3D-printed hierarchical scaffold for localized isoniazid/rifampin drug delivery and osteoarticular tuberculosis therapy

Zhu Min a,1, Li Kun b,1, Zhu Yufang a,*, Zhang Jianhua a, Ye Xiaoqian b,*

a School of Materials Science and Engineering, University of Shanghai for Science and Technology, Shanghai 200093, China
b Department of Orthopedics, Changzheng Hospital of Second Military Medical University, Shanghai 200003, China

A R T I C L E   I N F O

Article info

Received 15 October 2014
Received in revised form 26 December 2014
Accepted 22 January 2015
Available online 31 January 2015

Keywords:
Mesoporous bioceramics
Multi-drug delivery
Localized drug release
Long-term antituberculosis therapy

Abstract

After surgical treatment of osteoarticular tuberculosis (TB), it is necessary to fill the surgical defect with an implant, which combines the merits of osseous regeneration and local multi-drug therapy so as to avoid drug resistance and side effects. In this study, a 3D-printed macro/meso-porous composite scaffold is fabricated. High dosages of isoniazid (INH)/rifampin (RFP) anti-TB drugs are loaded into chemically modified mesoporous bioactive ceramics in advance, which are then bound with poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) through a 3D printing procedure. The composite scaffolds show greatly prolonged drug release time compared to commercial calcium phosphate scaffolds either in vitro or in vivo. In addition, the drug concentrations on the periphery tissues of defect are maintained above INH/RFP minimal inhibitory concentrations even up to 12 weeks post-surgery, while they are extremely low in blood. Examinations of certain serum enzymes suggest no harm to hepatic or renal functions. Micro-CT evaluations and histology results also indicate partly degradation of the composite scaffolds and new bone growth in the cavity. These results suggest promising applications of our hierarchical composite scaffold in bone regeneration and local anti-TB therapy after osteoarticular TB debridement surgery.

© 2015 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.

1. Introduction

In the past two decades, there were millions of new tuberculosis (TB) cases annually, which continues to be an occupational health concern around the world. Thereinto, osteoarticular TB, which is a persistent inflammatory form of the TB that affects joints, account for approximately about 10–11% of extrapulmonary TB cases.

Among the common treatments toward osteoarticular TB, long-term antitubercular drug (ATD) administration usually has unsatisfactory therapeutic outcomes relating to poor patient compliance as well as the emergence of drug resistance [1]. So far, the outcome of therapy could be improved with the introduction of nanotechnology-related rational drug delivery systems releasing the ATD in a slow and sustained manner, which allows reduction in frequency and dosing numbers and increase in overall drug efficiency [2–4]. However, the osseous focal point is poor in blood supply and it is not easy for blood-carrying either free ATD or ATD-contained nano-vehicles to reach the focus [5,6]. Therefore the tubercle bacillus living in the osseous focus or survived through debridement operations was difficult to be killed completely by traditional systemic chemotherapy and easy to become latent bacillus which would reproduce rapidly once in comorbid conditions.

An alternative general regulation has been surgical involvement due to the developments in fixation equipment and technology [7,8]. However, a 6–9 month course of multiple drugs is still regretfully obligatory to consolidate the curative effect in addition to surgery [9]. And the residual cavity after debridement should be repaired as well, otherwise it will give rise to rapid propagation of the residual tuberculosis germs when it was filled by blood clot [10]. Therefore, orthopedic consultants are more inclined toward the use of sustained drug-discharge implanted scaffold, which would on the one hand recover the remaining empty and on the other hand, offer sustained local drug release to desired places.

As already noted, much attention has been paid to poly (lactide-co-glycolide) (PLGA) as a base compound of micro-particles for pulmonary ATD delivery [11–14]. However, pure PLGA or other degradable and biocompatible polymer implants embedded into the bone defect cavities would induce the significant decrease of pH values by the acidic degradation products [15,16]. The decreased pH values could result in drug resistance of tubercle...
bacillus [17]. Alternatively, mesoporous silica-based materials [18–21], may provide a more advantageous choice for controlled and localized ATD delivery without causing significant pH value decreases, owing to the extensive nanoporous structure.

