Protocols

Effect of hierarchical pore structure on ALP expression of MC3T3-E1 cells on bioglass films

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A B S T R A C T

Hierarchical porous bioglass films on the tantalum were designed to enhance osteointegration of metal- 
imic implants. The films were prepared by a sol-gel method using P123 as the mesopore template and 
polystyrene microsphere as the nanopore template. The films with 5.4 nm mesopores and 100 nm 
nanopores (MBG-100) elicited an obviously elongated morphology of the cultured MC3T3-E1 cells, as 
a result, a higher alkaline phosphatase level was expressed. It is suggested that the nanopores play an 
important role in regulating cellular behavior by initial protein adsorption through nanopore curvatures. 
The mesopores were proven very effective for loading rhBMP-2, and the rhBMP-2 loaded on MBG-100 
films showed a better function of enhancing osteogenic differentiation, which is attributed to that the 
nanopore structure could expedite rhBMP-2 release and provide a microenvironment for intensifying 
the interaction of rhBMP-2 with the cells. Hence, the cell osteogenic differentiation can be enhanced by 
hierarchical porous bioglass films through both the porous structure and rhBMP-2 induction.

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1. Introduction

Numerous surface modifications have been developed to improve osteoconductive and osteoinductive of metallic implants such as tantalum (Ta) alloy and titanium (Ti) alloy via structural and compositional variations [1–7].

Surface topography has been shown to be an effective cue for the regulation of cell behaviours including cell attachment, migration, proliferation and differentiation [3]. Various topographic features such as pores [8–12], pillars [13–16] and tubes [17–19] with dimensions have been fabricated to regulate cell behaviours. Pore structure performed well in supporting and even controlling the growth and proliferation of cells by altering the pore dimensions. The porosity and pore size can be accurately tuned ranging from nanoscale to microscale. Recently, studies about cell interaction on nanopore (pore size <100 nm) have attracted considerable interest. The pore sizes span a range of sizes important in biology, a small DNA fragment in the order of a few tens of nanometers (nm), important extracellular matrix (ECM) proteins like Fibronectin and albumin generally in the 100 nm range [20]. Besides, cell filopodia is of several nanometers in diameter reacting to similar level of topography [21]. Researches show that cells can sense and be influenced by nanopore down to just a few nanometers (~5 nm). While the pore size had different effects on cell adhesion with different cells according to previous results. Thus, a pore size gradient approach could usually help optimize pore size for certain desired cell behaviors.

In respect of compositional modifications, such as bioceramics coating, biopolymer film and biomolecules grafting are extensively adopted [22]. The incorporation of growth factors is an effective strategy to accelerate osteoinduction. In particular, bone morpho-genetic protein-2 (BMP-2) is the most notable growth factor to promote the differentiation of osteoblasts and induce bone formation [23,24]. A lot of documents reported that biomaterials loaded with bone growth factors exhibited enhanced bone repair effect on the osteoinduction of biomaterials [25]. Small molecules are more effectively trapped in pores exhibiting a size smaller than 10 nm. Mesoporous bioactive glass (MBG) possesses higher specific surface area, pore volume, and apatite mineralization activity due to its well-ordered mesoporous structure compared to other bioactive coating [26]. Reasonably, MBG film on the metallic implants has turn out to be a desired carrier for rhBMP-2 through molecule entrapment in mesopore. Nevertheless, as far as we know, the flat and single surface of MBG film could rarely be modified with topography, which still remains a significant challenge for better bone regeneration [27].

Then, how to combining the porous structure with biological factors to improve the bone integration of metal bone implant is

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challenging nowadays. In this work, we design hierarchical porous bioglass films on the surface of tantalum, which possesses mesopore to carry rhBMP-2 through high pore volume and surface area and nanopore structure in the 100 nm scale to regulate cell response. With this material, we are closer to a functional biomaterial with both osteoconductivity and bone growth factor – delivery functions to promote osteogenesis.

The designed films were prepared by sol-gel method using polyisostyrone (PS) microsphere as the nanopore template. The effect of porous structure on cell behaviors was evaluated through culturing MC3T3-E1 cells. The ALP activity was measured to assess cellular osteogenic differentiation on the influence of porous structure as well as rhBMP-2. The initial extracellular proteins adsorption on the films was detected to explore the relations between pore structure and cell adhesion and differentiation.

