Bletilla striata Polysaccharide Cryogel Scaffold for Spatial Control of Foreign-body Reaction

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Research Article

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Abstract

Background

Implantation of a biomaterial may induce the foreign-body reaction to the host tissue that determines the outcome of the integration and the biological performance of the implant. The level of foreign-body reaction can be modulated by material properties.

Methods

First, we synthesized methacrylated *Bletilla striata* Polysaccharide (BSP-MA) and constructed a series of open porous cryogels utilizing this material via the freezing-thawing treatment of solvent-precursors systems. Second, Pore size and rheology were measured to characterize the material properties of cryogels. Live/dead staining of cells and CCK-8 was performed to test the cytocompatibility of the scaffolds. In addition, the Real-Time qPCR experiments were carried for *in vitro* tests. Finally, the BSP scaffolds were implanted subcutaneously to verify the foreign-body reaction between host tissue and materials.

Results

Our data demonstrated that cryogels with different pore sizes and modulus can be fabricated by just adjusting the concentration. Besides, the cryogels show well cytocompatibility in the *in vitro* experiments and exhibited upregulated expression levels of pro-inflammation-related genes (*Tnfa* and *Il1b*) with the increase of pore size. *In vivo* experiments further proved that with the increase of pore size, more immune cells infiltrated into the inner zone of materials. The foreign-body reaction and the distribution of immune-regulatory cells could be modulated by tuning the material microstructure.

Conclusions

Collectively, our findings revealed *Bletilla striata* polysaccharide cryogel scaffold with different pore sizes can spatially control foreign-body reaction. The microstructure of cryogels could differentially guide the distribution of inflammatory cells, affect the formation of blood vessels and fibrous capsules, which eventually influence the material-tissue integration. This work demonstrates a practical strategy to regulate foreign body response and promote the performance of medical devices.

Background

The rapid development of regenerative medicine has brought much promise to tissue maintenance, repair and host defense.[1–4] As a prominent tool in regenerative medicine, the tissue engineering has been an active field of scientific research for nearly three decades. [5, 6] The key to develop tissue engineering is the design of applicable bioactive materials. With the development materials science and tissue engineering, The range and degree of biomaterial sophistication has also dramatically increase.[7, 8] In addition to the basic characters of biocompatibility, biodegradability, mechanical properties, porosity,

considering of the further application in clinical, biological activity has been placed in an important position.[9][10] For example: cell adhesion and growth, vascularization, and biological recognition. Therefore, polysaccharides, as a kind of biological polymers, have come to the stage.[11]

Chinese medicines, as resources repository, have been widely applied in tissue repair and been proved effective in the past years. [12] Among them, *Bletilla striata*, has been used as an astringent hemostatic medicinal for thousands of years.[13, 14] The medicinal part is generally regarded to be its pseudobulbs and it has the effects of restraining bleeding, stopping bleeding, reducing swelling, resolving mass and promoting tissue regeneration. The effective component of it has been proved by modern pharmacology to be *Bletilla striata* polysaccharide (BSP).[15] In general, Chinese medicine derived polysaccharides are barely used in biomaterials. However, natural polysaccharides are demonstrated to be a potential biomaterial and have advanced interaction with tissue in diverse ways. EUP3, as a polysaccharide derived from *Eucommia ulmoides*, is demonstrated to have an extraordinary interaction with platelet-derived growth factor-BB thus leading to a Growth Factor-affinitive scaffold.[16] Besides, glucomannan (GM) polysaccharide derived from konjac was proved to modulate the action of macrophage[17]. Beyond that, the *Bletilla striata* polysaccharide we mentioned above has already been designed into scaffold and showed the capacity to promote angiogenesis.[18] Nevertheless, it's obvious that there are many defects as well. First, it is difficult to extract pure and homogeneous polysaccharide from natural production, thus stable processes and appropriate characterization methods need to be established. Second, since nature derived polysaccharides are not suitable to be used as biomaterials directly, the modification and design strategy of polysaccharides become key issues.

Related researches have been done before and discovered that glucomannan can stimulate macrophages to produce pro-regenerative cytokines thus promoting angiogenesis and tissue repairing. [19] However, the BSP still has challenges to be a three-dimensional scaffold for tissue engineering, the most common problem is the foreign-body response(FBR), which is a result of the wound healing response altered by the presence of a foreign body.[20–23] The properties of the implantation are provided to effect the degree of reaction. In this study, we modified the polysaccharide with a well-defined structure into a series of three-dimensional scaffolds with only one parameter changing: pore size, and investigated the FBR and its potential application as tissue engineering scaffolds.

