Preparation and characterization of a novel galactomannan based bioink for 3D bioprinting

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Abstract: The limited types of bioink significantly inhibit 3D bioprinting applied to tissue engineering. In this study, galactomannan (GM) was modified by a two steps modification method, by which GM was oxidized and then methacrylated. FTIR results showed that the two steps modification of GM is successful, and the prepared compound was named as OxGM-MA that was used to test whether it can be applied as a 3D bioprinting bioink. Compared with GM, the solubility of OxGM-MA increased and 4% (w/v) OxGM-MA solution was applied to further tests. This GM based bioink, the 4% (w/v) OxGM-MA solution, displayed a low viscosity, which is 8.5 mPa·s, and is able to solidified by blue light irradiation. Additionally, its light solidified hydrogel displayed a pore network structure with the compressive strength of 52.3±6.7 kPa, which is strong enough for tissue engineering operations. By carrying chondrocytes, the GM based bioink was printed into a hydrogel with designed shape, which was created with CAD software previously. Following bioprinting, chondrocytes within the printed hydrogel exhibited good viability during 3 days incubation with cell culture medium, indicating the good printability and biocompatibility of the novel GM based bioink. It suggested that the novel GM based bioink can be potentially applied to cartilage tissue engineering in the future.

1. Introduction
Three-dimensional (3D) bioprinting enables tight control of structure-property relationships and can print specific shapes according to application requirements, which make 3D bioprinting become a versatile and potent tool for biomedical and clinical applications. The performance and functionality of 3D bioprinting are largely determined by the biochemical, rheological, and material properties of the bioink[1]. High viscosity bioink will reduce the survival rate of cells after printing, while low viscosity bioink cannot stably maintain the specific shape of hydrogel after printing[2]. Since very few bioinks are available for 3D bioprinting, it is necessary to develop new bioinks[3].

Cartilage is an aneural, avascular and alymphatic tissue, which makes it have very limited self-repairing ability[4]. Scaffold-based tissue engineering (TE) is a promising strategy for the regeneration of articular cartilage compared with other treatment strategies[5]. Hydrogel is a three-dimentional structure, and more than 80% its components is water, that is similar to natural...
cartilage\textsuperscript{[6]}. Hydrogel with living chondrocytes obtained by 3D bioprinting seems to be a feasible approach for cartilage tissue engineering.

Galactomannans (GMs) are featured with 1, 4-linked-β-D-mannopyranosyl back-bone, which is substituted to varying degrees at O-6 by α-D-galactopyranosyl residue\textsuperscript{[7]}. GMs are widespread non-ionic polysaccharides and displayed a good biocompatibility\textsuperscript{[8]}. By using L929 fibroblasts, Everton et al demonstrated that no cytotoxicity of GM was observed\textsuperscript{[9]}. But the high viscosity and slow degradation of GM solution limit its application in 3D bioprinting\textsuperscript{[10]}. Oju Jeon et al oxidized alginate to form an open-chain adduct, which makes it easier to hydrolyze and higher water solubility, and we speculated that oxidized GM may enhances its water solubility as well\textsuperscript{[11]}

In this study, GM is firstly oxidized to improve its solubility, and subsequently GM is grafted with methacrylic acid to obtain photo-crosslink ability. By carrying rabbit chondrocytes, prepared GM based bioink was tested with a 3D printing machine to form a hydrogel with designed shape.

2. Materials and methods

2.1 Materials

Galactomannan (GM, 99%) was purchased from Henan Kangzhiwang Biological Technology Co. Ltd (Henan, China). Sodium periodate (NaIO\textsubscript{4}) was purchased from Sangon Biotech (Shanghai, China). Methacrylate anhydride (MA, 94%), cellulose dialysis membranes (MWCO 3.5 kDa) and penicillin/streptomycin (P/S) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenyl(2,4,6-trimethylbenzoyl) phosphinate (LAP) was purchased from Shanghai Yuchang New Materials Co (Shanghai, China). Fluorescein diacetate (FDA) was purchased from Shanghai Yisheng Biotechnology Co. Ltd (Shanghai, China). Distilled water (dH\textsubscript{2}O) and double distilled water (ddH\textsubscript{2}O) were prepared from SZ-93 automatic double pure water distiller. Phosphate buffer saline (PBS, pH 7.4) was prepared by adding 8 g NaCl, 0.2 g KCl, 1.44 g Na\textsubscript{2}HPO\textsubscript{4} and 0.24 g KH\textsubscript{2}PO\textsubscript{4} to 1 L double distilled water (ddH\textsubscript{2}O). High glucose DMEM and FBS was purchased from Gibco (Waltham, MA, USA).