2. Experiment

2.1. Preparation of hierarchical porous bioglass films

Hierarchical porous bioglass films were prepared by two steps, a sol-gel mesoporous bioglass (MBG) sol process and then a PS template method. Firstly, amphiphilic triblock copolymer poly(ethylene glycol)-block-polypoly(ethylene glycol)-block (ethylene glycol) (P123) was stirring dissolved in deionized water as the mesopore template. After that hydrochloric was added into the solution (molar ratio of P123/HCl/H2O = 0.0141/95.8). Subsequently, precursor sol was prepared by mixing tetraethyl orthosilicate (TEOS, Aladdin, AR), calcium nitrate tetrahydrate (Ca(NO3)2, Aladdin, AR), triethyl phosphate (TEP, Aladdin, AR), and tetraethyl titanate (TBOT, Aladdin, AR), with the molar ratio of SiO2/CaO/P2O5/TiO2 = 80/5/5/10. The MBG precursor sol was aging at 4 °C for 24 h. Secondly, polystyrene (PS) microspheres (Wakely Microsphere, Shanghai) with the diameter of 25 nm, 50 nm, and 100 nm were added into MBG precursor solution in a certain quality proportion. The mixed liquor, none PS added MBG sol included, was spin coated on tantalum (Ta) substrates (1 x 1 cm) at 7000 rpm for 40 s, afterward the spinning-coated films were placed at 4 °C and in the humidity of 75.7% for 24 h, and finally calcinated at 400 °C for 5 h at a rising rate of 1 °C/min. In this way, four designed hierarchical porous bioglass films were prepared and ready to use. Each film was composed of one mesopore in the range of 10 nm and gradient nanopore with the diameter of 0, 25 nm, 50 nm, and 100 nm, denoted as MBG, MBG-25, MBG-50, and MBG-100 film.

2.2. SEM observation

SEM images of the surface morphology of the films and initial adhesion of cells on different films were captured using Field-emission scanning electron microscopy (FE-SEM, Hitachi, operating voltage at 3 kV).

2.3. Contact angle measurement

Surface hydrophobicity of the films was determined by measuring the water contact angle with one drop of 1 μl of deionized water using Contact angle (OCA 20, Dataphysics) at room temperature. Three measurements were performed on each substrate to evaluate the average contact angle.

2.4. Pore characterized by BET analysis

N2 adsorption/desorption isotherms were obtained at −196 °C using an automatic surface area analyzer (AUTOSORB-1 C, BEL, U.S.) under continuous adsorption conditions. Prior to the N2 adsorption/desorption study, MBG films detached from the substrates were heated to 160 °C and the gasses were removed to 0.13 Pa at room temperature [28]. The total pore volume was calculated by a t-plot analysis. The Barrett–Joyner–Halenda (BJH) method was used to calculate mesopore size distribution.

2.5. Protein adsorption

Experiments were performed to verify the protein adsorption on different films. Bovine serum albumin (BSA, 95%, Sigma-Aldrich) and fibronectin (Fn, 2 mg/ml, Beijing Solarbio Science & Technology) were used, and dissolved in phosphate buffer solution (PBS) at pH 7.4 to form a solution with a concentration of 1 mg/l and 5 μg/ml according to their proportions in the human plasma albumin respectively. The nano-microstructured surfaces were soaked in the solution for 24 h to adsorb the protein. The amount of BSA adsorption was measured by BCA Protein Assay Kit (Beyotime Institute of Biotechnologe, Shanghai, China), and the optical density (OD) of supernatant liquid at 560 nm with a microplate reader (Multi-skan MK3). The amount of Fn adsorption was measured by Fn ELISA Kit (Beijing Solarbio Science & Technology Co., Ltd.), and the optical density (OD) of supernatant liquid at 450 nm with a microplate reader (Multiskan MK3). And then the OD value was converted to concentration using the standard curve line.

2.6. Loading and of rhBMP-2

Solution of 2 μg/ml recombinant human bone morphogenetic protein-2 (rhBMP-2) (Shanghai Rebone Biomaterials Co., Ltd.) was prepared by dissolving the protein in deionized water. RhBMP-2 adsorption was carried out respectively by soaking and dropping methods.