Our group has successfully fabricated a composite scaffold composed of mesoporous silica nanoparticles (MSN) and bioactive glass coatings in a porous β-TCP bio ceramic, which showed greater INH and RFP simultaneous encapsulation capability [22]. More importantly, the mesostructure of nanoparticles together with bioactive glass and/or hydroxyapatite deposits played vital functions in prolonging the drugs’ release in both in vitro and in vivo analyses. The therapeutic quantity of both INH and RFP for treating TB in vivo can be sustained for an extended period over 6 weeks without major long-term lesions to the liver and kidney. However, the lasting duration of drug release still leaves much to be desired. Relying on simple physical adsorption into the mesopores, MSN/BG/β-TCP composites lack capability to adjust drug loading amounts in a broader range as well as the release profile. Surface modification is believed to be the most common and effective method to regulate drug delivery behaviors of mesoporous materials [23–25]. On basis of the drug’s structural formula, different chemical groups such as amino-, carboxy-, thiol-groups, etc., have been grafted onto mesopore surface through reactions to silane coupling agents. The affinities between drug molecules and the functional groups on the pore wall offer feasibility to control the release rate of drugs [26,27]. The functionalization also shows influence in the pore size, mesophase structure and then affects delivery kinetics [23]. Moreover, another problem of MSN/BG/β-TCP scaffold is that MSN and dense calcium phosphate bioceramics can hardly induce apatite formation owing to low solubility, which thus is not beneficial for following osteogenesis procedure. Mesoporous bioactive glasses (MBG), being suggestive of potential use in bone tissue engineering recently, have superior apatite formation ability due to their significantly increased surface area [28,29]. However, previous studies by several groups have indicated the brittle characteristic of pure MBG scaffolds. For instance, Shi et al. and Yun et al. reported the preparation of hierarchically MBG scaffolds by using the nonionic triblock copolymer and polyurethane as co-templates to achieve nanoporosity (<10 nm) and macro porosity (>100 μm) respectively, and their compressive modulus was less than 1 MPa [30–32]. As a solution, composites of biodegradable polymers (PCL, PLGA, alginate, collagen and so on) and MBG ceramics were developed to increase the mechanical stability [33,34]. In addition, an alternative 3D plotting method is developed to promote mechanical strength [35,36]. For instance, our group had 3D-printed MBG scaffolds with a few amounts of polymer adhesives, and they evidently showed improved strength (ca. 2–12 MPa) compared to polyurethane foam templated MBG scaffolds (ca. 50 kPa). The significant advantage of this technique is that the architectures of the scaffolds can be precisely controlled by layer by layer plotting under ambient conditions. And a combination of different materials and drugs can be conveniently obtained though simply adjusting the pastes for printing. Thus, it is promising to construct proper MBG-based composite scaffolds through 3D printing approach and then solve the commonly existing issues for inorganic scaffold materials, for example, uncontrollable pore architecture, low strength and high brittleness.

Therefore, a mesoporous bio ceramic based composite scaffold was designed and fabricated by 3D printing technique herein to meet the requirements of advanced osteoarticular TB therapy. Carboxylic MBG and methyl-functionalized MSN were prepared specifically to load the first line ATD INH and RFP, respectively. PhBHx polymer was used as adhesives in 3D printing to bind all bioceramic and drug powders. A commercially available calcium phosphate scaffold with an irregular porous structure was used as reference to examine drug delivery behaviors and osteogenesis properties of the hierarchical composite scaffold in this study. Particular compositions and mesoporous features were shown to achieve an optimized long-term sustained drug release so that it was able to eradicate the residual tubercle bacillus.

2. Materials and methods

2.1. Preparation, functionalization and characterizations of MBG and MSNs

MBG (molar ratio of SiO2:CaO:P2O5 = 80:15:5) and MSN powders were synthesized respectively according to previous reports [37,38]. Methyl triethoxysilane (MTES, 99%, Sigma–Aldrich) and triethoxysilylpropylsuccinic anhydride (TESPSA, 94%, Sigma–Aldrich) were used for grafting methyl groups onto MSN and carboxylic groups onto MBG. For the modification procedure, 0.5 g of template-free mesoporous powder was refluxed in 50 ml of dry toluene solution containing a certain amount of MTES or TESPSA at 80 °C for 16 h. After cooling to room temperature, the powder was collected by filtration, washed with toluene and distilled water, then dried in vacuum at 80 °C to obtain carboxylic MBG (MBG-COOH) or methyllic MSN (MSN-CH3). The modified products were ground and sieved to reduce the particle size to less than 50 μm.

The morphology and microstructure of MBG, MBG-COOH, MSN, and MSN-CH3 were observed by transmission electronic microscopy (TEM, JEOL 2010, Japan). The surface area and pore parameters were obtained through calculations from N2 adsorption-desorption isotherms (Micrometrics Tristar 3200, 77 K).