2.2 The two steps GM modification method

The GM was oxidized following the method described by Yan S et al\textsuperscript{[12]}. 1 g GM (monosaccharide unit is 0.0062 mol) was dissolved in 200 mL of dH\textsubscript{2}O and continuously stirred for 12 h. In order to obtain polysaccharide with theoretical oxidation degree of 25%, sodium periodate with a molar ratio of 1:4 to monosaccharide units in polysaccharide was added to the solution to react for 6 hours in the dark. Then ethylene glycol with the same molar was added for 1 hour to end the reaction. The solution was dialyzed with dH\textsubscript{2}O in dialysis membrane for three days to remove the excessive ions. Then the reaction product was freeze-dried to obtain crude oxidized GM (OxGM). In order to ensure the solubility of the product, the crude OxGM was dissolved in 3% PBS and heated in a water bath at 50 °C for 1 hour. The supernatant obtained by centrifugation (7000 rpm, 10 min) was dialyzed in dH\textsubscript{2}O with dialysis membrane for three days, and finally lyophilized to obtain the finished product OxGM.

OxGM (0.6 g) was dissolved in 120 ml PBS (pH 7.4) by stirring electromagnetically for 3 hours. MA (0.27 g) in 20 mL PBS of which the mass ratio to OxGM is 45% was dropwise added to the OxGM solution. The reaction lasted for eight hours at room temperature in the dark. At the same time, the pH of the reaction solution was maintained between 8 and 9 by adding 5 M NaOH. Next, the solution was dialyzed with dH\textsubscript{2}O in dialysis membrane for three days to remove the excessive ions. In the end, the solution was lyophilized for three days by the freeze dryer and the resulting product was named as OxGM-MA.

2.3 Characteration of OxGM and OxGM-MA

FTIR spectra of samples were recorded at room temperature using Fourier transform infrared spectrometer (Thermo Nicolet Nexus 470, USA). The samples were prepared by the KBr disc method. The degree of oxidation (the percentage of oxidized monosaccharide units) was determined by the
amount of aldehyde following the method used by Zhao & Heindel[13]. These characterization methods were used to determine the structure and chemical modification degree of hydrogels.

2.4 Preparation and Characterization of hydrogels

2.4.1 Preparation of OxGM-MA hydrogels. OxGM-MA hydrogel was prepared with 4% (w/v) OxGM-MA in PBS (pH 7.4) by employing LAP (0.5% w/v) as photo initiator via photo-polymerization. The solution was placed under blue light (405 nm) source for 10 seconds to obtain hydrogel in the mold, which was named as OxGM-MA-4.

2.4.2 Scanning electron microscopy, pore size and porosity of lyophilized hydrogels. OxGM-MA-4 was lyophilized at -80 °C for two days. The lyophilized hydrogels were cut with a blade and pasted on silicon chip with conductive glue. Then the lyophilized hydrogels were coated with a thin layer of gold before observation. The microstructures of lyophilized hydrogels were observed by scanning electron microscopy (SEM) at an accelerating voltage of 30 kV (FEI Quanta 200, FEI Company, Czech Republic). The average pore size of lyophilized hydrogels was measured by using ImageJ.

2.4.3 Porosity. The porosity of lyophilized hydrogels was tested by true density meter (JW-M100A, JWGB SCI. and TECH, China). The weight (W) and the volume (V) of lyophilized hydrogels were measured. The true density (ρt) of lyophilized hydrogels was measured by a true density meter when the lyophilized hydrogel was put into the sample cup. The porosity of lyophilized hydrogels (α) were obtained following the equation:

\[
α% = \left(1 - \frac{ρ_s}{ρ_t}\right) × 100\%
\]  

where \(ρ_s = \frac{W}{V}\)

2.4.4 Swelling. The swelling rates of hydrogels were determined gravimetrically by the following method. The hydrogels were formed by blue light in a mold and then immersed in enough dH2O. The excessive of water on the surface of collected sample was absorbed with filter paper before weighed at predefined time intervals. The swelling ratios of hydrogels (SD) were calculated following the equation:

\[
SD% = \left(\frac{W_s - W_d}{W_d}\right) × 100\%
\]

where \(W_d\) represents the dry weight of hydrogels and \(W_s\) is the weight of swollen hydrogels.

2.4.5 Rheological properties of OxGM-MA. The viscosity of OxGM-MA was measured by rotational rheometer (HakkeMars 60, Germany) for 60 s at 25 °C. At constant temperature in shear mode, 5mL OxGM-MA solution was measured with No.60 rotor. All measurements were repeated three times.

2.4.6 Mechanical testing. The mechanical properties of OxGM-MA hydrogels were measured by universal mechanical testing machine (SHIMANZU, AGS-N, Japan). The strain gauge of the testing machine is 20 N, and the precision is 0.0001 N. Compression rate of strain gauge is 1 mm/min, and displacement accuracy is 0.0001 mm. The diameter of the hydrogels used for the test was 4.2 mm, and the height was 4 mm. The compression was finished when the hydrogel structure was defective.
2.5 3D printing tests.

