Colloids and Surfaces B: Biointerfaces 173 (2019) 18–26

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Surface functionalization of halloysite nanotubes with supermagnetic iron oxide, chitosan and 2-D calcium-phosphate nanoflakes for synergistic osteoconduction enhancement of human adipose tissue-derived mesenchymal stem cells

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ARTICLE INFO

Keywords:
Halloysite nanotubes
Multifunctional nanoscaffolds
Osteoconduction
hADMSCs
Bone tissue engineering
Calcium phosphate nanoflakes

ABSTRACT

Halloysite nanotubes (HNTs) are known to be the highly emerging materials in nano-medicinal applications. However, comprehensive exploitation of HNTs for the regenerative medicinal applications is still necessary to be done. Therefore, towards enhancing the osteogenic potential of human adipose tissue-derived mesenchymal stem cells (hADMSCs), this study synthesized a novel and multifunctional nanoscaffold of chitosan (CTs) functionalized supermagnetic halloysite nanotubes (M-HNTs) decorated with the calcium phosphate 2-D nanoflakes (*CaP*) (termed as; M-HNTs-CTs-*CaP*). Stepwise modified nanoscaffolds were characterized by FE-SEM, FE-SEM-EDS, FE-HR-TEM, XPS, FT-IR and VSM analyses. The hADMSCs osteogenic potential was confirmed by calcification (Alizarin Red S staining), phosphate quantification and immunocytochemistry. Nanoscaffolds; *CaP*, M-HNTs-*CaP* and M-HNTs-CTs-*CaP* were significantly enhanced and up-regulated osteogenic potential compared to the HNTs, M-HNTs, M-HNTs-CTs. Among the nanoscaffolds studied, M-HNTs-CTs-*CaP* exhibited highest osteogenesis, due to the enhanced *CaP* distribution on M-HNTs-CTs surface, and synergistic osteoconduction contributed from Fe_3O_4, chitosan and *CaP*. Moreover, immunocytochemistry analysis and morphologically observation showed well differentiated osteoblast on the M-HNTs-CTs-*CaP* surface. Therefore, M-HNTs-CTs-*CaP* found to have a strong osteogenic potential of hADMSCs, and might be serve as highly applicable, next generation nanoscaffold for bone tissue engineering application.

1. Introduction

Bone tissue disorders and damage caused by either physical trauma or degenerative diseases significantly influences the individual's quality of life [1]. Effective approaches to engineer new functional bone tissue is the most challenging fields [2], because there have several limitations to repair the bone tissue by re-establishing their function and structure [1,3,4]. Stem cells are capable of self-renewal and can differentiate into various lineages, such as adipocyte, osteoblasts, chondrocytes and neuronal cells [3]. Especially, human adipose tissue-derived mesenchymal stem cells (hADMSCs) can be promising application in the field of regenerative medicine due to its available abundant supply of mesenchymal stem cells from adipose tissue [5,6]. Recently, it has been reported that several nanomaterial's are known to be provide a physical microenvironments to enhance osteogenic potential of hMSCs [4,5]. To achieve the successful bone tissue engineering, it is further necessary to develop regenerative therapies by using a stem cells and nanomaterial's with unique properties and functions.

Recently, the clay material halloysite nanotubes (HNTs) were well-known for their extremely distinctive surface modification properties [7–16], and might be highlighted for remarkable tissue engineering potential in the near future. Beyond the extensive availability, HNTs exhibit excellent mechanical properties and biocompatibility [13,17]. Looking at the extensive studies has been done on the expensive carbon nanotubes and graphenes, HNTs are still undermined for its potential in tissue engineering application [4,14]. Therefore, there is a very high scope, to tune pristine HNTs surface with osteoconductive biopolymers and nanomaterials, to enhance efficacy of osteogenic differentiation.

