Biomaterials 149 (2017) 12–28

Contents lists available at ScienceDirect

Biomaterials

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

Nanolayered hybrid mediates synergistic co-delivery of ligand and ligation activator for inducing stem cell differentiation and tissue healing

Heemin Kang [a, 1], Minkyu Kim [b, c, 1], Qian Feng [a, 1], Sien Lin [d], Kongchang Wei [a], Rui Li [a], Chan Ju Choi [b], Tae-Hyun Kim [b], Gang Li [d], Jae-Min Oh [b, **], Liming Bian [a, e, f, g, h, *]

[a] Department of Biomedical Engineering, The Chinese University of Hong Kong, Hong Kong, China
[b] Department of Chemistry and Medical Chemistry, College of Science and Technology, Yonsei University, Wonju, Republic of Korea
[c] Future Industries Institute, Division of Information Technology, Engineering and Environment, University of South Australia, Mawson Lakes, SA, 5095, Australia
[d] Department of Orthopaedics and Traumatology, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China
[e] Shun Hing Institute of Advanced Engineering, The Chinese University of Hong Kong, Hong Kong, China
[f] Shenzhen Research Institute, The Chinese University of Hong Kong, Hong Kong, China
[g] China Orthopedic Regenerative Medicine Group (CORMed), Hangzhou, China
[h] Centre for Novel Biomaterials, The Chinese University of Hong Kong, Hong Kong, China

ARTICLE INFO

Article history:
Received 2 August 2017
Received in revised form
23 September 2017
Accepted 28 September 2017
Available online 29 September 2017

Keywords:
Synergistic ligation
Nanohybrid
Ligation activator
Stem cell differentiation
Tissue repair
Minimally invasive surgery

ABSTRACT

Cellular behaviors, such as differentiation, are regulated by complex ligation processes involving cell surface receptors, which can be activated by various divalent metal cations. The design of nanoparticle for co-delivery of ligand and ligation activator can offer a novel strategy to synergistically stimulate ligation processes *in vivo*. Here, we present a novel layered double hydroxide (LDH)-based nanohybrid (MgFe-Ado-LDH), composed of layered MgFe hydroxide nanocarriers sandwiching the adenosine cargo molecule, maintained through an electrostatic balance, to co-deliver the adenosine (Ado) ligand from the interlayer spacing and the Mg^{2+} ion (ligation activator) through the dissolution of the MgFe nanocarrier itself. Our findings demonstrate that the MgFe-Ado-LDH nanohybrid promoted osteogenic differentiation of stem cells through the synergistic activation of adenosine A2b receptor (A2bR) by the dual delivery of adenosine and Mg^{2+} ions, outperforming direct supplementation of adenosine alone. Furthermore, the injection of the MgFe-Ado-LDH nanohybrid and stem cells embedded within hydrogels promoted the healing of rat tibial bone defects through the rapid formation of fully integrated neo-bone tissue through the activation of A2bR. The newly formed bone tissue displayed the key features of native bone, including calcification, mature tissue morphology, and vascularization. This study demonstrates a novel and effective strategy of bifunctional nanocarrier-mediated delivery of ligand (cargo molecule) and activation of its ligation to receptor by the nanocarrier itself for synergistically inducing stem cell differentiation and tissue healing *in vivo*, thus offering novel design of biomaterials for regenerative medicine.

1. Introduction

Cellular behaviors and functions are regulated by the intricate ligation processes of surface transmembrane receptors [1,2]. A cascade of intracellular signaling [3,4] is mediated by surface receptors upon binding to a variety of extracellular ligands, such as hormones [2], chemokines [5], growth factors [6], and cell adhesion molecules [7]. The binding of ligand that activates the receptor to regulate cellular responses [8] is a complex process that is modulated by other factors.

The ligation to cell surface receptors is known to be regulated by various metal ions. It has been suggested that cell surface receptors possess binding sites for metal ions and the binding of those metal

* Corresponding author.Department of Biomedical Engineering, The Chinese University of Hong Kong, N.T, Hong Kong, China.
** Corresponding author.
E-mail addresses: jaemin.oh@yonsei.ac.kr (J.-M. Oh), lbian@cuhk.edu.hk (L. Bian).
[1] Contributed equally to this work.

https://doi.org/10.1016/j.biomaterials.2017.09.035
0142-9612/

ions activate the ligation process [9]. The ligations to G protein-coupled surface receptors have been shown to be stimulated by the binding of a number of metal ions, such as Cu^{2+} [10], Zn^{2+} [10,11], and Ni^{2+} ions [12], along with Mg^{2+} ion exhibiting potent 4–1500 fold stimulations of ligation [13–15]. The ligands for G protein-coupled receptors include adenosine, a small molecule that occurs naturally in the body, and has been used clinically as a vasodilator and neuromodulator [16,17]. The ligation of adenosine to adenosine A2b receptor (A2bR) is known to recruit A2bR to the plasma membrane, stimulate its expression, and activate intracellular signaling [18,19]. Recently, adenosine has been shown to stimulate the expression of A2bR and osteogenic differentiation of stem cells through its ligation to A2bR [20–22]. A2bR has been shown to play a dominant role in the osteogenic differentiation of stem cells and bone repair, *in vitro* and *in vivo* [20–24]. Furthermore, it was reported that Mg^{2+} ion supplementation stimulates the binding of ligand to the adenosine receptor 10-fold compared with that in the non-supplemented control [15]. Such previous reports imply that the development of novel biomaterials that can simultaneously deliver ligand molecules and ligation stimulators, such as the adenosine ligand and Mg^{2+} ion, could offer a promising approach to the synergistic activation of ligand receptors and thus regulate cellular responses, such as stem cell differentiation, both *in vitro* and *in vivo*.