Methods

1. Materials

Bletilla striata (China Pharmaceutical Corporation-Canton, Guangzhou, China); *Bletilla striata* polysaccharide (BSP) was prepared following our laboratory established and reported protocol; Fetal bovine serum and DMEM medium were obtained from Life Technologies; Calcium AM/PI kit was purchased from shanghaiyisheng (China); CCK8 kit; GoTaq 2-Step RT-qPCR system was purchased from Promega; TRIzol Reagent was obtained from Sigma-Aldrich; Other chemicals and reagents were purchased from Sigma-Aldrich unless otherwise stated.

The primers of relevant genes were listed as follows

Mouse beta-actin: Forward: 5'-GCTGGTCGTCGACAACGGCTC-3'

Reverse: 5'-CAAACATGATCTGGGTCATCTTTTC-3'

Mouse *Nos2*: Forward: 5'-CCAAGCCCTCACCTACTTCC-3'

Reverse: 5'-CTCTGAGGGCTGACACAAGG-3';

Mouse *Il1b*: Forward: 5'-GCAACTGTTCTGAACTCAACT-3'

Reverse: 5'-ATCTTTTGGGGTCCGTCAACT-3';

Mouse *Tnfa*: Forward: 5'-ACGGCATGGATCTCAAAGAC-3'

Reverse: 5'-AGATAGCAAATCGGCTGACG-3';

Mouse *Mrc1*: Forward: 5'-GTGGTCCTCCTGATTGTGATAG-3'

Reverse: 5'-CACTTGTTCTGGAAGTCAAGTA-3';

Mouse *Tgfb*: Forward: 5'-TGGAGCAACATGTGGAAGTCA-3'

Reverse: 5'-TGCCGTACAAGTCCAGTGAC-3';

Mouse *Arg1*: Forward: 5'-CAGAAGAATGGAAGAGTCAG-3'

Reverse: 5'-CAGATATGCAGGGAGTCACC-3';

Mouse *Osm*: Forward: 5'-AACTCTTCCTCTCAGCTCCT-3'

Reverse: 5'-TGTGTTTCAAGTTTTGGAGGC-3';

Mouse *Vegfa*: Forward: 5'-GTTTCAAGAGCGGAGAAAGCAT-3'

Reverse: 5'-TCACATCTGCAAGTACGTTTC-3';

2. Synthesis of methacrylated BSP

Oxidation of BSP: the C6 primary hydroxyls of *Bletilla striata* polysaccharide are oxidized to C6 carboxylate groups by TEMPO/NaClO/NaClO₂ oxidation system in sodium acetate buffer (0.2 M, pH 5.0). After stirring at 40 °C for 24h, oxidation was quenched by adding excessive ethanol. The precipitate of oxidated BSP was collected by centrifugation. Then, oxidized products were dialyzed (MWCO: 3,500) with milli-Q water, and lyophilized.

Preparation of methacrylated BSP: oxidized BSP was dissolved in a buffer solution (1% w/v, pH 6.5) of 50 mM 2-morpholinoethanesulfonic acid (MES). N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (molar ratio of NHS:EDC =1:2) were added to the solution to activate the carboxylic acid groups of the oxidized BSP. After activation for 5 min, AEMA (molar ratio of NHS: EDC: AEMA= 1:2:1) was added to the mixture and the reaction was maintained at room temperature for 24 h. The precipitate of methacrylated BSP was collected by centrifugation. Then, oxidized products were dialyzed (MWCO: 3,500) with milli-Q water, and lyophilized.

3. Characterization of methacrylated BSP

Characterization of oxidized BSP: oxidized BSP was dissolved in deuterated dimethylsulfoxide (DMSO-d₆), and the ¹³C NMR spectra of these glucomannan/DMSO solutions were recorded. Carboxylate content of oxidized BSP was determined by potentiometric titration method. 0.1M HCl was added to methacrylated BSP solution and set pH value in the range of 2.5-3.0, then record HCl volume. 0.1M NaOH solution was added up to pH11 and record NaOH volume.

Characterization of methacrylated BSP: methacrylated BSP was characterized by ¹H- NMR analysis and the efficiency of BSP methacrylation was determined from ¹H-NMR spectra based on the ratio of the integrals for the internal standard protons to the methylene protons of methacrylate.