Chitosan is a naturally biodegradable biopolymer, and known to be effectively used in antibacterial, anti-fungal, anti-tumour and

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https://doi.org/10.1016/j.colsurfb.2018.09.045
Received 6 February 2018; Received in revised form 19 August 2018; Accepted 19 September 2018
Available online 20 September 2018
0927-7765/

immunostimulating formulations [16]. Chitosan scaffolds were known to be used for the osteoconduction of hMSCs [19]. However, pure chitosan scaffolds possesses mechanical fragility and low biological resistance [16]. Therefore, coating chitosan over highly efficient backbone material such as HNTs provides a very suitable option for osteoconduction [15]. The calcium phosphate (*CaP*) is constitutes the primary inorganic component of the bone matrix [19]. The exceptional osteoconductive property demonstrated by *CaP* nanomaterials, marks them as a promising materials for bone regeneration [19]. However, the osteoconductive potential of coated *CaP* nanomaterials has been affected by many factors, including the size of *CaP* nanoparticle, *CaP* crystallinity, surface energy and surface pattern [20]. Therefore, synthesis of highly crystalline, 2-D surfaced *CaP* nanostructures makes them a superb candidate for osteogenesis. Additionally, coating *CaP* more uniformly on HNTs makes them multidimensional. Moreover, along with the proposed enhancement in osteogenesis by modified HNTs, it is also equally important, to locate, track and externally operate the treated nanoscaffolds and the target cells [21]. This property was enabled by the supermagnetic Fe_3O_4 [21]. In additional, Fe_3O_4 nanoparticles was accelerated osteogenic differentiation of mesenchymal stem cells via modulation of long noncoding RNA INZEB2 [22]. However, there has no report to imply such a multifunctional modification of HNTs with Fe_3O_4, chitosan and *CaP* nanoparticle loading.

Therefore, in this study, HNTs were super-magnetized (M-HNTs) and chitosan (CTs) functionalized (M-HNTs-CTs) for loading of the *CaP* nanoflakes (termed as; M-HNTs-CTs-*CaP*). The stepwise modified nanoscaffolds were structurally characterized, and examined for enhancement of the hADMSCs osteoconduction by ARS staining, calcification, phosphate quantification and osteoblast-specific markers ALP and OCN expressions. Therefore, decorating HNTs tunable surface; with supermagnetic Fe_3O_4, the chitosan an osteoconductive biopolymer, and a bone-matrix mimetic *CaP* nanostructures; can hypotheses as a highly novel, multifunctional and potential nanotubular-scaffolds, for synergistically accelerating the osteoconductive potential of hADMSCs.

2. Materials and methods

2.1. Synthesis of M-HNTs-CTs

The synthesis of M-HNTs-CTs was done in the two steps. In first step, pristine HNTs were supermagnetically tuned with the Fe_3O_4, with a low cost and facile reduction precipitation method [23]. In typical synthesis process, pristine HNTs 0.1–1 g was taken in 100 ml deionized water. This mixture was ultrasonicated for 1 h. Then, 50 ml of $FeCl_3.6H_2O$ (3%) was added and mixed thoroughly for the 15 min. The further addition of 30 ml of Na_2SO_3 (0.7%) gave the mixture typical wine red color. As soon as the wine red color turns to back to yellow, the precipitant 1 M NaOH solution 30 ml were added to the mixture. The developed black colored precipitate of M-HNTs was magnetically separated. The separated M-HNTs were washed several times with the water and freeze dried. The freeze dried powder was grounded in mortar and pestle for the further use. In the second step of M-HNTs-CTs synthesis, M-HNTs were hybridized with the chitosan. 1 gm of M-HNTs was taken in 100 ml of deionized water. This mixture was kept refluxing for 15 min at 50 °C. The 100 ml of 1% chitosan (prepared in the 1% acetic acid solution) was added dropwise in the mixture. The 2 ml of GTA were added to the mixture after 15 min of the mixing. This M-HNTs-chitosan mixture was continuously refluxed for 24 h at 50 °C. The obtained chitosan modified M-HNTs (M-HNTs-CTs) were magnetically separated and washed thoroughly with deionized water. The obtained M-HNTs-CTs were freeze dried and powdered in mortar and pestle for the further use.