2.2. Antituberculosis agent loading process

Functionalized MBG-COOH and MSN-CH3 were loaded with ATD INH and RFP respectively through impregnation.

Briefly, INH was dissolved in ultrapure water to obtain a solution of 50 mg/ml and RFP was dissolved in acetone to make a solution of 50 mg/ml. 1 g MBG-COOH were immersed into a 10 ml INH solution and 1 g MSN-CH3 was immersed into a 20 ml sealed RFP solution. After stirring for one day under room temperature, the drug solutions were evaporated completely and drug-loaded mesoporous powders were dried spontaneously.

In addition, commercial calcium phosphate scaffolds (CaP, BAM, Engineering Research Center in Biomaterials, Sichuan) as control were loaded with both INH and RFP by completely absorbing drops of drug solutions into them.

2.3. 3D printing of the composite scaffolds

Proper composite pastes were firstly prepared as follows: 0.2 g of PhBHx (Mw = 9.5 × 10^4) powders was dissolved in 2 ml of chloroform and dimethyl sulfoxide (DMSO) mixture solvents (volume ratio of CHCl3:DMSO = 20:80) to form a transparent polymer solution. A mixture of sieved MBG-COOH-INH and MSN-CH3-RFP materials was added into the solution and well stirred to produce homogenous pastes. The scaffolds were fabricated using a 4th generation 3D-Bioplotter system (Fig. S1, EnvisionTEC, Germany) under the guide of supporting computer workstations. The 3D models of scaffolds were designed by CAD. The prepared pastes were extruded from the dispensers through the conic plastic nozzles (25 gauge) by applying compressed air to manufacture a cylinder scaffold of φ = 6 mm × h = 8 mm. The printing unit moved with a constant speed along the x- or y-axis. An interval between strands of 700 μm was used on each layer. The final printed scaffolds were named as MPHs.
2.4. In vitro drug release test

In vitro releases of RFP and INH from one MPHS scaffold (weighed) and the control CaP scaffolds were carried out at 37 °C in SBF solution (mass/volume ratio equaled to 1 g/20 ml), respectively. The release medium was withdrawn at pre-determined time intervals, and replaced with a fresh soaking medium each time. Then the concentrations were determined by UV–vis spectrophotometer by measuring the maximum absorbance at the wavelengths of 262 nm for INH and 480 nm for RFP, respectively. The calibration curves were obtained using solutions of INH and RFP respectively in the same concentration ranges before determination.

Surface morphologies of MPHS were observed by SEM (FEI Quanta 450) after 3 days immersing in SBF to investigate the apoptosis formation.

2.5. In vitro cellular evaluation

Primary human bone marrow stromal cells (hBMSCs) were isolated as previously described [39]. The use of human samples was approved by the ethics committee of Sixth People’s Hospital, Shanghai Jiao Tong University School of Medicine. Subcultured hBMSCs at passage 4–10 were adopted in all in vitro cellular experiments.

2.5.1. Cell attachment

In order to assess cell adhesion and cell/biomaterial interaction, a 100 μl aliquot of hBMSC suspension containing 1 x 10⁵ cells were directly seeded on the testing MPHS scaffolds. After 4 h pre-adhesion, DMEM culture medium (GIBCO, Invitrogen Pty Ltd., Australia) supplemented with 10% fetal calf serum (FCS, InVitro Technology, Australia) was added and the plate was kept in humidified culture conditions. At day 3, the cell–scaffold constructs were rinsed with PBS, fixed with 2.5% glutaraldehyde in PBS for 1 h, washed by PBS containing 4% (w/v) sucrose and post fixed in 1% osmium tetroxide in PBS followed by sequential dehydration in graded ethanol (50%, 70%, 90%, 95%, 100%) and hexamethyldisilazane (HMDS). The specimens were sputter-coated with gold for SEM observation.

2.5.2. Cytotoxicity

A Cell Counting Kit-8 assay (Dojindo Molecular Technologies, Inc., Japan) was performed to evaluate the cytotoxicity of MPHS and CaP scaffolds. Briefly, 360 μl of culture medium and 40 μl of CCK-8 solution were added to each well at days 1, 3, and 7 and incubated at 37 °C for another 4 h. An aliquot of 100 μl was taken out, transferred to another 96 well plate and the light absorbances were measured at 450 nm with a microplate reader (Bio-Rad 680, USA).

