Biomaterials 52 (2015) 1–13

Contents lists available at ScienceDirect

Biomaterials

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

Gene therapy for nucleus pulposus regeneration by heme oxygenase-1 plasmid DNA carried by mixed polyplex micelles with thermo-responsive heterogeneous coronas

Ganjun Feng [a], Hongying Chen [b], Junjie Li [c], Qiang Huang [b], Melanie J. Gupte [d], Hao Liu [a], Yueming Song [a], Zhishen Ge [c, *]

[a] Department of Orthopedic Surgery, West China Hospital, Sichuan University, Chengdu 610041, China
[b] Technology Center for Public Research, West China Hospital, Sichuan University, Chengdu 610041, China
[c] CAS Key Laboratory of Soft Matter Chemistry, Department of Polymer Science and Engineering, University of Science and Technology of China, Hefei 230026, China
[d] Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109, USA

ARTICLE INFO

Article history:
Received 6 December 2014
Received in revised form
28 January 2015
Accepted 1 February 2015
Available online

Keywords:
Nucleus pulposus regeneration
Gene therapy
Nonviral gene carrier
Polyplex micelles
Thermo-responsive

ABSTRACT

Safe and high-efficiency gene therapy for nucleus pulposus (NP) regeneration was urgently desired to treat disc degeneration-associated diseases. In this work, an efficient nonviral cationic block copolymer gene delivery system was used to deliver therapeutic plasmid DNA (pDNA), which was prepared via complexation between the mixed cationic block copolymers, poly(ethylene glycol)-*block*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoehtyl]aspartamide} [PEG-*b*-PAsp(DET)] and poly(*N*-isopropylacrylamide)-*block*-PAsp(DET) [PNIPAM-*b*-PAsp(DET)], and pDNA at 25 °C. The mixed polyplex micelles (MPMs) containing heterogeneous coronas with hydrophobic and hydrophilic microdomains coexisting could be obtained upon heating from 25 to 37 °C, which showed high tolerability against nuclease and strong resistance towards protein adsorption. The gene transfection efficiency of MPMs in NP cells was significantly higher than that of regular polyplex micelles prepared from sole block copolymer of PEG-*b*-PAsp(DET) (SPMs) in *in vitro* and *in vivo* evaluation due to the synergistic effect of improved colloidal stability and low cytotoxicity. High expression of heme oxygenase-1 (HO-1) in NP cells transfected by MPMs loading HO-1 pDNA significantly decreased the expression activity of matrix metalloproteinases 3 (MMP-3) and cyclo-oxygenase-2 (COX-2) induced by interleukin-1β (IL-1β), and simultaneously increased the NP phenotype-associated genes such as aggrecan, type II collagen, and SOX-9. Moreover, the therapeutic effects of MPMs loading pDNA were tested to treat disc degeneration induced by stab injury. The results demonstrated that administration of HO-1 pDNA carried by MPMs in rat tail discs apparently reduced inflammatory responses induced by need stab and increased glycosaminoglycan (GAG) content, finally achieving better therapeutic efficacy as compared with SPMs. Consequently, MPMs loading HO-1 pDNA were demonstrated to be potential as a safe and high-efficiency nonviral gene delivery system for retarding or regenerating the degenerative discs.

1. Introduction

Disc degeneration is associated with low back pain and many related diseases [1]. It is believed that the degenerative process begins from the inner nucleus pulposus (NP) with a decrease of cell number and loss of proteoglycans, followed by the outer annulus fibrosus (AF) with radial fissures [2]. In the degenerative NP, phenotypes of NP cells change so that they could not maintain anabolism and catabolism of extracellular matrix (ECM) in balance. Current treatment approaches such as symptomatic treatment or surgical treatment can alleviate the symptoms in a short term but fail to address the underlying problems. One promising treatment method is genetic modification of disc cells through controlled and specific delivery of genetic materials (DNA or RNA) [3]. Several investigators have reported successful gene transfer to the intervertebral disc tissues based on viral gene delivery systems including

* Corresponding author. Tel.: +86 551 3603670.
E-mail address: gezs@ustc.edu.cn (Z. Ge).

http://dx.doi.org/10.1016/j.biomaterials.2015.02.024
0142-9612/

adenovirus, adeno-associated virus, and lentivirus [4–8]. However, the devastating side effects concerning viral gene delivery systems have also been realized. These concerns were further magnified particularly with respect to the proximity of the disc to vital neurovascular structures. In view of the serious adverse factors associated with viral gene therapy, non-viral vectors are preferred to be utilized to treat chronic and non-lethal diseases such as intervertebral disc degeneration.

