Novel Resorbable Drug-Carrying Nano-Scaffolds for Treating Bone Diseases at Targeted Sites

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ABSTRACT

This study explored the amino-silane chemistry method for immobilizing the peptide DIF-7c derived from bone morphogenetic proteins (BMPs) to nanophase hydroxyapatite (nano-HA) to promote drug loading efficiency and to achieve controlled release at local disease sites. For this purpose, nano-HA was synthesized by a wet chemistry precipitation method and the model peptide DIF-7c was chemically bonded to nano-HA through amino-silane chemistry. The nano-HA/peptide conjugate was then dispersed in poly(lactide-co-glycolide) (PLGA) solutions to create an implantable scaffold by a solvent-casting technique. The results demonstrated that drug loading efficiency was improved and long term drug release up to 52 days was achieved on these drug-carrying nanocomposite scaffolds.

Keywords: controlled drug delivery, nanocomposites, scaffolds, orthopedic applications, bone diseases.

1 INTRODUCTION

Pharmaceutical agents are often required to stimulate new bone formation for the treatment of bone injuries or diseases (such as bone fracture, osteoporosis and osteosarcoma). However, conventional systemic administrations (such as oral and intravenous administration) of these agents can not effectively reach targeted sites and, thus, they can cause non-specific bone formation in areas not affected by injury or disease. Moreover, even if intentionally delivered or implanted locally to the damaged bone tissue, these agents tend to rapidly diffuse into adjacent tissues due to weak physical bonding to their drug carriers, which limits their potential to promote prolonged bone formation in targeted areas of bone. Therefore, this study explored the chemical method for immobilizing peptides derived from BMPs (bone morphogenetic proteins) to nano-HA (nanophase hydroxyapatite) to promote drug loading efficiency and to achieve controlled release at local disease sites. In addition, the use of nano-HA can increase peptide or protein loading efficiency considering that nano-HA has much larger surface area and much more exposed reaction sites for chemical bonding.

2 MATERIALS AND METHODS

2.1 Synthesis of Nanocrystalline HA

Nanophase HA was synthesized using a wet chemistry precipitation method by mixing solutions of calcium nitrate (Sigma) and ammonium phosphate (Sigma) in an alkaline pH region. Specifically, a 1 M calcium nitrate solution and a 0.6 M ammonium phosphate solution were prepared by dissolving their respective solid state powders in deionized (DI) water separately. The produced ammonium phosphate solution was mixed with DI water which had been adjusted to pH 10 by ammonium hydride. The pre-made 1 M calcium nitrate solution was then added into the mixture of ammonium phosphate and ammonium hydride at a rate of 3.6 ml/min. Precipitation occurred as soon as the calcium nitrate was added.

Precipitation continued for 10 minutes at room temperature with constant stirring. The supernatant was collected, centrifuged to reduce 75% of the solution volume and placed into to a 125 ml Teflon liner (Parr Instrument). The Teflon liner was sealed tightly in a Parr acid digestion bomb 4748 (Parr Instrument) and treated hydrothermally at 200 °C for 20 hours to obtain nanocrystalline HA. The hydrothermal treatment has a great advantage to prepare a stoichiometric, ultrafine HA powder with a homogeneous shape and size distribution due to higher applied pressures than atmospheric. After the hydrothermal treatment, nano-HA particles were rinsed with DI water and dried in an oven at 80 °C for 12 hours.

2.2 Design and Synthesis of the Model Peptide DIF-7c

The BMPs have several hundred amino acids, approximately 2~3 nm, depending on the conformation, which are too large and complex to be chemically functionalized onto nanomaterials. These complex secondary structures of the proteins are prone to degradation and as a result, these proteins tend to lose their bioactivity quickly in aqueous physiological conditions. Moreover, short peptides can be attached to drug carriers more efficiently due to their small size. Therefore, it is proposed in this study to deliver short peptides that were

derived from bioactive regions of BMP-7 (osteogenic protein-1), instead of the whole BMP proteins, by chemically functionalizing them onto nano-HA.