2.5.3. ALP activity evaluation

To assess ALP activity of hBMSCs grown on the composite scaffolds, 1 x 10⁵ hBMSCs were seeded on each scaffold (n = 3) and cultured in a 24-well plate for 5, 10 and 14 days. At the predetermined time point, the culture medium was decanted and the cell layer washed gently three times with PBS followed by washing once in cold 50 mM Tris buffer, and then hBMSCs were lysed in 200 μl 0.2% Triton X-100. Lysates were sonicated after being centrifuged at 14,000 rpm for 15 min at 40 °C. 50 μl supernatant was mixed with 150 μl working solution according to the manufacturer’s protocol (Beyotime). The conversion of p-nitrophenyl phosphate into p-nitrophenol in the presence of ALP was determined by measuring the absorbance at 405 nm with a microplate reader (Bio-Rad 680, USA). The results were expressed in μM of p-nitrophenol produced per min per mg of protein.

2.6. Animal studies

2.6.1. Surgical procedures

Forty-eight young New Zealand rabbits (provided by Shanghai Second Military Medical University, Laboratory Animal Center, male or female, 2–2.5 kg body weight) were randomly divided into 16 groups (n = 3), 8 groups each for MPHS and CaP scaffolds. The use of animals and the experimental protocols was approved by the Institutional Animal Welfare Committee of Shanghai Second Military Medical University. Prior to surgery, the blood samples were collected from ear veins of five randomly selected normal rabbits. Briefly, when under general anesthesia by injecting 3% Nembutal (30 mg/kg) via the ear vein, the right femoral recipient site of rabbit was approached by a sharp scalpel, and the disto-medial metaphysis of the femur was approached and cleared of soft tissue with a Woodson or Freer periosteal elevator. Thereafter, a surgical drill (6 mm outer diameter) was used to prepare an 8 mm deep defect for the scaffold insertion. One drug-laden shaped MPHS sample or one CaP scaffold was respectively embedded into the groove. A picture was taken to explain the surgery procedure in Fig. S1 (Supplementary materials). Following surgery, surrounding soft tissues were carefully closed. All the rabbits were monitored after surgery and fed standardly.

2.6.2. In vivo drug distributions in periphery tissues and blood

At 1, 3, 7, 14, 28, 42, 63 and 84 days post-surgery, 2 ml blood samples were collected through heart puncture after all the rabbits were anesthetized. Then the rabbits were sacrificed humanely, and samples of bones, musculature and fibrous tissue clinging to the groove (local tissues, 0.5 cm away from the closer end of the groove) were collected as well. 1 g of local tissues was ground, added into 2 ml 10% methanol and homogenized into a suspension. The suspension and blood samples were centrifuged to collect the supernantant fluids for INH and RFP concentration tests by High Performance Liquid Chromatography.

2.6.3. Hepatic and renal function tests

The supernatants of blood samples including the blank control specimens obtained were directly placed in an Automatic biochemistry analyzer (OlympusAU2700). Contents of serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Blood Urea Nitrogen (BUN) and Creatinine (Cr) were examined.

2.6.4. Osteogenesis evaluation

After harvesting at each time point, the femurs were removed intact and fixed in 4% freshly prepared formaldehyde for 24 h at 4 °C. The μ-CT imaging system (μCT50, Scanco Medical, Bassersdorf, Switzerland) was used to evaluate osteogenesis within the defect region. The visualization of the bone was generated by reconstruction of isosurface renderings using the 3D Creator software.

After dehydration in ascending concentrations of alcohol, from 75% to 100%, the undecalced specimens were embedded in poly-methylmethacrylate (PMMA) and 150-mm-thick sections in the orientation of either transverse or sagittal surface were obtained using a microtome (Leica, Hamburg, Germany). The sections were then polished to a final thickness of approximately 40 mm and stained with van Gieson’s picrofuchsin to identify new bone formation. Red indicated new bone formation, and the scaffolds showed black.

2.7. Statistical analysis

The data were collected from three separate experiments and expressed as mean ± standard deviation. The one-way ANOVA and Student–Newman–Keuls post hoc tests were used to determine the level of significance, and P values <0.05 were considered to be significant.
3. Results

3.1. Mesoporous powder characterizations

The TEM images in Fig. 1 illustrated the mesoporous microstructure and particle morphology before and after chemical modification. As-synthesized MBG powders showed a highly ordered parallel mesopore organization (Fig. 1a1), which was consistent with the reported result [38]. Similarly, the MSN with size distribution at around 300 nm exhibited one dimensional cylindrical mesopores (Fig. 1b1). After functionalization, the mesopore arrangement of both samples as well as particle dimensions of MSN did not change a lot (Fig. 1a2 and b2).