Notably, cationic polymers-based nonviral gene delivery systems have been proposed as safer alternatives to viral vectors due to low immunogenicity, low cost for high-scale manufacture, and tunable surface and structural properties [9–13]. Poly(ethylene glycol) (PEG)-*block*-polycation block copolymers can complex with DNA via electrostatic interactions forming polyplex micelles with well-defined core-shell architecture [14,15]. The polyplex micelles preserve high colloidal stability in the physiological milieu due to PEG stealth function. Therefore, the polyplex micelles were recognized as highly promising candidates for *in vivo* application of gene therapy. Notably, degradable cationic polymer, poly{*N*-[*N*-(2-aminoethyl)-2-aminoehtyl] aspartamide} [PAsp(DET)], has been confirmed by Kataoka et al. to show high *in vitro* gene transfection efficiency with minimal toxicity [16–18]. Block copolymer, PEG-*b*-PAsp(DET), have been explored for *in vivo* applications as nonviral gene delivery vectors [19–21]. However, dense PEG shell reduces affinity of PEG-*b*-PAsp(DET)/DNA polyplex micelles to cell membrane so that limited transfection efficiency attenuates the ultimate therapeutic potency. On the other hand, PEG-*b*-PAsp(DET)/DNA polyplex micelles suffered from protein adsorption and premature dissociation in *in vivo* medium due to degradation of ubiquitous nuclease and exchange reaction of anionic macromolecules [15,22,23]. To further develop the carriers with both colloidal and complex stability simultaneously maintaining high-efficiency gene transfection activity for *in vivo* gene delivery, we have developed thermo-responsive mixed polyplex micelles via complexation between pDNA and mixed block copolymers [24]. The polyplex micelles with heterogeneous surfaces through simple heating from 20 °C to 37 °C have been confirmed to exhibit prominent performance as nonviral systemic gene delivery vectors due to high stability in biological environment and high-efficiency gene transfection activity in cancer cells.

On the other hand, cumulative evidences indicated that systemic proinflammatory cytokine interleukin-1β (IL-1β) plays a central role in both physiological and pathological conditions of disc degeneration [25,26]. IL-1β regulates the production of matrix metalloproteinases (MMPs), an important family of degradative enzymes implicated in intervertebral disc degeneration. MMP-3 as one of the most important MMPs involved in disc degeneration, is thought to play a critical role in cartilage destruction regulated by IL-1β. Therefore, agents that can down-regulate or inhibit the expression of MMP-3 are promising candidates for intervertebral disc regeneration therapy. Heme oxygenase-1 (HO-1) is heme degrading enzymes expressed in mammalian species. The main biologic function of HO-1 is to convert toxic hemes into antioxidants, which was considered to be essential to cope with various cellular stresses regulating cellular iron metabolism. In clinical conditions, the level of HO-1 expression has been thought to be associated with resistance to tissue injury. It has been reported that induction of HO-1 results in protective effects against inflammatory and degradative responses induced by IL-1β [27–29]. However, whether HO-1 expressed by pDNA could regenerate intervertebral disc has not been tested.

In this work, we explored gene therapy of disc degeneration using mixed polyplex micelles as the nonviral gene delivery vectors formed from HO-1 plasmid DNA (pDNA) and the mixture of PEG-*b*-PAsp(DET) and poly(*N*-isopropylacrylamide)-*block*-PAsp(DET) [PNIPAM-*b*-PAsp(DET)] (Fig. 1). With the temperature increasing from room temperature (25 °C) to body temperature (37 °C), the more compact polyplex micelles with heterogeneous hydrophobic/hydrophilic coronas could be formed. In general, two fundamental scientific questions were explored: 1) whether the mixed polyplex micelle system would be a safe and efficient non-viral vector for NP cells, which is the optimal therapeutic targets for treatment of disc degeneration; 2) whether overexpressed HO-1 enzymes by HO-1 pDNA could decrease the catabolic effects of IL-1β on extracellular matrix thus regenerating intervertebral disc. Experiments on the basis of *in vitro* NP cells and *in vivo* rat-tail disc degeneration models were performed.