The FTIR spectra of lyophilized pure BSP and BSP-MA were obtained on KBr pellet performed on a FTIR spectrophotometer (MAGNA IR560, Nicolet). All spectra were recorded with the resolution of 4 cm⁻¹ in the range 400-4000 cm⁻¹.

4. Methacrylated BSP scaffolds preparation

GM scaffolds preparation: methacrylated BSP solution (2%-10%) was synthesized using deionized water as a solvent. Then add tetramethylethylenediamine (TEMED) [0.5% (wt/vol)] and ammonium persulfate (APS) [0.25% (wt/vol)] to the methacrylated BSP solution which was precooled to 4 °C to decrease the rate of polymerization. After a complete incubation in -20 °C refrigerator for one night, the cryogels were put at room temperature to remove ice crystals and washed with milli-Q water.

5. Pore size and rheology measurement of methacrylated BSP scaffolds

GM scaffolds was stained with fluorescent dyes (FITC). A solution of FITC, 1mM in 20 mM Na-carbonate buffer (pH 9.4) was applied to the stained scaffold for 24 h and thoroughly washed with buffer and water. The stained cryogels were sectioned into slices. Samples were examined by confocal laser scanning microscopy (CLSM) (Leica TCS SP8), using a 20× objective and excitation and emission wavelengths 488 and 530 nm. ImageJ software (<http://rsb.info.nih.gov/ij/>) was used to analyze images to obtain the pore size and pore size distribution.

Flow and deformation of materials in response to applied force can be studied by rheology. Elastic modulus and elastic nature of the material is defined as storage modulus (G'). The dissipation (viscous) of the flow is represented by loss modulus (G''). The visco-elasticity behavior or phase angle is the difference between the storage and loss modulus. The cryogels used in experiments had 1.5 cm diameter and 2 mm thickness cylindrical shape. Amplitude sweep (strain sweep) test was applied from 0.01 to 100% at the constant frequency of 1 Hz to determine linear viscoelastic region. Then, frequency sweep measurement was performed from 0.01 to 100 Hz at a controlled strain of 0.2% to investigate the modulus change related to the oscillatory frequency.

6. Cell Culture

RAW 264.7, a murine monocyte/macrophage cell line, and human umbilical vein endothelial cells (HUVECs) were purchased from the ATCC (American Type Culture Collection). Cells were cultured in DMEM high glucose medium and RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin under 5% CO_2 at 37 °C. Cells were passaged after reaching 80% confluence.

7. Live/dead staining of cells in scaffolds

RAW 264.7 macrophages and HUVECs were washed by PBS. After cell counting, the cells were centrifuged and re-suspended in culture medium solution at a final concentration of 5×10^6 cells per milliliter. 3D scaffolds were sterilized in 75% ethanol for one night. Next, the cell solution was added to the 3D scaffolds and cells can be absorbed into the 3D scaffolds. The cell-laden cryogels were then placed in the atmosphere of 37° C with 5% CO_2 for 6 h to allow cells attachment inside the scaffold.

A live/dead assay was performed to test cell viability in cryogels. Scaffolds loaded with cells in triplicate were incubated with the mixture dye solution containing 1 μL of Calcein-AM and 0.5 μL propidium iodide (PI) in 1 mL of PBS. After 30min incubation, the cryogels were rinsed with PBS and cells were imaged by confocal laser scanning microscopy (CLSM) (Leica TCS SP8). Green fluorescence represents live cells and red fluorescence was related to dead cells.

8. Cell proliferation assay in scaffolds

RAW 264.7 macrophages and HUVECs were seeded in scaffolds as previously mentioned. Scaffolds were placed in a 96-well plate at the density of 2×10^5 cells/scaffold. 6 h after seeding, the cell-laden scaffolds were rinsed with PBS and transferred to another new well to remove the unattached cells. To evaluate cell proliferation, at day 1 and 3, the culture medium was replaced with the cell counting kit-8 (CCK-8) working solution and incubated at 37 °C for 3 h. The CCK-8 solution was collected and the absorbance value was measured with the multi-plate reader at wavelength of 450 nm.

9. Cell infiltration and distribution in scaffolds

RAW 264.7 macrophages were seeded in scaffolds as previously mentioned. Calcein-AM solution was added to the sample. After 30 min incubation at 37 °C, the cell-laden cryogels were observed by CLSM. All

images were generated by optical sectioning in the z-direction. Optical sections each of 10µm were taken to produce a 250µm z-stack for image processing.