For loading rhBMP-2 by soaking, films were immersed into 1 ml rhBMP-2 solution for 24 h; for loading rhBMP-2 by dropping, a 100 μl aliquot of rhBMP-2 solution was added dropwise onto the films. Then, the films with loading rhBMP-2 were dried at 37 °C. The amount of rhBMP-2 was monitored by a BMP-2 Quantikine ELISA Kit (Shanghai Yanjin Co., Ltd.). The absorbancy was measured on a microplate reader (Thermo, Multiskan MK3) at 405 nm and the corresponding rhBMP-2 concentration was calculated based on the calibration curve obtained. The in vitro loading/release of rhBMP-2 study was undertaken in triplicate for each preparation.

2.7. Culture of cells

MC3T3-E1 cells (pre-osteoblast, CRL-2594, ATCC) were cultured in various alpha-modified Minimum Essential Medium (AlphaMEM, Gibco) supplemented with 1% antibiotic solution containing 10,000 units/ml penicillin and 10 mg/ml streptomycin (Gibco), 1% sodium pyruvate (Gibco), and 1% MEM non-essential amino acids (Gibco), 10% fetal bovine serum (PBS, PAA, Australia) under a humidified 5% CO2 atmosphere at 37 °C. Sub-confluent cells growing on tissue culture polystyrene (TCPs) were trypsinized with 0.25% trypsin containing 1 mM Ethylene Diamine Tetraacetic Acid (EDTA) (Gibco), and were subcultured on different films.

2.8. Cell viability

Cell viability was determined by AlamarBlueTM method after culturing 1, 3, 7 days. The MC3T3-E1 cells with a density of 2.0 x 10^4 cells/ml were seeded on the samples. At each test time point, the samples were rinsed with PBS twice and 500 μl fresh medium containing 5% AlamarBlueTM was added. After incubating for 4 h, the absorbance values of 100 μl medium were recorded at 570 and 600 nm. The results were calculated following the instruction of AlamarBlueTM assay.
2.9. Morphology of cell adhesion

After culturing for 24 h, the samples were rinsed with PBS twice and fixed with 2.5% glutaraldehyde. After that, the cells were dehydrated on samples with gradient ethanol solutions (30, 50, 75, 90, 95 and 100 v/v% in sequence) for 10 min each. Afterwards, the samples were dried in hexamethyl disilazane ethanol solution. SEM was employed to observe cell morphology.

2.10. Alkaline phosphatase (ALP) activity

The MC3T3-E1 cells with a density of $2.0 \times 10^4$ cells/mL (for 7 days) or $1.0 \times 10^4$ cells/mL (for 14 days) were seeded on various films (three replicates) under a humidified atmosphere of 5% CO$_2$ at 37°C. After incubation for 7 or 14 days, culture medium was removed, the samples were rinsed with PBS for three times. The cells cultured on samples were removed in new 24-well culture plates and then lysed with Cellytic Buffer (Sigma). ALP (ab65834, abcam) expression was assessed by the LabAssayTM ALP Kit (Beyotime Institute of Biotechnology, Shanghai, China) at a wavelength of 405 nm and total protein contents tested in a BCA protein assay.

2.11. Statistical analysis

All values are expressed as mean± standard deviation. Statistical analyses were carried out by analysis of variance (one-way ANOVA) and Scheffe’s post hoc test with software (SPSS Inc., SPSS 16.0) for multiple comparison tests. Differences were considered statistically significant for *p < 0.05 and **p < 0.01. Error bars represent means ± SD for n = 3.

3. Results

3.1. Characterizations of the films

3.1.1. SEM observation

The MBG films (Fig. 1a) appeared as a flat surface with continuous ordered mesopores. The MBG films derived from the sols with PS nanospheres had a uniformly nanosized pore distribution. Hierarchical pores in the films had been formed, and the nanopore size in MBG-25, MBG-50 and MBG-100 films was 25, 50 and 100 nm (Fig. 1), respectively. Besides, the thickness for these films was almost same at ~320 nm (Fig. 1a2).

