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Wu , Chengtie, Fan, Wei, Chang, Jiang, & Xiao, Yin (2011) Mussel-inspired porous SiO2 scaffolds with improved mineralization and cytocompatibility for drug delivery and bone tissue engineering. *Journal of Materials Chemistry*, *21*, pp. 18300-18307.

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http://dx.doi.org/10.1039/C1JM12770E

Mussel-inspired porous SiO_2 scaffolds with improved mineralization and cytocompatibility for drug delivery and bone tissue engineering

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Porous SiO_2 scaffolds with mesopore structure (named as MS scaffolds) have been proposed as suitable for bone tissue engineering due to their excellent drug-delivery ability; however, the mineralization and cytocompatibility of MS scaffolds are far from optimal for bone tissue engineering, and it is also unclear how the delivery of drugs from MS scaffolds affects osteoblastic cells. The aims of the present study were to improve the mineralization and cytocompatibility of MS scaffolds by coating mussel-inspired polydopamine on the pore walls of scaffolds. The effects of polydopamine modification on MS scaffolds was investigated with respect to apatite mineralization and the attachment, proliferation and differentiation of bone marrow stromal cells (BMSCs), as was the release profile of the drug dexamethasone (DEX). Our results show that polydopamine can readily coat the pore walls of MS scaffolds and that polydopamine-modified MS scaffolds have a significantly improved apatite-mineralization ability as well as better attachment and proliferation of BMSCs in the scaffolds, compared to controls. Polydopamine modification did not alter the release profile of DEX from MS scaffolds but the sustained delivery of DEX significantly improved alkaline phosphatase (ALP) activity of BMSCs in the scaffolds. These results suggest that polydopamine modification is a viable option to enhance the bioactivity of bone tissue engineering scaffolds and, further, that DEX-loaded polydopamine MS scaffolds have potential uses as a release system to enhance the osteogenic properties of bone tissue engineering applications.

Key words: Porous scaffold; polydopamine; mineralization; drug delivery; proliferation; differentiation

1. Introduction

Mesoporous materials have many features which make them ideal for applications such as catalysis, adsorption/separation, biomedical and tissue engineering; these features include a large surface area, ordered mesoporous structure, tunable pore size and volume, and well-defined surface property.[1-6] The study of mesoporous materials, as a system for targeted drug delivery for bone tissue engineering applications, has been a hot area of research for the past decade, as these materials are able to load drugs and subsequently release them in a localized and controlled way.[7-12] The purpose of these drugs is to act as signals to attract bone-forming cells to the site of injury and porous SiO_2 scaffolds, with their porous structure and large pore size (several hundred micrometers), have also been developed with this aim in mind.[13, 14] The large-pore structure of MS scaffolds is essential to allow bone cell ingrowth and the mesopore structure of MS scaffolds can deliver drugs to stimulate bone-forming cells. Pure MS scaffolds generally have too slow *in vitro* mineralization to be considered a bioactive bone graft material and its cytocompatibility is far from optimal.[13, 15, 16] Although MS have been used as a drug delivery platform, it remains unclear whether the delivery of drug from MS scaffolds will have a positive effect on the proliferation and differentiation of bone-forming cells. In an effort to improve the *in vitro* bioactivity of MS scaffolds, scaffolds have been prepared in multicomponent SiO_2-CaO-P_2O_5,[17-19] SiO_2-CaO,[20] SiO_2-P_2O_5[21] and SiO_2-SrO[9] systems. The preparation of multicomponent systems with highly ordred mesoporous structures requires careful selection of the chemical components, such as inorganic precursor and surfactant molecules.[22] In addition, the incorporation of other components into MS always leads to a decrease of surface area and pore volume.[23]