The nitrogen adsorption/desorption isotherms (Fig. 2a) of the MBG series samples were identified as type IV isotherms with the same hysteresis loops of H1 type associated with the characteristic cylindrical pores, in accordance with the p6mm mesoporous structure of MBG materials. And MSN series samples (Fig. 2b) showed type IV isotherm curve as well with a step between 0.2 and 0.3 of $P/P_0$ indicating an ordered mesoporous structure. After drug encapsulation, the loops disappeared for both MBG and MSN, and their surface areas dropped to 10.1 and 63.0 m$^2$/g, respectively. All pore data are summarized in Table 1.

3.2. Composite scaffold characterizations

The SEM observation at low magnification of cylindrical MPHS scaffold in Fig. 3a revealed that the printing direction of strands followed an alternative X–Y pattern as designed in advance, which thus resulted in a well-defined square open pore structure from the top view. The strands in the first layer were rather straight and equal in width, while in the second layer or below the strands were slightly squished due to unfinished drying before upper layer plotting. Zoomed-in images gave more details of the surface of MPHS scaffold. Relative large MBG particles and MSN nanoparticles were bound tightly with PHBHHx in between. Moreover, plenty of MSN were embedded in the polymer, which was probably attributed to fast evaporation of organic solvent during 3D printing. The mean compressive strength value of MPHS scaffold was calculated to be around 3.15 ± 0.69 MPa as illustrated in Fig. S2 (Supplementary materials) [40].

3.3. Bioactivity and cellular responses of the composite scaffold

The image in Fig. 4a showed the surface morphologies of MPHS scaffolds after soaking in SBF solution for 3 days. A significant evolution from the relative smooth surface (Fig. 3b) to rough HA deposition layer was observed. Additional magnified picture in Fig. S3 (Supplementary materials) confirmed the formation of plate-like apatite crystal aggregates, and the energy diffraction spectra showed a reasonable Ca/P molar ratio of about 1.53 corresponding to calcium-deficient carbonated hydroxypatite, which was close to the value of 1.67 of formal apatite.

The surface of composite MPHS scaffold was able to support hBMSC cell attachment as well as growth. In Fig. 4b, hBMSCs on strands all presented well-extended morphology after being cultured with MPHS scaffold for 3 days. It is obvious that the phenotype of hBMSCs were maintained. Fig. S4 gave an additional observation of cells penetrating into the macro pores of MPHS scaffold and adhering to the surface well.
The proliferation of hBMSCs cultured on both CaP and MPHS scaffolds was determined by CCK-8 assay and the results are shown in Fig. 4c. An increase in absorbance from day 1 to day 7 was recorded indicating that all scaffolds supported cell proliferation, although the MPHS sample showed a slightly lower absorbance compared to commercial CaP scaffold which might be due to the possible residual organic solvent. Similar results of ALP activity to cell proliferation were obtained. As presented in Fig. 4d, ALP activity also followed a steady rise along with a longer culture time and both of MPHS and CaP scaffolds showed good hBMSC osteogenic differentiation.

### 3.4. In vitro drug release

According to the UV–vis examination and calculations, the drug loading efficiency was as high as about 71% for INH and 63.7% for RFP. The release profiles of INH and RFP from MPHS composites and CaP scaffolds are shown in Fig. 5. Both INH and RFP drugs were almost completely released from the macropores of CaP scaffold only within 3 days. In contrast, MPHS presented an extraordinarily sustained release pattern of both drugs. Taking INH for an example, a slight initial burst release of INH was observed during the first day which accounted for about 25% of the total loaded from the MPHS scaffolds. It is noticed that such a burst release percentage is significantly smaller than that of CaP or our reported composite scaffold [22], which can be most probably attributed to the polymer encapsulation. To confirm, the drug release patterns in an early stage of 1 week from dry MPHS pastes and printed MPHS scaffold are plotted in Fig. S5 (Supplementary materials). There were no significant differences of drug release rates between these two composites with only different formations, which demonstrated that few free drugs were exposed to release medium. Subsequently, the release process of MPHS samples lasted for more than 12 weeks. About 80% of loaded drugs, both INH and RFP, were freed out on the 84th day according to Fig. 5.