10. Real-Time qPCR

RAW 264.7 macrophages were seeded in scaffolds at a seeding density of 5×10^4 cells/scaffold. Cell-laden scaffolds were rinsed with PBS and transferred to another new well to remove the unattached cells and 1.5 ml of new culture medium was added. Then, samples were incubated in CO₂ incubator for 24h.

RNA was isolated by kit. RNA was reverse-transcribed into cDNA. Quantitative real time PCR (q-PCR) measurements were performed using a SYBR Green RT-PCR kit. Marker genes including *Tnfa*, *Il1b*, *Nos*, *Mrc1*, *Tgfb*, *Arg1*, *Vegfb* and *Osm* were selected for analysis with the primer sequences using the 2^{-ΔΔCt} relative quantification method.

11. Implantation of cryogel scaffolds

Male C57BL/6J mice (6-8 weeks) were used with cryogel scaffolds subcutaneous implanted in the back for assessing the host response and the biocompatibility of cryogel scaffolds. All procedures were approved by the Animal Ethics Committee, University of Macau. We divided 18 mice into three groups and treated them with hydrogel and BA2 and BA8 respectively embedded. The mice in each group were divided into 2 days and 14 days.

Before surgery, the mice were anesthetized with intraperitoneal injection of sodium pentobarbital (70 mg/kg) and then the dorsal hair was shaved. After the sterilization of skin with 75% ethanol, two independent incisions were made on the back, and the scaffolds were embedded in and then the wound were sewn up.

12. Histology Analysis

After two days and 2 weeks housing, the mice were sacrificed and the implants along with the 2 cm × 2 cm skin tissue samples were collected and immediately fixed in 4 vol.% formalin and dehydrated by gradient ethanol before embedding in paraffin wax. These samples were cross-sectioned into 6 µm for histological analysis. The sections were deparaffinized and rehydrated for Hematoxylin–eosin (H&E), Masson's trichrome staining. Besides, the deparaffinized and rehydrated sections were blocked and stained by anti-VEGF, anti-CD31 and anti-CD86 for immunohistochemistry. The images were recorded by a light microscope (BX51; Olympus). ImageJ software was utilized to quantify the fibrous capsule thickness, immunohistochemistry staining (with the assistant of IHC Toolbox plugin), and obtain the cell coordinate datasets followed by calculating the minimum cell distance in R language.

13. Statistical analysis

Statistical differences among samples were studied through t-test or the one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. The data presented as the mean ± standard deviation

was obtained based on at least three independent replicates. Significance was set to $p < 0.05$. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Results

1. Synthesis and characterization of glucomannan derivatives

There is no report of methacrylated on BSP up to now. Therefore, we first propose a modification method of BSP to MA. C6-OH groups of BSP are oxidized to C6 carboxylate groups by TEMPO/NaClO/NaClO₂ oxidation system (Fig. 1a). [24] The ¹³C-NMR spectrum with the peak at 180ppm indicated that C6 primary hydroxyls were successfully oxidized to carboxylate groups (Fig. 1b). Determined by potentiometric titration method, the carboxylate content of C6-OH was 2.12 mmol/g and oxidation ratio of C6-OH was 37.06%. (Fig. 1d) Then these carboxylate groups are linked with AEMA to introduce a carbon-carbon double bond, which can be crosslinked via chemical method, TEMED/APS initiation system based on an autocatalytic reaction (Fig. 1a). At last, BSP was successfully methacrylated.

The ¹H-NMR spectra of methacrylated BSP exhibit peaks of vinyl methylene and methylene protons that were newly formed by the reaction with AEMA, which are located at δ 6.2, 5.7 and 2.9 respectively (Fig. 1b). The FTIR also confirms the successful synthesis of methacrylated BSP (Fig. 1c): the single peak of 1600cm⁻¹ presented the stretching frequency of C=C in the alkene. These results showed that BSP was successfully methacrylated. To calculate the efficiency of BSP methacrylation, the ¹H-NMR spectra were recorded on using tetramethylsilane (TMS) as internal standard. The efficiency of BSP methacrylation was calculated to be 20.66% based on the ratio of the integrals for the internal standard protons to the methylene protons of methacrylate (Fig. 1b).

2. Fabrication and characterization of methacrylated BSP scaffolds

The process of methacrylation makes the BSP crosslinking to form hydrogel through simply ultraviolet(UV) radiation. [25] The BSP still displays liquid status after 15 seconds UV radiation, however, the BSP-MA with different concentration 0.5%, 1% and 2% were tested to show the different capacity of gelation after the same radiation time. (Fig. 2a) The 2% BSP-MA is shown to change the liquid state to solid state obviously demonstrating the capacity of BSP-MA gelation. However, the pore size in hydrogel is much smaller than cells which is not suitable for cell infiltration and angiogenesis.