Nanoparticle-mediated co-delivery of various molecules has recently received substantial attention owing to their great capabilities in mediating the controlled release of multiple cargo molecules for a sustained period, which is critical for *in vivo* applications. In particular, nanocarriers have been designed to co-deliver small molecular drugs with DNA [25,26], proteins [27], or siRNAs, as reported by us and others [28–33], or DNA with siRNA [34] to simultaneously and/or synergistically elicit various biological responses. However, there has been no reported approach of utilizing nanocarrier-mediated co-delivery of a ligand molecule and a ligation stimulator, such as a metal ion, to synergistically induce biological responses, both *in vitro* and *in vivo*, possibly due to the difficulty of simply loading metal ions (ligation activators) into the nanocarriers for their sustained delivery. Layered double hydroxide (LDH) nanoparticles [35,36] consist of alternating positively charged metal hydroxide layers with interlayer anionic molecules, which are electrostatically stabilized and can be reversibly exchanged with other anions or polar moieties [37]. The distinctive exchange ability of anionic/polar molecules in biocompatible LDH nanoparticles has been utilized for various applications, such as catalysis [38–41] and delivery nanocarriers [42]. Furthermore, the metal hydroxide layers in LDH as inorganic nanocarriers [43] can include various ligation stimulators, such as Mn^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, and Mg^{2+} ions, in their framework, which can be dissolved and released [35,44], but such delivery of the constituent of the nanocarrier itself has not been utilized for biomedical applications. Hence, harnessing LDH nanoparticles to co-deliver both ligand cargo molecules and metal ions as ligation stimulator, offer a novel and versatile strategy for synergistically inducing ligation-mediated biological responses, such as stem cell differentiation, both *in vitro* and *in vivo*.

In this study, we developed a bifunctional MgFe-LDH-based nanohybrid harboring adenosine (MgFe-Ado-LDH nanohybrid) that can co-deliver adenosine ligand molecules and Mg^{2+} ions in a sustained fashion as shown graphically in Scheme 1. We hypothesized that adenosine and Mg^{2+} ions released from the MgFe-Ado-LDH nanohybrid may synergistically activate A2bR to promote the expression of A2bR and the osteogenic differentiation of the treated human mesenchymal stem cells (hMSCs). Our findings show that the MgFe-Ado-LDH nanohybrid embedded within injectable hydrogel induced the osteogenic differentiation of stem cells through the activation of A2bR, more efficiently than direct supplementation with adenosine alone. Furthermore, the MgFe-LDH nanoparticles were shown to synergistically activate A2bR by adenosine and the delivered Mg^{2+} ions, for inducing stem cell osteogenesis. We also show that the injection of the MgFe-Ado-LDH nanohybrid with stem cells expedited the healing of long bone defects through the formation of vascularized, bridged, and mature neo-bone tissue through the activation of A2bR.

2. Materials and methods

2.1. Preparation of MgFe-layered double hydroxide (MgFe-LDH) nanoprecursor

MgFe-LDH nanoprecursor as metal cation-based hydroxide layers was synthesized through coprecipitation method. Briefly, mixed metal nitrate solution (0.13 M $Mg(NO_3)_2 \cdot 6H_2O$ and 0.07 M $Fe(NO_3)_3 \cdot 9H_2O$, Sigma-Aldrich) in deionized (DI) water was titrated to pH 9.5 by using alkaline solution (0.5 M NaOH and 0.15 M $NaHCO_3$) and kept at 25 °C under vigorous stirring for 24 h. The reaction mixture was then filtered and washed with deionized (DI) water, yielding brown precipitates that were subsequently lyophilized.

2.2. Synthesis of MgFe-Ado-LDH nanohybrid

MgFe-Ado-LDH nanohybrid that includes both MgFe-LDH nanoprecursor as metal cation nanolayers and adenosine (Ado) ligand as cargo molecule was prepared through reconstruction method based on the memory effect of stacked hydroxide nanolayers. The dried MgFe-LDH nanoprecursor was calcined in alumina crucible at 400 °C for 8 h to obtain metal oxides. To optimize adenosine incorporation into the interlayer of MgFe-LDH, 70 mM adenosine solution in DI water was titrated to various pH (8, 11, or 13) by using 4 M NaOH. 0.025% (w/v) calcined MgFe-LDH (approximately 1.4 mM) was then dispersed into the 70 mM adenosine solution at 65 °C under an inert atmosphere and kept under stirring for 3 d. The resulting solution was centrifuged, washed with DI water, and then lyophilized.

2.3. Powder X-ray diffraction (PXRD)

To examine the intercalation of adenosine into MgFe-LDH nanoprecursor for MgFe-LDH nanohybrid by change in crystalline peaks, MgFe-Ado-LDH nanohybrids synthesized at various pH (8, 11, and 13) along with MgFe-LDH nanoprecursor were characterized by PXRD. Samples were prepared by stacking lyophilized powders on a poly(methyl methacrylate) holder. PXRD diffraction spectra were obtained by using D2 Phaser with LYNXEYETM detector (Bruker) with Ni-filtered Cu-Kα radiation ($\lambda = 1.5418$ Å) with a scanning range (2θ) from 5° to 30° and scanning step of 0.02°/s.

2.4. Compositional analysis

Chemical compositions of MgFe-Ado-LDH nanohybrids (synthesized at pH 8, 11, and 13) along with MgFe-LDH nanoprecursor were determined by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) for Mg^{2+} and Fe^{3+} ions, CNHS Elemental Analyzer (EA) for carbon and nitrogen elements, and High Performance Liquid Chromatography (HPLC) for adenosine.

2.5. Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS)

Field emission-scanning electron microscopy (FE-SEM) was