3.1.2. Pore characterized by BET analysis

The pore characteristic of MBG and MBG-100 films was measured as shown in Table 1 and the corresponding parameters were summarized in Table 1. MBG film possessed a type IV isotherm with a type H1 hysteresis loop (Fig. 2a1), an average pore size of 5.4 nm (Fig. 2b1), a BET surface area of 300.4 m$^2$/g and a total pore volume of 0.36 cm$^3$/g, which corresponds to a good mesoporous character. For MBG-100 film, the area of hysteresis loop increased in the isotherms between partial pressures $p/p_0$ of 0.4–0.95, indicating larger pores existing in the film (Fig. 2a2). Moreover, the physical properties including pore size distribution curve, the surface area and pore volume were very similar with those of MBG film (Table 1).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>MBG</th>
<th>MBG-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multipoint BET (m$^2$/g)</td>
<td>300.4</td>
<td>359.9</td>
</tr>
<tr>
<td>Total Pore Volume (cm$^3$/g)</td>
<td>0.36</td>
<td>0.52</td>
</tr>
<tr>
<td>Average Pore Size (nm)</td>
<td>5.4</td>
<td>5.7</td>
</tr>
</tbody>
</table>

These further verified the resulting films with characteristic of hierarchical porous structure.

3.1.3. Contact angle measurement

Contact angle was determined as a quantitative evaluation for hydrophilicity of the films, the results were showed in Fig. 4. MBG film had a contact angle of 33° to exhibit hydrophilic, MBG-25 and MBG-50 films both had a contact angle of about 48°, and MBG-100 film had a contact angle of 55.7°. The films became less hydrophilic with bigger nanopores.

3.1.4. Protein adsorption

Albumin is the most abundant protein in ECM, and fibronectin (Fn) is reported to promote cell adhesion and growth. The adsorption of two proteins on the films was detected to support understanding of the interaction between cells and different surfaces. The results of albumin and fibronectin (Fn) adsorption on the films were illustrated in Fig. 4a and b, respectively. It was obvious that the adsorption of both albumin and fibronectin became larger with increasing nanopore size (MBG-100 > MBG-50 > MBG-25 > MBG film). Albumin of 3.18 µg/cm$^2$ and fibronectin of 154 ng/cm$^2$ were adsorbed on MBG-100 film.

3.1.5. Loading amount of rhBMP-2 on the films

RhBMP-2, which can induce cell osteogenic differentiation, was adopted to load in the films in order to evaluate the role of hierarchical porous structure in further promoting the formation of bone tissue.

The loading capacity of rhBMP-2 in these films was measured and listed in Table 2. MBG film had the highest loading amount of 555 ng/cm$^2$, MBG-100 film had a relatively high loading amount of 321 ng/cm$^2$ although the loading amount decreased with increasing nanopore size.

It should be noted that rhBMP-2 loading amount decreased with increasing nanopore size while the adsorption of BSA and Fn was the opposite. It was suggested that rhBMP-2 were loaded into films through a protein entrapment since the size of 5.4 nm mesopore was matched with that of rhBMP-2 molecule (7.5*3.5*3 nm). Though the BSA molecule is ellipsoid of 4–6 nm, the 1 mg/mL concentration of BSA soaking solution (2 µg/mL for rhBMP-2 soaking) was so high concentrated that BSA was mainly adsorbed on the surface depending on the radius of curvature and hydrophobicity instead of mesopores. Besides, Fn molecule is rod-like structure of 140*2 nm, too big for mesopore, mainly adsorbed on the surface too. Therefore, the load of rhBMP-2 is different from the surface adsorption of BSA and Fn, which resulted in the difference.

<table>
<thead>
<tr>
<th>Amount/ng cm$^2$</th>
<th>MBG</th>
<th>MBG-25</th>
<th>MBG-50</th>
<th>MBG-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhBMP-2 loading</td>
<td>555</td>
<td>542</td>
<td>472</td>
<td>421</td>
</tr>
</tbody>
</table>

These further verified the resulting films with characteristic of hierarchical porous structure.

3.2. Cellular morphology, adhesion and proliferation

Cell morphology on the films (Fig. 5) was examined via SEM after culturing for 24 h. MC3T3-E1 cells cultured on MBG films had a more rounded shape, with little filopodia (Fig. 5a2–d2), whereas the cells cultured on MBG-50 and MBG-100 films appeared increasingly elongated cytoskeleton and showed many filopodia with increasing pore diameter of the films (Fig. 5).