Cryogels are gel matrices in which polymerization occurs at subzero temperatures and solvent crystals are defrosted to form interconnected macropores network.[26] BSP cryogels were prepared by freezing and thawing (Fig. 2b). The freezing step causes the ice crystals to form and occupy space, while the subsequent thawing step causes the ice crystals to melt, thus forming large interconnected pores. It shows the macroscopical performance of cryogel BSP-MA, the three colors represent three different concentrations of BSP-MA. (Fig. 2a) It's obvious that the red one with lowest BSP-MA concentration performs sort of collapse. To explore the concentration effect on material properties, we prepared cryogels with four concentration of BSP-MA and named BA2(2%) BA4(4%), BA6 (6%) and BA8(8%).

(Fig. 2c) The concentration can affect the mechanical properties by affecting the pore size[27]. As a result, the cryogels were stained with FITC and pore size and frequency were measured. It's showed that BSP cryogels contained tunable pore size of 169.49,89.22~48.29 and 19.51 μ m (Fig. 2d), The pore size gradually decreases with the increase of concentration. The BA2 and BA4 shows the largest pore size.

Next, to observe the inner network and analyze the structure and distribution of the pore in each cryogel group, we measured the swelling ratios and the interconnected porosity. We found that through adjusting the concentration of the initiator and the precursor BSP-MA solution, we developed cryogels with tunable pore size. With the increase of concentration of the precursor BSP-MA solution, porosity was decreased, and some of them were even about 90% (Fig. 2f), while the swelling ratios of cryogels declined from 25 to 13.

Rheological properties were also investigated to characterized the cryogels to confirm the dimensional stability of the inner work.[28] Rheology test demonstrated that cryogels of different concentration exhibited diverse stiffness at a range from 709.1576 (storage modulus, Pa) to 7803.308 (storage modulus, Pa) (Fig. 2e). As a result, the modulus regulation can be achieved by adjusting pore size, which is a key factor for biomaterial application [29].

3.Cytocompatibility analysis of the cryogel-based scaffolds

To evaluate the cytocompatibility of the cryogels as a biomaterial, we chose BA2 and BA4 cryogels to culture cells due to their relatively large pore size, which is proved to be suitable for cell infiltration and tissue growth. As control, the same concentration of hydrogel was also prepared. As the previous work showed that BSP could regulate macrophages and promote angiogenesis, RAW 264.7 cells and HUVECs were chosen to seed in BA2 and BA4 to evaluate cell distribution and viability. After 6h and 72h of culture, RAW 264.7 cells and HUVECs proliferated well in the BA2 and BA4 scaffolds without any significant difference (Fig. 3a). Compared with cells seeded on hydrogels, OD values of cells increased by around 1.5 times after culturing for 3 days (Fig. 3a).

To further compare the cell infiltrating between hydrogel and cryogel, we use live/dead staining. RAW 264.7 cells could infiltrate even up to 450 μ m depth in cryogel while the hydrogel can only infiltrate up to 200 μ m after culturing for 1 day, indicating the construction of interconnected macropores (Fig. 3b). Besides, both BA2 and BA4 cells had high survival rates at 6h and 72h in RAW 264.7 cells and HUVACs. (Fig. 3c) To quantification the ratio of live/dead cells, we used Image J to analyze the fluorescence. (Fig. 3d) For HUVACs, the BA2 shows 93.3% live ratio in 6h and 90.1% in 72h, BA4 shows 71.5% in 6h and 93.3% in 72h. For RAW 264.7 cells, the BA2 shows 95.2% live ratio in 6h and 94.1% in 72h, BA4 shows 87.6% in 6h and 96.5% in 72h. These results demonstrated that BA2 and BA4 cryogels were excellent substrates for cell growth.

4.GM biomaterial based mechanical modulation of macrophages

The above work proves that by only adjusting the concentration, the cryogel can be adjusted to have different mechanical properties, such as pore size, modulus, etc. Besides, cytocompatibility experiments have proved that cryogels are suitable for cells growth. Therefore, in order to investigate the FBR, the expression levels of macrophage related genes were measured by RT-qPCR performing.