Chen et al. investigated three short peptides derived from bioactive regions of BMP-7 [1]. These three peptides were composed of 10 amino acids and were designated as peptide a (SNVILKKYRN), b (KPCCAPTQLN) and c (AISVLYFDDS). The results showed that peptide b increased osteoblast proliferation while peptide a and c promoted osteoblast differentiation (e.g. mineralization) [1].

In this study, the peptide c (AISVLYFDDS) was chosen and further modified at its N-terminal with a cysteine-containing spacer to ease chemical conjugation onto the nano-HA particles using amino-silane chemistry followed by a maleimide cross-linker molecule. The peptide with a 12 amino-acid sequence of CKAISVLYFDDS was used as the model peptide and termed as DIF-7c.

The peptide DIF-7c was obtained as carboxyl terminal acids to more than 98.2% purity according to the HPLC profile provided by the manufacturer (GenScript Corporation, USA). The molecular weight of the peptide DIF-7c was 1360.56 g/mol.

2.3 Peptide Loading onto Nano-HA/PLGA Composites

As mentioned, the difficulties of drug delivery lie in the efficient loading and controlled release. In this study, a chemical bonding method was used for improving the efficacy of drug delivery.

2.3.1. Preparation of HA_Ps_PLGA Drug Carriers

For chemical bonding, nano-HA was functionalized through amino-silane chemistry under dry conditions to avoid surface contamination and, thus, ensure stability of the peptide [2,3]. First, nano-HA was silanized in 3-aminopropyltriethoxysilane (APTES; Sigma 440140) in anhydrous hexane (Sigma 296090). Second, for substituting a hetero-bifunctional cross-linker for the terminal amine, the silanized nano-HA was coupled with N-succinimidyl-3-maleimido propionate (SMP; also called 3-Maleimidopropionic acid N-hydroxysuccinimide ester, Sigma 358657) in anhydrous N,N-dimethylformamide (DMF; Sigma 494488). Third, the peptide DIF-7c was immobilized onto nano-HA in anhydrous DMF through a reaction between the outer maleimide group with the thiol group of cysteine present in the terminal of DIF-7c. The nano-HA and model peptide conjugates that were bonded using amino-silane chemistry were termed as HA_Ps.

PLGA pellets (50/50 wt.% poly(DL-lactide/glycolide, Polysciences, Inc., Warrington, PA) was dissolved in an organic solvent at 40 °C for 40 minutes. The obtained HA_Ps nanoparticles were then added into PLGA solution. The weight ratio of HA_Ps to PLGA was 30/70. The mixture was sonicated for 10 min at controlled powers to achieve a uniform dispersion of HA_Ps in PLGA. After sonication, the mixture was cast into a Teflon mold, evaporated in air at room temperature for 24 hours, and dried in a vacuum oven at room temperature for 48 hours. These HA_Ps_PLGA films were then cut into 1 cm × 1 cm squares for use in material characterizations and *in vitro* studies.

2.3.2 Preparation of Controls

PLGA with the peptide were used as polymer controls. For this purpose, PLGA was first dissolved in an organic solvent at 40 °C for 40 minutes; and the peptide was added into PLGA solution after PLGA was completely dissolved. The PLGA_peptide mixture was then cast into a Teflon mold, evaporated in air at room temperature for 24 hours, and dried in an air vacuum chamber at room temperature for 48 hours. These PLGA_peptide films were then cut into 1 cm × 1 cm squares and termed as PLGA_P for use in material characterizations and in vitro studies. HA_Ps was also used as ceramic controls.

2.4 Characterization of Drug Loading: CBQCA Assay

A novel 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA, Molecular Probes) fluorescence technique was used to characterize the loading of the peptide onto the nano-HA. This technique could provide ultrasensitive detection of primary amines. Inherently CBQCA is a non-fluorescence molecule, but it becomes highly fluorescent upon reaction with amine groups in the presence of cyanide molecules. CBQCA reacts specifically with primary amines to form conjugates that are highly fluorescent and the sensitivity of detection of CBQCA conjugates could reach the attomole range (10^{-18} moles).