Mussels (*Bivalvia mollusca*) are marine organisms and have the ability to attach to virtually any type of inorganic and organic surfaces.[24] Clues to the mussel's adhesive versatility may lie in the amino acid composition of proteins found near the plaque-substrate interface, which are rich in 3,4-dihydroxy-L-Phenylalamine (DOPA) and lysine amino acids.[24, 25] Inspired by this property in mussels, Lee et al. developed polydopamine coatings on different material surface, which was confirmed to be an efficient way of improving hydrophilicity, graft organic molecules and improved surface mineralization.[24, 26-28] Polydopamine is a polymer by the polymerization of dopamine, which occurs in a manner that is reminiscent of melanin formation, involving oxidation of catechol to quinone, and further reacts with amines and other catechols/quinones to form an adherent polymer. Dopamine is classified as a catecholamine (a class of molecules that serve as neurotransmitters and hormones). It is a monoamine (a compound containing nitrogen formed from ammonia by replacement of one or more of the hydrogen atoms by hydrocarbon radicals). Dopamine is formed by the decarboxylation (removal of a carboxyl group) from dopa. Polydopamine contains a great number of bioactive groups, such as catechol moieties, OH^- and NH_2^- and will therefore bind strongly to metal ions.[29] For this reason, we hypothesized that polydopamine modification of MS scaffolds would improve the mineralization and cytocompatibility of scaffolds. One aim of this study was therefore to coat a layer of polydopamine on the pore walls of MS scaffolds in order to improve their mineralization capacity and enhance the attachment and proliferation of BMSCs in the scaffolds. The other aim was to evaluate dexamethasone (DEX) delivery in MS-based scaffold system and investigate its effect on the response of BMSCs.

2. Experimental Section

2.1 Preparation and characterization of polydopamine-modified porous SiO_2 scaffolds.

Porous SiO_2 scaffolds were prepared using co-templates of nonionic block polymer P123 (EO20-PO70-EO20) (Sigma Aldrich) and polyurethane sponges. P123 is used to produce mesoporous structures (mesopore size: several nanometers) and polyurethane sponges are used to create large pores (large pore size: several hundred micrometers) as described in our previous publication.[13] Typically, 6.67 g of P123 (Mw=5800, Sigma Aldrich), 13.83 g of tetraethyl orthosilicate (TEOS, Sigma Aldrich) and 1.67 g of 0.5 M HCl were dissolved in 100 g of ethanol and stirred at room temperature for one day. The polyurethane sponges (25 ppi) were cleaned and completely immersed into this solution for 10 min, then transferred to a petri dish; excess solution was removed and the remainder evaporated at room temperature for 24 h. This procedure was repeated five times. Once the samples were completely dry, they were calcined at 700^{o}C for 5 h yielding the porous MS scaffolds.

To prepare polydopamine-modified MS scaffolds, dopamine hydrochloride was first dissolved in 10 mM Tris-HCl (pH 8.5) with the concentration of 2 mg/mL according to the publication.[24] Then, the prepared MS scaffolds (5×5×4 mm) were soaked in dopamine/tris solution for 6 and 24 h (hereafter referred to as MSD6h and MSD24h, respectively). The color of the dopamine/tris solution becomes dark due to the pH-induced oxidation of dopamine. After soaking, porous MSD6h and MSD24h scaffolds were rinsed in water and dried by N_2 gas. The surface morphology, large-pore structure, and surface composition of MS scaffolds before and after polydopamine modification were characterized by scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The scaffolds were

then crashed into powders. The inner mesopore-structure of the powders was characterized by transmission electron microscopy (TEM). The mesopore size distribution in the pore walls of MS scaffolds, before and after polydopamine modification, was measured through BJH analysis of N_2-adsorption-desorption by using the crashed powder samples.

The open-pore porosity of the scaffolds was measured according to the following formulation $P = (W_2-W_1)/(W_2-W_3) \times 100\%$,[30, 31] where W_1 is the dry weight of the scaffolds, W_2 is the weight of scaffolds saturated with water, and W_3 is the weight of scaffolds suspended in water. Three samples were used for repeating this testing.