We evaluated the pro-inflammation related genes (*Tnfa*, *Il1b*) and anti-inflammation related genes (*Mrc1*, *Tgfb*) and repair related genes (*Vegfb* and *Osm*) (Fig.4). in RAW264.7 after 24h culture. It's exhibited increased expression levels of pro-inflammation related genes including *Tnfa* and *Il1b*. (Fig.4a) Specifically, with the increase of pore size, the upregulation of *Tnfa* and *Il1b* increases obviously from BA8 to BA2 and BA6 has the similar low level with BA8. It's common that the proinflammatory cytokines increase because of the cryogels intervention. However, it is worth noting that the proinflammatory cytokines gradually decreased as the pore size became smaller, which preliminarily proved that regulating the pore size can modulate inflammatory reaction. The *Tgfb* as a pleiotropic cytokine were upregulated at the comparable level regardless of the pore size. (Fig.4b) Meanwhile, anti-inflammation related genes such as *Mrc1* was downregulated. (Fig.4b) In addition, the expression of angiogenesis-associated gene *Vegfb* and osteogenesis-related gene *Osm* genes were increased at the same time (Fig.4c). Therefore, we found that our materials can promote the M1 polarization of macrophage, and the degree of pro-inflammation decrease gradually with the decrease of the pore size of cryogels.

Figure 4 Gene expression of RAW 264.7 macrophages response to BSP based cryogels with varying stiffness. qPCR analysis of gene: *Tnfa* (a), *Il1b* (a), *Mrc1* (b), *Tgfb* (b), *Vegfb* (c) and *Osm* (c) expression of macrophages in response to BA2 – BA8 cryogels. Statistical analysis: Error bars represent standard error (n = 3). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

5. *In vivo* foreign-body reaction modulated by pore size

To evaluate the biocompatibility and foreign-body reaction of the materials *in vivo*, we implanted the materials: BA8 and BA2 subcutaneously and hydrogel as control for 2 days and 14 days. What we found is that we can modulate the pore size of materials to regulate the FBR.

Firstly, the distribution of immune cells can be regulated by pore size of materials. It can be shown that the materials still have similar morphology as measured *in vitro* after implantation, which is that BA2 has interconnected macro pores and BA8 has smaller pores, while hydrogel as a whole material has no obvious pores. (Fig. 5)

We use Hematoxylin-eosin (H&E) staining and Masson' trichrome (M&T) to show the distribution of immune cells [30]. It is obvious that host response exists in the implantation site. After 2 days, for BA2, the materials with the largest pore size, the immune cells are distributed in both inner and edge of materials (Fig.5 and 6). The quantitative data further demonstrated immune cells distribution is compatible between inner and edge of the materials (Fig.6b). BA8, with the smaller pore size, immune cells infiltrated both inner and edge as well, but it's showed that the concentration at the edge of the

materials (Fig.6c). For hydrogel, with no macro pore, immune cells only exist at the edge of the material, with little inner presence. After 14 days, the immune response was generally diminished and the distribution of both BA2 and BA8 is more even but hydrogel still edge-concentrated. The above results indicated that the pore size can mediate the host response by regulating the distribution of immune cells. At the initial stage of material implantation, the bigger the pore size is, the easier it is for immune cells to infiltrate into the inner of the materials. With the decrease of the pore size, the immune cells are distributed to the edge of the materials. Immunohistochemical staining of CD86 was also performed to identify the location of macrophages in the cryogels (Fig.7a). The distribution of macrophages is similar with it of overall immune cells. Notably, BA2 material shows higher density of macrophages than BA8, which was consistent with the levels of inflammatory cytokines *in vitro*.

Figure 5 Immune cell infiltration in materials. H&E staining images of (a)BA2 subcutaneously implanted in mice for 2 days and 14 days (b), and BA8 implanted for 2 days (c) and 14 days (d), hydrogel implanted for 2 days (e) and 14 days (f). the yellow triangle shows the location of fibroblasts. The red star shows the angiogenesis.

The formation of fibrous capsule can be modulated by pore size of materials.[31] In tissues exposed to the system's immune system, lymphocytes and fibroblasts develop fibrous capsules to fight against biological materials. We evaluate the thickness of the fibrous capsule as an indicator of the FBR. M&T staining (Fig. 6) and the quantification (Fig. 7e and 7f) showed that BA8 has the thinner fibrous capsule than BA2, and hydrogel has the thickest fibrous capsule. This may be due to the larger pore size of BA2, which leads to more macrophages to enter the material, while the macrophages of BA8 and hydrogel concentrating at the edge secrete cytokines to induce collagen deposition by the fibroblasts and form fibrous capsule.[32]

Fig. 6 The cell distribution and the formation of fibrous capsule in tissue surrounding the BA2, BA8 and hydrogel. (a) shows the M&T staining of BA2, BA8 and hydrogel after 2 days and 14 days implantation. (b) shows the quantification of the immune cells density. (c) (d) shows the quantification of cell distribution. (e) M&T staining of fibrous capsule and the thickness quantification. The green arrows show the immune cells infiltration and the blue crosses show the materials and the purple arrows show the thickness and location of fibrous capsule.