To gain a more quantitative understanding of cell morphology, the cell length to diameter ratio was determined for the four porous films as listed in Table 3. The length of individual cells was determined by measuring the length of the longest processes from the
cell and dividing the diameter of the diameter of the cell body. The cell length to diameter ratio was found to be the largest on the MBG-100 film, and the smallest on the MBG film. But the difference was not significant between MBG-25 and MBG-50. These results suggested that cells reacted to the 100 nm pores by expanding and elongation. Therefore, MC3T3-E1 cells on MBG-100 films presented more active in cell motility and most elongated morphology.

A CCK-8 assay was employed to evaluate cellular adhesion (1-day culture) and proliferation (4-day culture). Fig. 6 displayed the adhesion and proliferation of MC3T3-E1 cells cultured on the films, respectively. Compared with that on the first day, the cell den-
sity increased several folds on the fourth day, showing that these films supported cellular proliferation of MC3T3-E1 cells well. However, there was no significant discrepancy in cellular adhesion and proliferation.

3.3. Cell osteogenic differentiation

3.3.1. ALP activity on the films without rhBMP-2

Alkaline phosphatase (ALP) activity was measured to evaluate the influence of the hierarchical porous structure on osteogenic functionality. Fig. 7 indicated that the ALP activities of the cells cultured on the hierarchical porous films were stronger than MBG films at 7th day, and ALP activity for MBG-100 film was highest at 14th day.

3.3.2. ALP activity on the films loaded with rhBMP-2

The films were loaded with the same amount of rhBMP-2 (300ng/cm²), and ALP activities of MC3T3-E1 cells cultured on the films were assayed as shown in Fig. 8.

Although the ALP activities for all films were distinctly enhanced after loading rhBMP-2, there was a significant difference (Fig. 8) for each other, as MBG-100 film gave the highest value at ALP activity.

Furthermore, the increment in ALP activity owing to rhBMP-2 contribution in the films was estimated by the data in Figs. 7 and 8, and listed in Table 4. The 7-day ALP activity increment for MBG-100 film was much higher than others (Table 4).

4. Discussion

Hierarchical porous bioglass films with 5.4 nm mesopores and nanopores of 25 nm, 50 nm or 100 nm were well established as shown in Figs. 1 and 2, and these films could provide a basis to regulate cell behaviors due to hierarchical porous nanotopography and to enhance rhBMP-2 loading for inducing osteogenesis due to mesopore structure.

Hierarchical porous bioglass films could cause the morphology of the cultured MC3T3-E1 cells to be elongated, the larger the nanopore size, the more intensely elongated (Fig. 5 and Table 3), indicating the nanopore structure in the films effectively played a role of modulating cellular behavior [21]. The osteogenic differentiation can be promoted when the cultured cells are regulated as an elongated morphology due to activating indirect mechanotransductive pathway [29]. In this work, the nanopore structure in the films is able to induce the elongated cell growth, as expected, the ALP activity of the cultured cells on the films increased with nanopore size of the films, and MBG-100 film showed a strongest role of enhancing osteogenic differentiation.

As is widely known, cells do not adhere and spread on the films until they sense the material by available extracellular proteins on the surface [30]. It is found that the phenomenon of adhesion and elongation is determined by the initial adsorption of proteins [31], namely fibronectin [32] and albumin [33–35]. On the hierarchical porous bioglass films, albumin and fibronectin adsorbed a lot owing to substantive attachment sites afforded by the mesopore [36], and the adsorption exhibited a gradually increasing trend with nanopore size of the films (Fig. 4). The more protein adsorption on MBG-100 film could be attributed to that it has more hydrophobic surface (Fig. 3) which favors protein adsorption.

Moreover, it was reported that the protein adsorption depended on the surface nanostructures, and more adsorption of albumin and fibronectin appeared on spherical surface with ∼100 nm scale.
Fig. 4. Adsorption of albumin (a) and fibronectin (b) on different films. Error bars represent means ± SD for n = 3, *p < 0.05, **p < 0.01, indicating a statistically significant difference.