3D printing machines used in this study are all based on the stereolithography (SLA) technology. SLA is printed layer by layer by controlling the shape of illumination. The technique is based on a photosensitive initiator molecule initiated free radical photo-polymerization. SLA presents several advantages such as short curing time, high printing accuracy, and energy saving.

The solution obtained by dissolving 4% OxGM-MA (w/v), 0.5% LAP (w/v) and 0.03% sodium citrate (w/v) in PBS was used as bioink. 3D printing machines is a digital light processing (DLP) micro 3D printing system (nanoArch S140, BMF Material Technology Inc, Shenzhen, China) equipped with a 405 nm laser.

Isolation and culture of rabbit chondrocytes was following our previous publication without any modification. Then the chondrocytes were uniformly mixed into the bioink at a concentration of 1.2×10⁷ cells/mL. The parameters of the printing process are shown in Table 1. The printed hydrogel was placed in a cell culturing plate containing culture solution for cell culture and then it was transferred to an incubator. The structures of printed hydrogels were observed by a digital microscope (Dino-Lite, ANMO ELECTRONICS Corporation). The growth morphology of chondrocytes were characterized at 1 and 3 days using FDA staining, and were observed with a fluorescence microscope (Olympus, IX73).

2.6 Statistical analysis

All data are stated as mean ± standard deviation of representative experiments performed in triplicate. Statistical analysis was conducted using origin 8.5 software. Statistically significant differences between groups were detected by one-way analysis of variance (ANOVA) at a confidence interval of 95%.

3. Results and discussion

3.1 Synthesis of the novel GM based bioink-OxGM-MA

The aqueous solution of pure GM exhibits a cloudy look and its viscosity is high. Moreover, its highest solubility is not more than 1% (w/v) at room temperature. Partial oxidation modification of GM with sodium periodate was carried out firstly to obtain oxidized GM, named as OxGM. Then OxGM was methacrylated to prepared the novel GM based compound, named as OxGM-MA, which was tested whether can be used as a bioink for 3D bioprinting.

FTIR spectrum shows difference between the spectrograms of GM and OxGM (Figure 1b). A weak absorption peak can still be seen near 1728 cm⁻¹, which is related to the C=O symmetrical vibration of aldehydes (Figure 1a). It proved that GM was successfully oxidized. The actual oxidation degree of OxGM measured by NaOH titration is 22.0%. And it is obvious that the absorption peak of C=O appears at 1718 cm⁻¹, which is the ester bond obtained after OxGM is modified by methacrylic anhydride (Figure 1a). It demonstrated that OxGM-MA was prepared successfully by applying the two steps modification method to GM.

![Figure 1](image-url)

Figure 1. (a) FTIR of GM, OxGM and OxGM-MA, (b) Partial enlarged spectrum of the FTIR of GM, OxGM.
3.2 Properties of the novel GM based bioink

To test whether the solubility of OxGM-MA was increased compared with GM, 4% (w/v) OxGM-MA solution was prepared successfully, but saturated GM solution only contains less than 1% (w/v) GM (Figure 2a and Figure 2b). This result indicated that the solubility of OxGM-MA is higher than that of GM.

According to our hypothesis, the viscosity of OxGM-MA solution is lower than that of GM. To validate this, 4% (w/v) OxGM-MA solution and 1% (w/v) GM solution were prepared, and it showed that the viscosity of GM solution is higher than that of OxGM-MA solution (Figure 2c and Figure 2d). By using rotational rheometer (HakkeMars 60, Germany), the viscosity of 4% (w/v) OxGM-MA solution is 8.5 mPa·s, which is suitable for 3D bioprinting (Figure 2e). The viscosity of 1% (w/v) GM solution is beyond the test range of this machine.

Since the photo-crosslink ability of bioink is very important for its printing efficiency, the photo-crosslink ability of the GM based bioink was examined. The solid hydrogels were rapidly formed after the GM based bioink, 4% (w/v) OxGM-MA solution, was irradiated by blue light for 10 seconds, demonstrating that the novel GM based bioink can be printed with SLA (Figure 3a).

The lyophilized light solidified OxGM-MA hydrogel displayed a porous structure inside and the pore connectivity were good (Figure 3b). Via the measurement and analysis of SEM images by ImageJ software, it is known that the pore size of lyophilized OxGM-MA hydrogel was 155±54 μm, which is suitable for cell growth and nutrients transporting for tissue engineering applications. Besides, the open porosity of lyophilized OxGM-MA hydrogel was 80.3%. In a word, the spatial structure of light solidified OxGM-MA hydrogel basically meets the basic performance requirements of tissue engineering scaffolds.