2.2. Synthesis of M-HNTs-CTs-CaP

CaP nanoparticles, M-HNTs-*CaP* and M-HNTs-CTs-*CaP* were synthesized by a double reverse microemulsion method [3]. In a typical synthesis process, 7 ml of cyclohexane mixed with 3 ml of Igepal CO-520, in 2 glass vials (50 ml), separately. This two tubes were labelled as microemulsion A and B. Then, 50–500 µl of $CaCl_2$ (100 mM) and Na_2HPO_4 (60 mM) degassed solutions were added in the microemulsion A and microemulsion B, respectively. Then, both microemulsion A and microemulsion B were refluxed continuously at 50 °C for 1 h. Next, microemulsion B were added dropwise into the microemulsion A and mixed for 5 min. This mixture formed microemulsion C and further added with the 100 µl of 1% Pluronic F-127. This mixture was refluxed continuously for 20 min at 50 °C. Finally, after the addition of 35 ml of ethanol (pH 7 by 1 M KOH), the white puffy precipitate *CaP* nanoparticles coated with Pluronic F-127 were observed. Similar process was used for tuning of M-HNTs and M-HNTs-CTs surface with *CaP* nanoparticles, except preparation of microemulsion B. In this type of microemulsion B preperation, the 50 mg of each M-HNTs and M-HNTs-CTs were added among with Na_2HPO_4 (60 mM) degassed solutions, respectively. The rest of the process was continued as abovementioned. The synthesized, *CaP* nanoparticles, and *CaP* loaded on M-HNTs (M-HNTs-*CaP*) and M-HNTs-CTs (M-HNTs-CTs -*CaP*) were further investigated for the osteoconductive potential. The materials used and characterizations studied were explained in supplementary materials methods S1 and methods S2.

2.3. hADMSCs culture and induction for osteogenic differentiation

The hADMSCs (CEFO, Korea) were cultured in ADMSC growth medium (CEFO) for 6 days. Cells were detached by using accutase (Millipore, USA), then experiments were performed. For osteogenic differentiation, biological passage number 4 was used. Osteogenic differentiation was induced by culturing cells for 14 and 21 day in osteogenic differentiation medium (OM); contained with DMEM-low glucose (Gibco, USA), 1% penicillin streptomycin (Gibco), 10% fetal bovine serum (Alphabioregen, USA), 0.1 µM dexamethasone (Sigma, USA), 10 mM β-glycerophosphate (Sigma), 50 µM L-ascorbic acid-2-phosphate (Sigma), and respective nanoscaffolds (20 µg); HNTs, M-HNTs, M-HNTs-CTs, *CaP* nanoflakes, M-HNTs-*CaP* and M-HNTs-CTs-*CaP*. The typical experiments for cell viability, SEM observation of developed osteoblasts and statistical analysis performed were explained in supplementary materials methods S3, S4 and S5.

2.4. Alizarin red S (ARS) staining

Osteogenic differentiation was evaluated by Alizarin Red S staining. Cells were washed twice with DPBS and fixed with 10% formalin (Sigma) for 15 min. After fixation, cells were washed with DPBS and Alizarin Red S (Sigma) staining solution was treated for 45 min at room temperature in dark. Then, cells were washed 4 times with distilled water and captured using microscope (Nikon, Japan). To quantify the remained Alizarin Red S stain on cells, dye was eluted by 10% cetylpiridium chloride (Sigma) and measured the absorbance at 570 nm.

2.5. Phosphate quantification

After 14 days of osteogenic differentiation with HNTs, M-HNTs, M-HNTs-CTs, *CaP* nanoflakes, M-HNTs-*CaP* and M-HNTs-CTs-*CaP*, the cells were lysed with RIPA buffer (Biosesang, Korea) then centrifuge at 16,200 rpm for 15 min. The supernatant collected and tested for phosphate content using phosphate colorimetric assay kit (Biovision, USA).

2.6. Immunocytochemistry

Cells on pre-coated 10 nm coverslips in 35 mm dish with poly-L-lysin (Sigma) were induced for osteogenic differentiation for 14 days with the *CaP* nanoparticles, M-HNTs-*CaP* and M-HNTs-CTs-*CaP*. Cells were fixed with 4% formaldehyde (Sigma), permeabilized with 0.25% Triton