2.2 In vitro mineralization of the scaffolds

SBF containing ion concentrations similar to those in human blood plasma was prepared according to the method described by Kokubo.[32] MBG scaffolds were soaked in SBF at 37°C for three days, and the ratio of the solution volume to the scaffold mass was 200 mL/g. Apatite mineralization on the surface of three scaffolds (MS, MS6h and MS24h) was determined by SEM and energy dispersive spectrometry (EDS) (Jeol JSM6510, Tokyo, Japan).

2.3 Drug delivery from the scaffolds

Dexamethasone (DEX, Sigma-Aldrich), is a synthetic and widely used glucocorticoid, which affects osteogenesis. It was dissolved in phosphate-buffered saline (PBS) to a concentration of 0.05 mg/mL. To investigate the effect of polydopamine modification on the loading and release of DEX, scaffolds (5×5×4 mm) were soaked 20 mL of DEX/PBS solution for 24 h at room temperature, then dried at 40°C for 24 h. At the same time, DEX was loaded into

MSD24h scaffolds (hereafter referred to as MSD-DEX) for cell culture experiments. The loading amount of DEX in the three scaffolds types (MS, MSD6h and MSD24h) was measured by the depletion method, by determining the difference of DEX concentration in the loading medium before and after loading by UV analysis.

DEX release was evaluated by placing one DEX-loaded scaffold into 4 mL of PBS (pH 7.4) at 37 ºC for 2, 5, 8, 24, 72, 168, 336 and 504 h. DEX release was determined by UV analysis. The accumulative release rate of DEX (%) was calculated with the following equation: DEX (%) = (total amount of DEX released / total loading amount of DEX in scaffolds) × 100%.

2.4 Morphology and proliferation of BMSCs on the scaffolds

Isolation and culture of BMSCs was conducted following previously published protocols.[6, 7, 33] Bone marrow aspirates were obtained from patients (mean age, 67 years) undergoing elective knee and hip replacement surgery. Informed consent was given by all patients involved and the research protocol had been approved by the Human Ethics Committees of Queensland University of Technology and The Prince Charles Hospital. As MSD6h scaffolds have no obvious mineralization in SBF, they were eliminated from further cell experiments. For cell culture, three scaffolds types (MS, MSD24h and MSD-DEX) were selected to evaluate how polydopamine modification, as well as DEX delivery, influenced the attachment, proliferation and differentiation of BMSCs on the MS scaffold.

BMSCs were cultured on 5×5×4 mm scaffolds placed in 24-well culture plates, at an initial density of 1×10^5 cells/scaffold. The cells were cultured for 1 and 7 days in DMEM culture medium (GIBCO) supplemented with 10% FCS, after which the scaffolds were removed from

the culture wells, rinsed in PBS, and then fixed with 1.25% glutaraldehyde, 4% paraformaldehyde, and 4% sucrose in PBS for 1 h. The fixative was removed by washing with buffer containing 4% (w/v) sucrose in PBS and post fixed in 1% osmium tetroxide in PBS followed by CO_2 critical-point drying. The specimens were coated with gold and the morphological characteristics of the attached cells determined using SEM.

To assess cell proliferation, an MTT assay was performed by adding 0.5 mg/mL of MTT solution (Sigma-Aldrich) to each scaffold and incubated at 37ºC to form formazan crystals. After 4 h, the media was removed and the formazan solubilized with dimethyl sulfoxide (DMSO). The absorbance of the formazan-DMSO solution was read at 495 nm on a plate reader. Results were expressed as the absorbance reading from each well minus the optical density value of blank wells.