CBQCA reagent solutions were prepared by dissolving the CBQCA (MW = 305.3 g/mol) in dimethylsulfoxide (DMSO, Sigma D2650) (10 mM). Potassium cyanide (KCN, MW = 65.1, Sigma 60178) was dissolved in DI water to give a 10 mM working solution. HA_Ps nanoparticles were exposed to CBQCA and potassium cyanide working solutions for 2 hours at room temperature, and were then visualized under a fluorescence microscope (LEICA DM5500B upright fluoresence microscope). Images were obtained using Image Pro software.

2.5 Surface Characterization

The HA_Ps_PLGA drug delivery systems and PLGA_P controls were characterized using a Field Emission Scanning Electron Microscope (FESEM, LEO 1530) at a 3 kV accelerating voltage. The specimens were sputter-coated with a thin layer of gold-palladium, using a Hummer I Sputter Coater (Technics) in a 100 mTorr vacuum argon environment for 3 min at 10 mA of current.

2.6 In Vitro Drug Release Profiles

In vitro peptide release kinetics were studied in PBS (pH=7.4). All samples of interest were incubated in PBS under standard cell culture conditions for 52 days. After 1, 3, 5, 7, 30, and 52 days, the supernatants were collected and analyzed. The peptide release from scaffolds into culture solution was determined using a micro-BCA assay (Pierce). Briefly, the peptide DIF-7c standards were prepared by a serial dilution and the working reagent was mixed according to the established protocol. Each standard and unknown sample were aliquoted in 150 µL into a microplate well and mixed thoroughly with the working reagent on a plate shaker for 30 seconds. The reactions were incubated at 37 °C for 2 hours. The microplates were cooled to room temperature and read the absorbance at 562 nm using a spectrophotometer. A standard curve was generated by plotting the average Blank-corrected 562 nm reading for each peptide standard versus its concentration in µg/mL. The peptide concentration in the supernatants was calculated according to the standard curve.