### 3.5. In vivo drug release

The released concentrations of the ATD with respect to time in periphery tissues or blood were monitored. The data are displayed in Fig. 6. Both INH and RFP drug concentrations reached maxima only in the third day from CaP scaffolds either in blood or in tissues. More importantly, the concentrations of drugs from CaP scaffold quickly dropped down to zero in 2 weeks, while those from MPHS composite scaffolds still remained above the effective minimum inhibitory concentrations (MICs, INH: 10 µg/ml; RFP: 5 µg/ml [41,42]) for even more than 8 weeks. For instance, released INH drug concentration in the periphery tissues reached its maxima in the seventh day and the value (about 105 µg/g) was remarkably high. Moreover, the concentration was kept in the range of scaffold [22], which can be most probably attributed to the polymer encapsulation. To confirm, the drug release patterns in an early stage of 1 week from dry MPHS pastes and printed MPHS scaffold are plotted in Fig. S5 (Supplementary materials). There were no significant differences of drug release rates between these two composites with only different formations, which demonstrated that few free drugs were exposed to release medium. Subsequently, the release process of MPHS samples lasted for more than 12 weeks. About 80% of loaded drugs, both INH and RFP, were freed out on the 84th day according to Fig. 5.
0.025–0.05 µg/ml [40,41] and could inhibit the growth of tubercular germs till 12 weeks later. It is noticed that INH drug level in the blood was in a much lower order of magnitude than that in tissue samples, and the concentration arrived at only 3.2 µg/ml which was supposed to be the maxima during our release test period. These results were consistent with the in vitro release profiles.

3.6. Hepatic and renal function tests

The hepatic and renal functions were tested by examining the values of some serum enzymes at different time points. The blood specimens collected from the ear vein of the rabbits without any post treatment were employed as control (Fig. 7, black dotted line). As Fig. 7 demonstrates, AST values of the MPHS group were detected to be slightly higher in the first 2 weeks, while recovered to normal levels of those of the control group later on. This phenomenon was understandable and consistent with the in vivo drug release features shown in Fig. 6. More generally, all of ALT, AST, BUN and Cr values of tested scaffold samples oscillated around the control line during the whole monitoring process, and the variations remained within the reasonable scope, which suggested that both MPHS composites and the CaP implant had no significant long-term harms to either liver or kidney according to medical principles.

3.7. Osteogenesis activity

For evaluating the responses of the tissue to the biomaterial and new bone formation, we performed micro-CT and histological analysis on the peripheral area of the implant. Representative transverse images of osteogenesis in the femoral defect for each group are shown in Fig. 8. The difference between the three groups was depicted visibly and showed that the scaffold-filled defects achieved more favorable osteogenesis and mineralization when compared to the unfilled defects, though there was still a small gap remaining at the drill incision site which was probably filled with granulation tissues. Specifically, the defects with either MPHS or CaP scaffold implanted were largely recovered with cortical bone and no cancellous bone was apparent until 12 weeks. When untreated, minimal amounts of cancellous bone scattered on the surface of the host bone. Both of the CaP porous scaffold and the tested MPHS scaffold were gradually connected to the surrounded tissues by the newly formed bone. Inside the area of implants, evident calcifications could be observed in MPHS and CaP scaffold indicating possible new bone generations. The sagittal images of MPHS samples are shown in Fig. S6 (Supplementary materials) to illustrate progressive degradation of the MPHS biomaterials with time. It is noticed that the regular porous structure of 3D printed MPHS scaffold was only partially kept 2 months post-surgery and almost lost after 3 months.

Under light microscopy, the undecalcified specimens of CaP and MPHS groups demonstrated no significant difference in the amounts of new bone formation. The bone regeneration commenced only from the periphery of the host bone or the interface between the scaffolds and the surrounded bone, whereas no obvious newly formed bone was found in the center of the defect site. On higher magnification, a line of cuboidal-shaped osteoblasts could often be seen along the scaffold surface to support progressive bone formation (see Fig. 9).

4. Discussion

In the treatments for patients with osteoarticular TB, surgical involvement and obligatory anti-TB multi-drug therapy have encountered several severe problems, such as significant side effect and unsatisfactory curative efficacy due to low targeted drug concentration at TB foci. In this report, we have designed a hierarchical macro/mesoporous composite scaffold (MPHS) to locally deliver...
sufficient bi-ATD in the articular TB foci and regenerate the surgical defect in the meantime. This system has superiorities in achieving higher dosages of INH/RFP drugs and more sustained drug release extending over about 3 months. Additionally, the 3D printing technique is advantageous to improve the mechanical strength and control the chemical compositions, morphology and structure of the scaffold, which is evidently more helpful in bone tissue repair.