2. Materials and methods

2.1. Materials

α-Methoxy-ω-amino-poly(ethylene glycol) (M_w 12,000) (PEG-NH_2) was obtained from Jenkem Technology Co., LTD (Beijing, China). *N*-isopropylacrylamide (NIPAM) was purified by recrystallization from a mixture of benzene and hexane (3/7, v/v). Azobis(isobutyronitrile) (AIBN) was recrystallized twice from methanol. *N*-Hydroxysuccinimide (NHS, 97%, Aldrich) was recrystallized from toluene prior to use. 4-Dimethylaminopyridine (DMAP, 99%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ≥ 99.5%), and *N*-boc-propanediamine (98%) were purchased from Energy Chemical (Shanghai, China) and used as received. Polyethylenimine (M_w, ~25 kDa, PEI25K, branched) was purchased from Sigma–Aldrich and used as received. β-Benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) was obtained from Chengdu Enlai Biological Technology Co., Ltd. (Chengdu, China) and recrystallized three times from THF/hexane. DNase I, cell culture lysis buffer, luciferase Assay System Kit, 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT), and enhanced BCA Protein Assay Kit were purchased from Beyotime Institute of Biotechnology (Nantong, China). The plasmid DNA (pDNA) encoding green fluorescent protein (GFP), luciferase (Luc), or heme oxygenase 1 (HO-1) with a CAG promoter was amplified in competent DH5α *Escherichia coli* and purified with a QIAGEN HiSpeed Plasmid MaxiKit (Germantown, MD). NP cells were obtained from 8-week-old male New Zeland white rabbits (Animal Center, Sichuan University). High-glucose DMEM medium was purchased from GIBCO, Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO) and β-actin were obtained from Sigma–Aldrich, USA. HO-1, IL-1β, and fluorescein isothiocyanate (FITC)-labeled mouse anti-goat secondary bodies were purchased from Santa Cruz Biotechnology, Santa Cruz, USA. Primers for HO-1, aggrecan, SOX-9, type II collagen, MMP-3, and cyclo-oxygenase-2 (COX-2) were purchased from Applied Biosystems, USA. Vectastain ABC was obtained from Vector Laboratories, USA. Sprague–Dawley rats (three months old) were obtained from Animal Center in Sichuan University.

2.2. Polymer synthesis

Block copolymer, PEG-*b*-PAsp(DET), was synthesized according to the procedures in the literature reports [16]. Briefly, PEG-*b*-PAsp(DET) block copolymer was prepared via the polymerization of monomer BLA-NCA initiated by PEG-NH_2, followed by aminolysis reaction to introduce diethylenetriamine molecules into the side chain of PBLA. The prepared polymers were determined to have a narrow unimodal molecular weight distribution (M_w/M_n = 1.07) by gel permeation chromatography. The degree of polymerization of PAsp(DET) segment was confirmed to be 64 on the basis of ^{1}H NMR analysis.

PNIPAM-*b*-PAsp(DET) block copolymer was synthesized via reversible addition-fragment chain transfer (RAFT) polymerization and subsequent ring open polymerization (ROP) (Scheme S1). First, end group functionalized polymer, PNIPAM-*NHBoc* was prepared by RAFT polymerization of NIPAM followed by complete deprotection to activate amino groups (Fig. S1). Subsequently, PNIPAM-*b*-PBLA, was obtained by ROP of BLA-NCA using PNIPAM-NH_2 precursor as a macromolecular initiator. Gel permeation chromatography (GPC) analysis revealed a relatively narrow and unimodal elution profile, thereby indicating successful preparation of block copolymers without presence of inactivated homopolymer precursor) (Fig. S2). The final block copolymer, PNIPAM-*b*-PAsp(DET), was fabricated by aminolysis by diethylenetriamine (DET), which could be denoted as $PNIPAM_{80}$-*b*-$PAsp(DET)_{34}$ on the basis of ^{1}H NMR analysis (Fig. S1). The detailed synthesis procedures and characterization results (GPC and ^{1}H NMR) were described in the Supporting information.

2.3. Preparation of polyplex micelles

The block copolymers, $PNIPAM_{80}$-*b*-$PAsp(DET)_{34}$ and PEG_{272}-*b*-$PAsp(DET)_{64}$, and pDNA were separately dissolved in 10 mM HEPES buffer (pH 7.4) as stock solutions. Mixed solution of $PNIPAM_{80}$-*b*-$PAsp(DET)_{34}$ and the PEG_{272}-*b*-$PAsp(DET)_{64}$ with PNIPAM/PEG weight ratio of 1:1 was added into a 2-fold volume of pDNA solution for complexation at various N/P ratios (molar ratio of amino groups in block