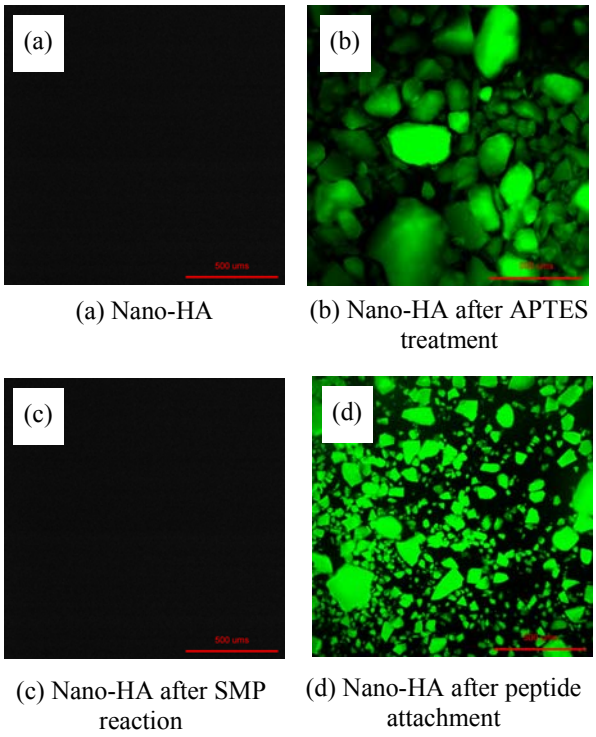
3 RESULTS AND DISCUSSION

3.1 Characterization of Drug Loading

The results of the CBQCA assay demonstrated the success of loading the peptide onto nano-HA chemically, as shown in Figure 1. Nano-HA with chemically loaded peptide produced very good fluorescence (Figure 1d), which indicated the successful attachment of the peptide onto nano-HA. Moreover, in the absence of the CBQCA, APTES treated nano-HA did not fluorescence (image not shown). In contrast, in the presence of CBQCA, APTES treated nano-HA did fluorescence (Figure 1b). Nano-HA after SMP reaction did not fluorescence (Figure 1c), indicating that the amine groups were completely covered by the SMP. The nano-HA (without peptide) did not show fluorescence (Figure 1a), which provided evidence that the CBQCA did not react with HA and only reacted with the amino groups.

3.2 Surface Characterization

Scanning electron micrographs suggest that the distribution of nano-HA particles was uniform in the HA_Ps_PLGA drug delivery systems after controlled sonication, even when these HA nanoparticles were chemically functionalized with the peptide DIF-7c (Figure 2a). PLGA_P maintained a very smooth surface similar to the PLGA (Figure 2b).



(a) Nano-HA

(b) Nano-HA after APTES treatment

(c) Nano-HA after SMP reaction

(d) Nano-HA after peptide attachment

Figure 1: The CBQCA analysis of nano-HA loaded with the model peptide DIF-7c by the chemical bonding method. Fluorescence images are: (a) nano-HA, (b) nano-HA after APTES treatment, (c) nano-HA after SMP reaction, and (d) nano-HA with the chemically attached peptide. Original magnifications are 10x. Scale bars are 500 µm.

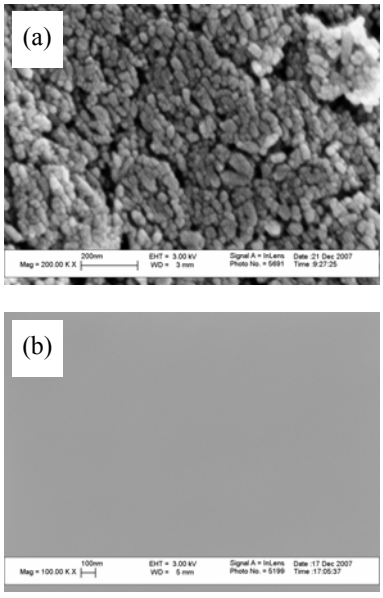


Figure 2: SEM images of (a) HA_Ps_PLGA and (b) PLGA_P. Magnification bars are 200 nm for (a) and 100 nm for (b).

3.3 In Vitro Drug Release Profiles

The release of peptide DIF-7c in vitro was studied for up to 52 days, as shown in Figure 3. In Figure 3(a), the single phase drug carriers, including PLGA_P and HA_Ps, all demonstrated one-phase release, although the major release happened at different time points for the HA carrier and the PLGA carrier. Specifically, the HA carrier (HA_Ps) started the peptide release at day 1, while the PLGA carrier did not release any peptide until day 7. At day 30, the HA carrier stopped the peptide release, while the PLGA carrier showed evidence of peptide release. At day 52, the PLGA carrier continuously showed peptide release, while HA carrier did not release any peptide. The HA carrier demonstrated continuous peptide release from day 1 to 7. The total amount of peptide released by the HA_Ps was greater than the PLGA_P during 52 days. It was speculated that the nano-HA had higher peptide loading efficiency compared to the PLGA. That is, chemical fictionalization permitted more peptide to be attached onto nano-HA compared to physical dispersion of peptide in PLGA. In Figure 3(b), the composite drug carrier (HA_Ps_PLGA) demonstrated two-phase release. At phase I (from day 1 to 7), the HA_Ps_PGA demonstrated continuous peptide release at a gradually decreased amount. At phase II, the HA_Ps_PLGA demonstrated increased peptide release from day 30 to 52.