Specifically, the fabrication of MPHS scaffold in this study is portrayed in Fig. 10, which also demonstrated schematically how the composites worked for improving the local ATD delivery. The influence factors could be summarized into three aspects: mesopore confinement of the drugs, pertinent surface modification and direct printing of polymer and ceramics.

INH and RFP are the most important two first line anti-TB drugs and the combined use of them is certainly better than individual use. Since the molecular size of each drug is less than 1 nm, either MBG (pore size ~ 4 nm) or MSN particles (pore size ~ 2.5 nm) have enough mesopore space for them. In case of too fast degradation of MBG and then induced scaffold collapse, we adopted a mixture of MBG and MSN mesoporous bioceramics as drug containers in the composite scaffold. As shown in Fig. 10, -COOH were anchored onto the surface of MBG, and INH molecule with the amine functional group is able to be connected firmly via ionic interactions. It is observed from N2 sorption isotherms that the mesopores were almost all blocked up after drug loading, which demonstrated great INH capacity in the -COOH modified pores. As for RFP molecules, they can hardly dissolve in water and are difficult to accommodate to the hydrophilic silica mesopores. Therefore, methyl-functionalized surfaces were created to develop a more hydrophobic environment and increase the affinities between RFP molecules and the mesopores. Similarly, results showed undetectable pore size and extremely small pore volume (0.05 cm3/g), indicating high RFP loading amounts. In addition, unabsorbed drugs were not discarded when the drug solution impregnation was completed. The free drugs together with drug-laden mesoporous powders were

Fig. 5. Release percentage curves of INH (a) and RFP (b) in SBF at 37°C from CaP scaffolds and MPHS scaffolds.

Fig. 6. Drug distributions of INH (1) and RFP (2) with respect to time in periphery tissues (a1, a2) and in blood (b1 and b2).
directly mixed with PHBHHx polymers to form proper pastes for 3D printing, thus the ultimate content of INH/RFP could be expediently adjusted. According to calculations and the operations in this study, a 0.8 mm × 0.6 mm cylindrical scaffold (weight average ~ 0.06 g) contained about 21% INH and 13.5% RFP of the total weight, as estimated be around 12.6 mg of INH and 8.1 mg of RFP in one implant. Owing to the relative enclosure space in the osteoarticular TB foci, we could assume drug retention over quite a long time and this INH/RFP drug reservoir would then hold up drug concentrations above the MICs. To compare with the conventional large oral dosage, the locally used drugs are much more efficient and targeted though the actual quantities are far lower. In addition, the CaP scaffolds could absorb very limited drugs through dropping drug solutions on them. For example, about 0.01 ml of

Fig. 7. Values of serum enzymes ALT, AST, BUN and Cr with respect to time of in vivo drug release from CaP and MPHS scaffolds, blood samples of rabbits before surgery were used as control (■ dotted line).

Fig. 8. Transverse micro-CT images of femoral defects at 1 day and 12 weeks post-implantation of control CaP scaffolds or MPHS scaffolds (scale bar: 2 mm).
Fig. 9. Histological evaluation of newly formed bone, the photomicrograph of new bone formation in repaired calvarial bone defects from undecalcified samples. The new bone appears red, and residual scaffold material appears black.

Fig. 10. Schematic illustrations of the MPHS system. Chemically modified mesoporous bioactive glasses or mesoporous silica nanoparticles were prepared firstly and then were loaded with anti-TB drugs INH/RFP, respectively. PHBHHx polymer was used as adhesives to bind all bioceramic and drug powders, and then the mixed pastes were fabricated into scaffolds via 3D printing technique.
INH or RFP solution could saturate a porous CaP scaffold with the same size of the experimented MPHS sample, which indicated that the maximum loading amounts of each drug was at most 1 mg in this study. More generally, for reported composite scaffolds that involve mesoporous phase adsorbed or coated on the surface, limited amounts of drugs were encapsulated and then the drugs will likely diffuse out from the bare surface quite soon. However, if the mesophas was directly mixed with polymers through common strategies like foaming or leaching, the fabrication process could usually involve large volumes of fluids or even high temperature treatment, as not beneficial for drug loading.