Fig. 5. SEM images of morphology of MC3T3-E1 cells after culturing on different films for 24 h. (a1 and a2) Cells on the MBG films; (b1 and b2) cells on the MBG-25 films; (c1 and c2) cells on the MBG-50 films; (d1 and d2) cells on the MBG-100 films.

Fig. 6. CCK-8 assay of MC3T3-E1 cells adhesion and proliferation on different films. Error bars represent means ± SD for n = 3, *p < 0.05, **p < 0.01, indicating a statistically significant difference.

Fig. 7. ALP activity expression of MC3T3-E1 cells on different films. Error bars represent means ± SD for n = 3, *p < 0.05, **p < 0.01, indicating a statistically significant difference.
Here, the nanopore edges in the films could also provide a similar spherical surface, it is supposed that the curvature of nanopore increases with nanopore size. 100 nm pores in the films may favor the interaction with the proteins, and result in cell spreading out with long and plentiful filopodia. Hence, appropriate edge curvature and spatial distribution of the nanopores in MBG-100 film are responsible for the appearance of the elongated morphology of the cultured cells on the film, eventually leading to higher osteogenic differentiation ability.

It has been considered that mesopores facilitate rhBMP-2 loading very well. In the present hierarchical porous bioglass films, MBG-100 film had the largest nanopore size (i.e. removal of mesoporous glass in the film to the most extent) and still a relatively high loading amount of 321 ng/cm² (Table 2) [39,40]. Besides, we have measured the rhBMP-2 release profile of the MBG film in previous study [41]. The results showed that 90% loaded rhBMP-2 didn’t released until 25th day, showing a good and sustained release behavior. Usually, the more rhBMP-2 carried in the film with same composition and thickness, the slower rhBMP-2 would be released. Many studies have shown that it needs a quick release the first day after entering the body and maintaining a larger release in the first two weeks for BMP-2 applications [42,43]. The MBG-100 film load capacity is slightly smaller than the MBG film, so the release rate will be slightly faster than the MBG film. It was estimated that the rhBMP-2 release could last more than two weeks time for the experimental films. Therefore, the larger pore structure of MBG–100 film increased the amount of rhBMP-2 released in the previous two weeks, which may partially enhance the osteogenic differentiation.

It turned out that MBG–100 film showed the largest 7-day ALP activity increment derived from rhBMP-2 contribution (Table 3) in comparison with other films loaded by the same rhBMP-2 amount. In addition to the above-mentioned more rhBMP-2 released on the MBG–100 films, a three-dimensional microenvironment of the 100 nm nanopores provided interspace for rhBMP-2 molecules remaining and more interaction spots with the attached cells [41]. As a consequence, it could be deduced that the larger pore structure of MBG–100 film not only expedited rhBMP-2 release, but also enhanced the induction of rhBMP-2 by porous structures to achieve the best osteogenic differentiation.

The present films demonstrate that the mesopores make for the rhBMP-2 loading amount and the nanoporous structure also favors rhBMP-2 function, the corporate role for rhBMP-2 could result from both increased release of rhBMP-2 and sufficient interaction between the attached cells and rhBMP-2 owing to the porous topography in MBG–100 films.

To sum up, the MBG–100 film applies mesopore for rhBMP capacity, and hierarchical pores including 100 nm nanopore for protein adsorption and cell elongation. The loaded rhBMP-2 and hierarchical porous topography on the MBG-100 film synergetically exert the most enhancement the bone differentiation.

5. Conclusions

In conclusion, hierarchical porous bioglass films with ordered mesopores of 5.4 nm and uniformly nanopores of 25 nm, 50 nm or 100 nm were prepared. MC3T3-E1 cells behaviors were regulated by the hierarchical porous nanotopography, exhibiting elongated morphology and higher ALP activity when cultured to 7th and 14th days, especially on the MBG-100 films. On account of that extracellular proteins namely albumin and fibronectin adsorbed the most on the MBG–100 films due to appropriate curvature. Besides, these films loaded the rhBMP-2 well attributed to the mesopores. And the MBG–100 films favors rhBMP-2 function to the maximum efficiency resulting from the increased rhBMP-2 release and better interaction with the attached cells by porous topography. Considering its high cytocompatibility, potential biodegradability, and potential biomolecule-delivery function, hierarchical porous bioglass film is a promising biomaterial for bone-tissue engineering.

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