A series of drug therapies are usually necessary after orthopedic surgeries to prevent either infection or inflammation or to induce appropriate natural tissue integration with the implants. Currently, drugs (such as antibiotics, anti-inflammatory drugs and bone growth factors) are typically administered either orally or intravenously. These routes of drug delivery often result in limited bioavailability, thus, requiring high dosages for drugs to be effective at the site of implantation. The ideal situation is delivering drugs directly at the interface of the implant and tissue. In other words, drug carrying scaffolds or implants (such as HA_Ps_PLGA) that are capable of controlling drug release may provide a promising approach for treating bone diseases at targeted sites.

4 CONCLUSIONS

Results of this study demonstrated three different drug release profiles achieved by using various drug carriers. The drug loading efficiency are related to the drug carriers and the loading methods. Single phase drug carriers (such as HA_Ps and PLGA_P) provided one-phase release profiles. The nanocomposite drug carrier (such as HA_Ps_PLGA) demonstrated a two-phase release profile. Importantly, a prolonged peptide release (up to 52 days) was achieved on the HA_Ps_PLGA drug delivery systems. The drug carriers and the drug loading methods are very important factors that should be considered when designing the next generation of drug carrying orthopedic prostheses for various clinical applications. The appropriate drug carriers and drug loading methods should be carefully chosen for specific applications. This study presented a useful guideline for designing more effective, controlled drug delivery systems according to requirements of specific applications.

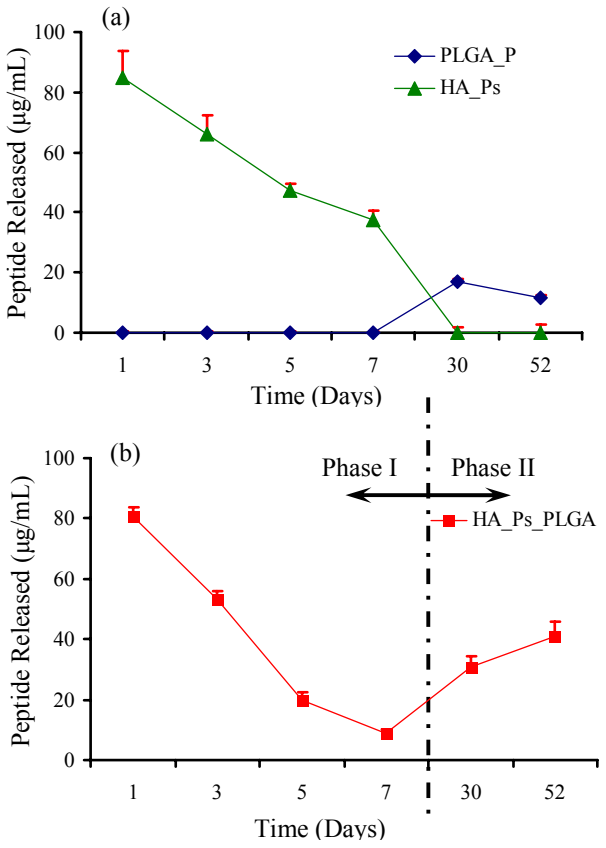


Figure 3: The amount of peptide DIF-7c released from the drug delivery systems of interest to this study. (a) Peptide released from the controls: PLGA_P and HA_Ps. (b) Peptide released from the nanocomposites: HA_Ps_PLGA. Values are mean ± SEM; N=3.

REFERENCES

[1] Chen Y, Webster TJ. Simple structure, easily functionalized and controlled release bioactive BMP-7 short peptides for orthopaedic applications. Journal of Oral Implantology, in press.

[2] Hong HG; Jiang M; Sligar SG; Bohn PW. Cysteine-specific surface tethering of genetically engineered cytochromes for fabrication of metalloprotein nanostructures. Langmuir. 10(1): 153-158; 1994.

[3] Balasundaram G, Sato M, Webster TJ. Using hydroxyapatite nanoparticles and decreased crystallinity to promote osteoblast adhesion similar to functionalizing with RGD. Biomaterials. 27(14): 2798-2805; 2006.