The drug release rate is one determining factor that influences the length of drug action time. Most of pure mesoporous silica-based scaffold without any modification or covering could only reach a sustained release in a week with usually a burst release (ca. 60%) at early stage, which is certainly not long enough for anti-TB therapy [43,44]. In this study, the burst release amounts accounted for only about 25% of all, and evidently the major released molecules at very beginning stemmed from the unloaded drug powders which were directly added into pastes. Thus it showed a remarkable reduction of burst release from MPHS scaffold. Chemical functionalization was the first solution we have taken to prolong release duration, which has been well documented to control the delivery rate. For instance, MCM-41 silica matrices with aminopropyl moieties lead to a decrease in the delivery rate of ibuprofen, and for ibuprofen loads in the same order of magnitude, the release time is almost five times higher for the functionalized sample [26,27]. Either steady chemical bonding (INH–COOH) or the hydrophobic interactions (RFP–CH3) would effectively retain the drug release. Secondly, tangled PHBHx polymers as adhesive commonly had wrapped the bioceramic powders and might block the mesopores. The polymer hindrance played a rather important role in preventing both free drug molecules that mixed with PHBHx directly and drugs occluded in the pore channels from fast release outward, which resulted in almost no burst release in the first three days similarly for either the composite pastes or the printed scaffolds (Fig. S5, Supplementary materials). Furthermore, in the later stage, apatite precipitations would also take part in retarding the drug release process (not discussed in detail) [21].

It is well-known that MICs of INH and RFP are 10 and 5 mg/ml, respectively, which were effective enough because they are considered to be toxic for the majority of the resident microorganisms. Correspondingly, keeping INH concentration in the range of 0.025–0.05 μg/ml and RFP in between 0.005 and 0.5 μg/ml could inhibit the growth of tuberculosis germs. In the in vitro simulated release test, we have adopted about 1 ml SBF release medium for one MPHS scaffold, and refreshed SBF at each time point. According to the results, the lowest drug concentrations were still above 12 μg/ml of INH and 8 μg/ml of RFP in the early period of frequently sampling. However, the control CaP scaffold did have more than 70% of INH and 60% of RFP washed out only in the first hour. In the later stage, the concentrations dramatically declined and there were thus hardly possibilities of long-term enough drugs remaining in the local site. The investigations of in vivo drug distributions had confirmed that, even after 12 weeks, INH and RFP concentrations were high enough and fell into the effective inhibitory ranges.

At a certain time point in vivo, it is not difficult to figure that the majority of released drugs were reserved in surrounded tissues rather than in blood. As a result, the low drug concentrations in blood did not bring about significant fluctuations of the relevant serum enzymes, such as ALT, AST, BUN and Cr (Fig. 7), which indicated no noticeable damage to the hepatic or renal functions of the rabbits. In another words, the drug delivery of MPHS scaffolds was nearly absolutely localized.

3D printing method herein had remarkably enhanced the compressive strength of the implant, so that the scaffold would not fall apart and the drug would not leak out. Moreover, the controllable interconnected macropores of MPHS scaffold were propitious to cell growth in and following new tissue generation. 3D printing is of great potential for scaffold fabrication with different materials and for different purposes.

5. Conclusions

An implantable antitubercular composite scaffold (MPHS), which also acted as an ATD delivery system was fabricated by 3D printing. The modified silica-based materials in MPHS scaffolds provided well-defined porous structures and microenvironment for loading high dosages of INH and RFP drugs. The MPHS composite scaffolds also showed an extraordinarily sustained co-release pattern of INH and RFP for over 84 days, with comparison that the complete in vitro release of all drugs lasted only for five days from commercial CaP scaffold. The drug concentrations of both INH and RFP in vivo can be maintained above the effective bacillus-killing values for an extra-long duration over 84 days as well without significant long-term lesions to liver and kidney. Furthermore, the MPHS scaffold showed good osteogenesis capability. Therefore, this approach suggests future innovative uses for the material which can release drugs over a really long period, in the treatment of TB and also in bone replacement surgeries to avoid internal infection and post-surgery complications.

Acknowledgments

Many thanks to the Engineering Research Center in Biomaterials (Sichuan University) for the supply of CaP scaffolds. We also greatly acknowledge the financial supports from the National Nature Science Foundation of China (51302170), Shanghai Nature Science Foundation (13ZR1458600), Innovation Program of Shanghai Municipal Education Commission (14YZ085), National High Technology Research and Development Program of the Science and Technology (863 Program, 2013AA032203) and Shanghai Nanometer Special Project (11nm0504200).

Min Zhu and Kun Li contributed equally to this work.

Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 2, 4–7, 9 and 10, are difficult to interpret in black and white. The full color images can be found in the on-line version, particularly at http://dx.doi.org/10.1016/j.actbio.2015.01.034.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.01.034.

References