2.5 Alkaline phosphatase (ALP) activity of BMSCs on the scaffolds

Osteogenic differentiation was assessed by measuring a time course of alkaline phosphatase (ALP) activity of BMSCs grown on the various scaffold types. Scaffolds were placed into 24-well plastic culture plates and seeded with $1x10^5$ BMSCs per scaffold. The cells were incubated at 37ºC in 5% CO_2 for 7 and 14 days and the medium changed every 3 days. On day 7 and 14, the samples were removed and ALP activity was measured. The scaffolds were irrigated with PBS three times to remove as much residual serum as possible and then 0.5 mL of 0.2% Triton® X-100 were placed on the scaffold sample to dissolve the cells. The solution was transferred into a 1.5 mL tube, and sonicated after which the samples were centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant transferred to fresh 1.5 mL tubes to which 100

μL 1 mol/L Tris-HCl, 20 μL 5 mmol/L $MgCl_2$, and 20 μL 5 mmol/L p-nitrophenyl phosphate was added. After 30 min incubation at 37°C the reaction was stopped by the addition of 50 μL of 1N NaOH. Using p-nitrophenol as a standard, the optical density was measured at 410 nm with a spectrophotometer. The ALP activity was expressed as the changed optical density (OD) value divided by the reaction time and the total protein quantity as measured by the BCA Protein assay kit (Thermo Scientific, Melbourne, Australia).

2.6 Statistical analysis

All data were expressed as means ± standard deviation (SD) and were analyzed using One-Way ANOVA with a Post Hoc test. A p-value < 0.05 was considered statistically significant

3. Results

3.1Characterization of the scaffolds

Optical graphs show that the color of MS scaffolds vary from white to brown and black after being modified by polydopamine at 0, 6 and 24 h, respectively (Figure 1). SEM analysis was used to observe the large-pore structure of the obtained scaffolds. Three scaffolds types (MS, MSD6h and MSD24h) have a highly porous structure with a similarly large-pore size of around 300 μm (Fig. 2a, c and e). Higher magnification images show that MS scaffolds have a smooth surface (Fig. 2b). There are a few polydopamine microparticles on the surface of MSD6h scaffolds (Fig. 2d). After coated for 24h, a rough polydopamine film is formed on pore walls of MSD24 scaffolds (Fig. 2f).

FTIR analysis confirms that polydopamine was coated on the pore walls of MS scaffolds (Fig. 3). There is an obvious peak at wavenumber 1497 cm^{-1} in the pattern of MSD24h scaffolds, which is assigned to C=C ring stretching and NH_2 from dopamine.[34, 35] In addition, at the wavenumber of 3340cm^{-1}, the peak intensity in MSD24h pattern is significantly higher than that in MS pattern, which may be assigned to NH_2 stretching models and OH^- groups from dopamine.[35-37]

TEM was used to observe the mesopore channel structures in the pore walls of scaffolds. Polydopamine modification did not change the ordering of mesoporous structure of MS scaffolds. TEM analysis shows that MS scaffolds both before and after polydopamine modification have a well-ordered mesopore channel structure and the mesopore size is around 5 nm (Fig. 4a and b). N_2-adsorption-desorption testing was used to analyze the nanopore size distribution of the mesopores in the inside of scaffold pore walls, which have indicated that polydopamine modification to MS scaffolds with different time did not change the pore distribution of mesopores, which is mainly distributed at 5 nm (Fig. 4c).

3.2 The in vitro mineralization of the scaffolds

There is no obvious apatite mineralization on the surface of MS (Fig. 5a and b) and MSD6h (Fig. 5c and d) scaffolds after soaking in SBF for 3 days. EDS analysis shows that only Si element was detected in the patterns of MS and MSD6h scaffolds after soaked in SBF (Fig. 5b and d); on the contrary, MSD24h scaffolds induce significant apatite mineralization in SBF. The apatite forms a layer of clusters on the surface of pore walls after soaked in SBF for 3 days (Fig. 5e). Higher magnification images show that the formed apatite clusters are

composed of net-like nanocrystals (Fig. 5f). The ratio of Ca/P for the formed apatite clusters is 1.59 (Fig. 5f).

3.3 Drug delivery of the scaffolds

The loading amount of DEX in each MS, MSD6h and MSD24h was 31.3±1.8, 35.3±1.7, and 31.8±4.7 μg, respectively. There was no obvious difference in the loading capacity of DEX in any of the three scaffolds types, nor were there any obvious differences in the release kinetic of DEX (Fig. 6). Even after 21 days (504h) of soaking in PBS, the scaffolds still maintained a sustained release of DEX, and the accumulative release reached around 95%. (Fig. 6).

