-expansion ratio was calculated by the following method. The lyophilized stent was put into PBS, the stent could soaked the liquid and self-expanded in the aqueous environment to their expanded states. The self-expansion ratio was defined as the ratio of the outer diameter of the expanded stents to the outer diameter of the lyophilized stents.

2.4 Cytotoxicity of test stents

Human umbilical vein endothelial cells (HUVECs) were cultured in Dulbecco’s modified eagle medium (DMEM) containing 10 % fetal bovine serum (FBS) with 5 % CO₂ at 37 °C. Briefly, cells (3×10⁴ cells/mL) were seeded in 96-well culture plates. Various concentrations (1000, 500, 250, 100, 50 μg/mL) of the stents’ extract liquid in cell culture medium were incubated for 24, 48, or 72 h followed by MTT assay.

2.5 Biodegradability in vitro

The degradability of test stents was evaluated using lysozyme (50 000 U/mg protein, EC 3.2.1.17, Solarbio). After enzymatic degradation, the formation of oligomers containing N-glucosamine units, due to the cleaved β-glycosidic bonds of HBC and chitosan, induced an increment in the free-amino-group content in the incubation medium, which can also be determined by the ninhydrin assay [19].

2.6 Statistical analysis

Comparison between two groups was analyzed by the one-tailed Student’s t-test using statistical software (SPSS, Chicago, Ill, USA). Data are presented as mean ± SD. A difference of p < 0.05 was considered statistically significant.

3 Results and discussion

3.1 Physical characteristics of test stents

Through the above-mentioned method, HBC and chitosan stent with spiral shape were synthesized. The colour of the stent changed from white to dark blue in the preparation process (Fig. 2). The inner structures of the HBC-G and CS-G were observed by SEM (Fig. 3). The inner structure of the HBC-G covered in long narrow strip with few nanoscale holes, whereas the CS-G exhibited porous structure. It is clear that the inner structure differences have significant implications in the property of mechanical and biological of polymeric stent. Compared with CS-G, the structure of HBC-G was more compact, meanwhile the smaller holes and smooth surface in HBC-G were beneficial for improving the mechanical strength.

The crosslinking time determines the crosslinking degree between HBC and genipin. In this study, the crosslinking time respectively were 1 h, 3 h, 6 h, 9 h, 12 h. With the increase of the crosslinking time, the crosslinking degree increased and the self-expansion ratio of test stents decreased (Fig. 4 and Table 1). The value of crosslinking degree was inversely proportional to that of self-expansion ratio. The higher crosslinking degree was beneficial for enhancing the mechanical strength of stent, but would increase its brittleness. Considering for the crosslinking degree and the self-expansion ratio, the stent with 3 h crosslinking time was chosen for the next experiments.
Table 1. Self-expansion ratio of HBC-G fabricated under different time periods (n = 3).

<table>
<thead>
<tr>
<th>Crosslinking Time (h)</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-expansion Ratio</td>
<td>2.42±0.01</td>
<td>2.21±0.02</td>
<td>1.33±0.01</td>
<td>1.36±0.05</td>
<td>1.27±0.03</td>
</tr>
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3.2 Protein absorption

The amount of BSA absorption in the HBC-G was 48.9 ± 4.2 μg/mL, about 62.3% of that in the CS-G (78.5 ± 5.6 μg/mL) (Fig. 5). BSA is a nature protein with negative charge, thus it is much easier to be spontaneously absorbed by the positively charged CS-G. Compared with chitosan, the free amino group on HBC was less due to the reaction between hydroxybutyl group and amino group on chitosan molecular chain. Therefore, HBC-G exhibited less BSA absorption than that of CS-G. In addition, the inner structure of the stent also plays an important role in the BSA absorption. The porous structure of CS-G increases the surface area thereby absorbing more BSA than the HBC-G.

3.3 Hemolysis test

Both CS-G and HBC-G presented higher hemolysis rates than their corresponding raw materials (Fig. 6). When the genipin reacted with HBC or CS, the formed stents became insoluble leading to more -NH₂ protonation and the amount of the positive charge increased, resulting in reinforcement of the strength between the stents and the red cell membrane. The highest hemolysis rate was induced by CS-G (2.05 ± 0.19 %), followed by CS (1.88 ± 0.21 %), HBC-G (1.19 ± 0.24 %) and HBC (0.41 ± 0.13 %). A mean hemolysis value from three test samples of 5% or less was considered acceptable. Nonetheless hemolysis rates of all samples were under 5%, which were in line with the national standard.

3.4 Cytocompatibility test

The MTT assay results (Fig. 7) were used to evaluate the cytocompatibility of HBC stent. The cell viability was measured by the relative cell growth. There was no significant difference between the absorbance of HBC-G and CS-G at high concentration. The viability of HUVECs was above 80% at any concentration of HBC-G, indicating that the HBC-G was non-cytotoxic to HUVECs.

3.5 Biodegradability in vitro

Lysozyme widely exists in cells and blood. The HBC and chitosan could be degraded by enzymatic hydrolysis; the primary enzyme is lysozyme which target β-1,4 glycosidic bond. Before this experiment, the crosslinking degrees of (i) HBC stent with 3 hours crosslinking time (3h-HBC-G) and (ii) with 12 hours crosslinking time (12h-HBC-G) and (iii) chitosan stent crosslinked for 3 hours (3h-CS-G) were evaluated, which were respectively 38.5%, 68.7%, 54.9% (Fig. 8). The stent with lower crosslinking degree could be more easily degraded. For stent with higher crosslinking degree, the degradation reaction was difficult due to the exist of large amount of CO-NH bonds. Additionally, the electrostatic repulsion is another important factor. With the
degradation of stent, more free –NH₂ groups were exposed and protonized in the solution, and the electrostatic repulsion among –NH₂ groups further accelerated the degradation of stent.

4 Conclusion
In this study, we successfully developed a polymeric stent through HBC crosslinking with genipin. The polymeric stent exhibited improved mechanical properties, favorable biocompatibility and biodegradability in vitro than its counterpart formed by chitosan and genipin. These results indicate that the HBC-genipin stent can serve as a platform for the construction of cardiovascular stent for the treatment of vascular restenosis.

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