Figure 2. (a) Morphology of 0.1 g GM, 0.05 g GM and 0.4 g OxGM-MA, (b) Morphology of 0.1 g GM (1%, w/v), 0.05 g GM (0.5%, w/v) and 0.4 g OxGM-MA (1%, w/v) dissolved in 10 mL PBS, (c) Morphology of 1 mL 1% GM and 4% OxGM-MA solution in the bottle, (d) Morphology of 1 mL 1% GM and 4% OxGM-MA solution when the bottle was put upside down for 10 seconds, (e) The effect of shear rate on the viscosity of 4% (w/v) OxGM-MA solution (25 ℃).
3.3 Properties of light solidified OxGM-MA hydrogels

The sample of light solidified GM based bioink were prepared by irradiation 4% OxGM-MA solution with blue light. Swelling means the amount of liquid that hydrogel can absorb or store. The swelling property of hydrogel also reflects its hydrophilicity and stability in water to some extent. It exhibited that the swelling degree of the light solidified OxGM-MA hydrogels stabilized after 24h incubation with water (Figure 4a). And the swelling degree of light solidified OxGM-MA hydrogel is about 1900%.

Hydrogel, as a kind of tissue engineering scaffold, is required to provide a sufficient mechanical strength for tissue engineering operations. Before reaching the yield stress, the deformation of the hydrogels increased with the increase of stress (Figure 4b). This showed that the hydrogel can undergo reversible deformation within the compressive strength. The compressive strengths of light solidified OxGM-MA hydrogels is 52.3±6.7 kPa that meets the requirement of cartilage tissue engineering operations.

3.4 Applying the novel GM based bioink to 3D printing

Since the important indexes to evaluate bioink is the properties of hydrogel obtained after 3D bioprinting, the GM based bioink was tested by employing the SLA, a common used 3D bioprinting machine. Rabbit chondrocytes suspension solution was prepared by suspending them in the PBS mixture including OxGM-MA (4%, w/v), LAP (0.5%, w/v), and then rabbit chondrocytes suspension solution was loaded into the ink-tank of SLA. Before applying 3D bioprinting, the optimized printing parameters of SLA only using the novel GM based bioink were explored, and the preliminary results
are shown at the upper line of the table 1. After rabbit chondrocytes were mixed into bioink, the transparency of the solution decreased, which resulted in fluctuation of printing parameters. The exposure intensity and exposure time had been adjusted, and the results were shown at the lower line of the table 1.

With the optimized printing parameters, the mixture of the GM based bioink, LAP and chondrocytes were printed into a product by 3D bioprinting. The printed hydrogel displayed the precise shape designed by CAD with the excellent outline (Figure 5a), which facilitates the high-precision manufacture. After 1 day of incubation with cell culture medium, the printed hydrogel was observed by FDA living cell staining. The outline of hydrogel was perfect, and the cells survived well and were well-distributed in hydrogel (Figure 5b). After three-day culture, chondrocytes showed chondrocyte phenotype. It could be seen from the figure 5(b) b4 that the cells became polygonal and formation of cell clusters. It illustrated that the novel GM based bioink with 3D bioprinting can be adopted for tissue engineering applications.

### Table 1. 3D printing parameters of SLA

| Printing Materials         | Exposure intensity (mW/cm²) | Exposure time (s) | Section number (100μm/layer) | Printing time (min) |
|----------------------------|-----------------------------|------------------|------------------------------|--------------------|
| The GM based bioink only   | 7                           | 5                | 10                           | 12                 |
| The GM based bioink+cells  | 10                          | 5                | 10                           | 15                 |

Figure 5. (a) Optical image of the hydrogel sample manufactured via 3D bioprinting with the novel GM based bioink and chondrocytes (scale bar, 0.5 mm), (b) FDA staining of samples prepared via 3D bioprinting with the novel GM based bioink and chondrocytes following 1 day (b1 and b2) and 3 days (b3 and b4) incubation (scale bar, b1, b3, 500 μm; b2, b4, 200 μm).

4. Conclusion

By using the two steps modification method, a novel GM based bioink, OxGM-MA, was prepared successfully according to the results of FTIR spectrum. Compared with GM, OxGM-MA displayed a higher solubility, and its solution showed a low viscosity and photo-crosslink ability that is suitable for 3D bioprinting applications. The internal porous structure and the mechanical properties of light solidified OxGM-MA hydrogel suggested that it is a proper scaffold for tissue engineering. By mixing chondrocytes with this novel GM based bioink, a hydrogel with CAD designed shape was accurately printed out with SLA. Chondrocytes within this hydrogel manufactured by 3D bioprinting are alive and proliferated following 3 days incubation with cell culture medium. It suggested that the novel GM based bioink is a promising bioink of 3D bioprinting for cartilage tissue engineering applications.

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