3.4 Attachment, morphology, proliferation, ALP activity of BMSCs on the scaffolds

BMSC attachment and morphology on the three scaffold types were examined by SEM (Fig. 7) and showed that after 7 days of culture, BMSC attachment was supported in all three (MS, MSD and DEX-MSD) scaffold types (see arrows). There are some cells visible on the pore walls of MS scaffolds (Fig. 7a); however, a greater number of cells attach on the polydopamine modified MS scaffolds (Fig. 7b) and similarly on the DEX-MSD scaffolds (Fig. 7c). It can be seen that the BMSCs on the MSD and DEX-MSD scaffolds form closer contact with their substrates by the presence of numerous filopodia (Fig. 7b and c).

MTT analysis shows cell proliferation on the MS, MSD and DEX-MSD scaffolds commensurate with increased time in culture (Fig. 8), and it is noteworthy that the proliferation of BMSCs is significantly higher on MSD and DEX-MSD than on pure MS scaffolds.

There is no obvious difference for the ALP activity of BMSCs on the three scaffold types after 7 days in culture; however, after 14 days in culture, the ALP activity of BMSCs on MSD-DEX scaffolds is significantly higher than that of both the MS and MSD scaffolds (Fig. 9).

4. Discussion

We have successfully prepared mussel-inspired MS scaffolds with large-size pore (300 μm) and well-ordered mesopores (5 nm) by coating polydopamine on the surface of pore walls, and investigated the effect of polydopamine modification on the mineralization, and the attachment, proliferation and differentiation of BMSCs in MS scaffolds. Our results provide evidence that such polydopamine modification significantly stimulated apatite mineralization and enhanced the attachment and proliferation of BMSCs in the scaffolds. Polydopamine modification did not influence the rate of DEX release from the scaffolds but it was found that DEX significantly improved the ALP activity of BMSCs cultured in and on the scaffolds. The current results strongly suggest that polydopamine-modified MS scaffolds represents a promising scaffold system with which to enhance mineralization and cell viability, as well as a drug release systems to enhance osteogenic properties and offers clear advantages in the development of a long-lasting and effective tissue engineering platform.

Porous MS scaffolds were prepared by using a polymer sponge method. We showed that MS scaffolds produced by this method have a porosity of 85%. The obtained scaffolds were characterized with two levels of pores by different characterization methods. SEM analysis has shown a large-pore size around 300μm in the scaffolds. To identify the mesopores, the

scaffolds were crashed into powders without large-pore size. TEM and N_2-adsorption-desorption analysis were mainly used to test the nano-size microstructure of the crashed powders, in which both of testing methods have indicated that the pore walls of the obtained scaffolds possess mesopores with a size of 5nm. The large pores in MS scaffolds greatly benefit tissue ingrowths and nutrient exchange.[38] The scaffolds are sufficiently robust to retain structural integrity when handled for *in vitro* experiments. In terms of bone tissue engineering applications, the main role of these scaffolds is to provide initial mechanical support for cells. In this respect, MS scaffolds are able to meet the requirements as a cell carrier for bone tissue engineering.

Mussel-inspired MS scaffolds have been prepared by modification of scaffolds using polydopamine. Lee, *et al.* originally coated polydopamine on a number of material surface, including noble metals, oxides, semiconductors and synthetic polymers,[24] whereas our focus was on coating polydopamine on tissue engineering scaffolds. It was found that the method used is a workable way to construct more bioactive MS scaffolds for tissue engineering applications. We confirmed that a uniform polydopamine coating formed on the surface of the scaffold-pore walls. This process may involve the oxidation of the catechol moiety of dopamine to a quinone, followed by polymerization in a manner reminiscent of melanin formation as described in previous publications.[24, 39] The polydopamine may then form strong covalent and noncovalent interactions with the substrates.[24, 40]