For the implantation of biomaterials, the tissue compatibility is also a very important consideration. [33, 34]The pore size can affect the integration of materials and tissues. Immunohistochemical staining of VEGF and CD31 was measured to evaluate the angiogenesis (Fig. 7c-e). It is obvious that the level of VEGF *in vivo* is consistent with that measured *in vitro*, which is that BA2 showed the high level contrast with BA8 at both 2 and 14 days. It's showed that angiogenesis at 14 day which indicated the formation of tissue in BA2 and BA8(Fig. 7e). In addition, BA8 has better tissue compatibility (Fig. 7), BA2, with large pore size, allowed macrophages to infiltrate to inner material thus leading more severe inflammation, which is not conducive to blood vessel growth and tissue integration. The schematic diagram showed of the probable immune cells infiltration.(Fig. 8)

Fig. 7 Inflammatory marker staining and qualification analysis of BA2, BA8 and hydrogel. (a) inflammatory staining and (b) qualification analysis of CD86 (c) inflammatory staining of VEGF and (d) qualification analysis (e) inflammatory staining of CD31. The red stars marked the angiogenesis.

Discussion

In this study, we have developed a new, natural polysaccharide based cryogel scaffold, which is effective to regulate host response. Notably, this polysaccharide is a Chinese medicine herb derived glucomannan, showing the high biocompatible and stability as a 3D scaffold.

Along with the repaid progress of tissue engineering normal biomaterial with biological functions and tunable physical properties are in high demand.[35] On the one hand, natural polysaccharides from ocean have been widely developed. [36–38] On the other hand, polymers from Chinese medicine herbs, despite the evidence bioactivity, still have much room for development, key obstacles include unclear composition and difficulties for material fabrication. [39–41] Here, for the first time, we made macroporous BSP gels suitable for cell culture just by freezing and thawing without adding any pore-forming agents.

The *in vitro* and *in vivo* data verified our hypothesis, which is the pore size can induce different level of host response. Firstly, our *in vitro* and *in vivo* data shows high consistency. The *in vivo* results: immunohistochemistry staining of CD86 and VEGF is agreed with the *in vitro* results of RT-PCR. Secondly, perhaps the most remarkable finding is that the material we successfully made can differentially guide to the distribution of immune cells, blood vessels, fibrous capsule. for example, the Fig. 5-7 show the macrophages and other immune cells distribution and amount is associated with the pore size of materials, BA2, with largest pore size, has more macrophages infiltrated and shows more internal distribution, which is also consist with the *in vitro* data: the upregulation of pro-inflammation related gene. In addition, Many researches have as proved that foreign-body reaction can be modulated by various material properties such as porosity. [42, 43]

These exciting findings suggest the future works for the development of BSP cryogel system. First, much work remains to do on the specific types, subtle types of the immune cells into the scaffold at different time points, also interest is that how these differential immune cells profiles lead to tissue remodeling and repairing. Second, as a prove of a consequence study, we implant the gels in health mice, we will evaluate the general potential in specific disease model in future. We believe our exploration will open up a new avenue for the development of Chinese medicine resource for broaden applications.

Conclusions

Taking together, our works revealed that *Bletilla striata* polysaccharide cryogel scaffold with different pore sizes can spatially control foreign-body reaction. The microstructure of cryogels could differentially guide the distribution of inflammatory cells, affect the formation of blood vessels and fibrous capsules,

which eventually influence the material-tissue integration. This work demonstrates a practical strategy to regulate foreign body response and promote the performance of medical devices.

Abbreviations

FBR: Foreign-body Reaction; BSP: *Bletilla striata* polysaccharide; GM: glucomannan; MES: 2-morpholinoethanesulfonic acid-NHS-N-hydroxysuccinimide-EDC-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; DMSO: dimethylsulfoxide; TEMED: tetramethylethylenediamine; APS: ammonium persulfate; CLSM: confocal laser scanning microscopy; HUVECs: human umbilical vein endothelial cells; ATCC: American Type Culture Collection; CCK-8: cell counting kit-8; q-PCR: Quantitative real time PCR; H&E: Hematoxylin-eosin; M&T: Masson' trichrome

Declarations

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Author Contributions

YMN designed the study. HQZ extracted and purified the natural polysaccharide with the assistant of DPX. HQZ performed major chemical modification and scaffold preparation. JXC contributed to biological and animal experiments. JXC and HQZ were the main drafters of the manuscript. YMN provided funding supports. All authors contributed to data analysis and manuscript drafting.