One of the most interesting results of this study was the fact that polydopamine modification of MS scaffolds significantly stimulated apatite mineralization in biological solutions. Apatite mineralization of bioactive materials plays an important role in the formation, growth, and

maintenance of the tissue-biomaterials interface.[32, 41] In addition, previous investigations have confirmed that a mineralized apatite layer has the capacity to enhance osteoblastic activity,[42, 43] possibly by binding serum proteins and growth factors to the substrate, which then stimulate cell proliferation and activate cell differentiation.[44] Apatite mineralization on biomaterials have two potential mechanisms: One is that bioactive glasses and ceramics release Na^+ or Ca^{2+} ions and the saturated ions in thus in the biological solution will induce the remineralization of apatite;[45, 46] the other mechanism is that the surface of biomaterials is negatively charged with OH^- which can also induce the apatite mineralization.[47, 48] In this study, we speculated that the OH^- and NH_2^- groups in polydopamine could contribute to a negative surface charge and in turn induce the Ca-P mineralization on the pore walls of MS scaffolds.

The other interesting result from this study was that polydopamine modification of the MS scaffolds significantly enhanced the attachment and proliferation of BMSCs. We hypothesize there are three potential factors which may contribute to the enhance cell attachment and proliferation in the scaffolds. The first factor is that polydopamine itself can act as a strong anchor between cells and substrates without any covalently grafting.[24] The second factor is that polydopamine contains OH^- and NH^{2-} groups. These chemical groups will improve the hydrophilicity and alter the surface charge of MS scaffolds, which may benefit the attachment and proliferation of BMSCs in scaffolds.[49] As we discussed above, apatite mineralization of biomaterials can enhance the osteoblastic activity. The fact that polydopamine-modified MS scaffolds have enhanced apatite mineralization may therefore be the third factor to benefit the attachment and proliferation of BMSCs. However, the ALP activity of BMSCs on MSD group

is lower than that on MS group. From the point of cell cycle view, the cells should proliferate firstly and then differentiate. Our results indicate that the BMSCs on MSD group have not switched from the proliferation to the differentiation status well.

Polydopamine modification did not change mesopore structure and pore-size distribution of the MS scaffolds; the polydopamine modified-MS scaffolds therefore maintained a sustained release of DEX. The sustained release of DEX from polydopamine-modified MS scaffolds significantly stimulated ALP activity of BMSCs. Earlier studies have reported that the incorporation of DEX into various polymer (PLGA, PCL or chitosan) scaffolds enhanced the differentiation of osteoblast-like cells.[50-54] Our study has indicated that DEX delivery could enhance the early-stage differentiation of BMSCs. Our recent study has also shown that DEX delivery from bioactive glasses scaffolds has significantly improved the ALP activity and bone-relative gene expression of osteoblasts.[31] Therefore, it is speculated that the DEX delivery in our current MSD scaffolds may promote the bone-relative gene expression of BMSCs. However, we will conduct more tests in the future to confirm our speculations.

5. Conclusions

Mussel-inspired MS scaffolds with hierarchically large pores (300 μm) and well-ordered mesopores (5 nm) were successfully prepared by coating on the surface of the pore walls MS scaffolds. These modified MS scaffolds have significantly enhanced the apatite mineralization ability, as well as attachment and proliferation of BMSCs, compared non modified scaffolds. The osteogenic drug, DEX, can efficiently be loaded and released from the MS scaffolds. The sustained release of DEX from these scaffolds significantly improved ALP activity of BMSCs.

This study indicates that DEX-loaded polydopamine coated MS scaffolds are excellent platforms for bone tissue engineering application with significantly enhanced osteogenic properties.

Acknowledgements

Funding for this study was provided by the Natural Science Foundation of China (Grant 30730034), Vice-chancellor Research Fellowship of QUT and One Hundred Talent Project, SIC-CAS. The authors wish to thank Dr Petr Formanek for helping TEM analysis and Mr Thor Friis for his assistance in the preparation of this manuscript.

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