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Availability of data and materials

Not applicable.

Declarations

Not applicable.

Ethics approval and consent to participate

The animal care and experimental procedures used in this study were approved by the Animal Ethics Committee, University of Macau.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interests.

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Figures

Figure 1

Preparation and characterization of methacrylated BSP. (a) The Chinese medicine *Bletilla striata* and Schematic illustration of methacrylated BSP synthesis procedures and the polymerization under the TEMED/APS initiation system. (b) ^{13}C -NMR and ^1H -NMR spectrum analysis of oxidized BSP and methacrylated BSP. (c) The FT-IR of BSP and BSP-MA. (d) The potentiometric titration to measure the degree of substitution.

Figure 2

Fabrication and characterization of BSP-based cryogels. (a) Fabrication of hydrogels and cryogels with different concentrations (b) Schematic depiction of cryogels preparation. (c) Confocal laser scanning fluorescence microscopy (CLSM) analysis of BA2 - BA8 cryogels (varying concentration of the precursor solution: 2%, 4%, 6%, 8%, (w/v) %) and average pore diameters and the pore size distributions. Scale bars: 100μm, 50μm, 20μm, 20μm. (d) Rheological analysis of BA2 - BA8 cryogels. Storage modulus (G') and

loss modulus (G'') of BA2 - BA8 cryogels on strain sweep measured at 1 Hz of frequency(left), frequency sweep measured at 0.2% of strain (right). (e) Interconnected porosity (n=3) and (f) swelling ratio (n=3) of BA2 - BA8 cryogels. Statistical analysis: Error bars represent standard error (n = 3). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3

Cytocompatibility analysis of cell-laden cryogel: (a) RAW 264.7 cell and HUVECs viability on BA2 hydrogels and cryogels and BA4 hydrogels and cryogels after 24h and 72h culture.(b) Infiltration and distribution images of RAW 264.7 cells seeded on hydrogel and cryogel for 24h. (c-d) Representative images and quantitative analysis of live/dead staining of RAW 264.7 macrophages and HUVECs in BA2 and BA4 cryogels after 6h and 72h of culture. Scale bar: 200μm. Statistical analysis: Error bars represent standard error (n = 3). T-test, *** p < 0.001.

Figure 4

Gene expression of RAW 264.7 macrophages response to BSP based cryogels with varying stiffness. qPCR analysis of gene: *Tnfa* (a), *Il1b*(a), *Mrc1* (b), *Tgfb* (b), *Vegfb* (c) and *Osm* (c) expression of macrophages in response to BA2 – BA8 cryogels. Statistical analysis: Error bars represent standard error (n = 3). One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Figure 5

Immune cell infiltration in materials. H&E staining images of (a)BA2 subcutaneously implanted in mice for 2 days and 14 days (b), and BA8 implanted for 2 days (c) and 14 days (d), hydrogel implanted for 2 days (e) and 14 days (f). the yellow triangle shows the location of fibroblasts. The red star shows the angiogenesis.

Figure 6

The cell distribution and the formation of fibrous capsule in tissue surrounding the BA2, BA8 and hydrogel. (a) shows the M&T staining of BA2, BA8 and hydrogel after 2 days and 14 days implantation. (b) shows the quantification of the immune cells density. (c) (d) shows the quantification of cell distribution. (e) M&Tstaining of fibrous capsule and the thickness quantification. The green arrows show the immune cells infiltration and the blue crosses show the materials and the purple arrows show the thickness and location of fibrous capsule.

Figure 7

Inflammatory marker staining and qualification analysis of BA2, BA8 and hydrogel. (a) inflammatory staining and (b) qualification analysis of CD86 inflammatory staining of VEGF and (d) qualification analysis (e) inflammatory staining of CD31. The red stars marked the angiogenesis.

Figure 8

The special control of foreign-body reaction by modulation the pore size. The immune cells, including macrophages, monocytes, natural killer cells, dendritic cells, T cells, B cells, fibroblasts diversely distributed in the materials with different pore size and the different level of fibrous capsule formation.