Colloids and Surfaces B: Biointerfaces 161 (2018) 129–138

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Full length article

Injectable deferoxamine nanoparticles loaded chitosan-hyaluronic acid coacervate hydrogel for therapeutic angiogenesis

S. Vignesh[a,1], A. Sivashanmugam[a,1], Annapoorna Mohandas[a], R. Janarthanan[b], Subramania Iyer[b], Shantikumar V. Nair[a], R. Jayakumar[a,*]

[a] Centre for Nanosciences and Molecular Medicine, Amrita University, Kochi 682041, India
[b] Department of Plastic and Reconstructive Surgery, Amrita Institute of Medical Sciences and Research Centre, Amrita University, Kochi 682041, India

ARTICLE INFO

Article history:
Received 8 June 2017
Received in revised form
11 September 2017
Accepted 10 October 2017
Available online 12 October 2017

Keywords:
Injectable hydrogel
Chitosan
Hyaluronic acid
Coacervate hydrogel
Deferoxamine
Angiogenesis
Neo-vascularization

ABSTRACT

In this study, an injectable chitosan-hyaluronic acid (CS-HA) based hydrogel was designed incorporating pro-angiogenic molecule, deferoxamine loaded PLGA nanoparticles (DFO NPs), for enhancing angiogenesis. DFO-NPs were prepared by double emulsion solvent diffusion technique and characterized for their physicochemical properties. The DLS and SEM analysis showed an average particle size of 220 ± 71 nm with spherical morphology and the encapsulation efficiency was found to be 30 ± 5%. An ECM mimicking chitosan-hyaluronic acid (CS-HA) coacervate hydrogel was prepared. Both free DFO and DFO NPs were entrapped into the prepared CS-HA composite hydrogel. The hydrogels were characterized by SEM, FTIR and Rheology. Addition of DFO NPs did not affect the injectablility and flowability of developed hydrogels. *In vitro* DFO release from the prepared composite hydrogels showed controlled release over a period of 10 days. Both the hydrogel systems showed excellent cyto-compatability and good cell proliferation for rASCs as well as HUVECs. The DFO and DFO NPs loaded composite hydrogels revealed effective tube formation in comparison with control hydrogels without DFO and DFO NPs. The *in vivo* angiogenic evaluation of the free DFO and DFO NPs (0.025%w/w) loaded composite hydrogels were studied by injecting the developed hydrogel subcutaneously into mice for 2–4 weeks. The DFO NPs loaded composite hydrogel had enhanced neovascularization when compared to control gels. Thus, the developed DFO NPs loaded composite hydrogel could potentially be used for therapeutic angiogenesis.

1. Introduction

Hydrogels are cross-linked 3D networks that retain high amount of water and are widely used in tissue engineering for regeneration of damaged tissues [1]. These gels can also be loaded with bare drug or nanoparticles encapsulating drug molecules, growth factors or any bioactive compounds that can be released in a sustained manner [2,3]. These injectable hydrogels have added advantages like moldability, lower invasiveness during delivery, increased bioadhesion and can provide a favorable microenvironment for the cells to survive, grow and proliferate [2]. They also have mechanical and structural properties similar to many tissues and can potentially mimic the native extracellular matrix (ECM) environment especially when synthesized from materials that are derived from native ECM. Hyaluronic acid (HA) is an polyanionic polysaccharide macromolecule comprised of repeating units of β-1,4-D-glucuronic acid and β-1, 3 N-acetyl-D-glucosamine disaccharides [4]. Apart from being ubiquitous in the human body, undifferentiated cells have maximum HA content during early embryogenesis which then decreases with the initiation of differentiation, wherein it plays a key role in regulating the angiogenic process [5]. It is found to be a major intracellular component of connective tissues and also a well-recognized ECM component [6]. Additionally, the half-life of HA ranges from hours to days in tissues due to its rapid turnover by the enzyme hyaluronidase. HA can be modified chemically so as to bear desired functional groups that might result in enhanced properties. Through different processing methods, HA based viscoelastic solutions, *in-situ* hydrogels, mechanically soft and tough hydrogels and scaffolds have been successfully prepared [7,8]. Chitosan (CS), a cationic, linear biopolymer obtained from alkaline deacetylation of chitin (a major component in exoskeleton of crustaceans, such as shrimps), is primarily composed of glucosamine units and few N-acetyl-glucosamine units depending on the degree of deacetylation [9]. CS and HA being oppositely charged can involve in complex

* Corresponding author.
E-mail addresses: jayakumar77@yahoo.com, rjayakumar@aims.amrita.edu (R. J.).
[1] These authors contributed equally.

https://doi.org/10.1016/j.colsurfb.2017.10.033
0927-7765/

formation through non-specific electrostatic interactions, which is termed as complex coacervation [10,11]. CS based nanoparticles are widely used as drug carriers and both CS, HA hydrogels find application in tissue engineering and regenerative medicine. CS and HA possess intrinsic properties such as non-toxicity, biocompatibility and biodegradability, making them excellent base material for biomedical research [12,13].

Deferoxamine (DFO) is a prolyl-hydroxylase inhibitor, having potential to convert the intracellular condition from normoxia to hypoxia by selectively chelating Fe (II) ions. This results in the accumulation of a stable intracellular Hypoxia Inducible Factor (HIF-1α) which inturn facilitates a multifaceted angiogenic response. The active signaling of HIF-1α results in upregulation of its transcriptional targets like vascular endothelial growth factor(VEGF) and angiopoietin-2 (ANG-2) [14,15]. DFO increases nuclear HIF-1α accumulation, upregulates expression of VEGF, enhances vascular growth and tissue perfusion in models of ischemic disease [16,17]. DFO being water soluble, low-molecular-weight drug has a very short vascular retention time period and the systemic administration of which, result in Fe(II) ions being randomly removed from the body [18]. A strategy that could be devised to circumvent this problem is to develop a local, confined DFO release system [19].

In this paper, we report the fabrication of an injectable hybrid coacervate CS- HA hydrogel system incorporating deferoxamine loaded poly (lactic-co-glycolicacid) nanoparticles (DFO NPs). The hydrogel system was effective in reducing the burst release of drug from the nanoparticles *in vitro*. The *in vivo* results showed enhanced neovascularization suggesting the developed system as a promising biomaterial for applications related to therapeutic angiogenesis.

2. Experimental

2.1. Materials

Chitosan (CS) (M.W.−100−150 kDa, DDA-85%Koyo Chemicals, Japan), Hyaluronic acid (HA) 140 kDa Qingdao HaitaoBiochemical Co Ltd (China), PLGA 50:50 (M.W. 20,000 Wako chemicals, Japan), poly(vinylalcohol) (PVA, MW 13,000–23,000), Deferoxaminemesylate salt powder (DFO) ≥92.5% and *Alpha*-Minimum Essential Medium (*Alpha-MEM*) were procured from Sigma-Aldrich USA, Ethyl acetate (EtAc) was purchased from Merck chemicals.Iscove's Modified Dulbecco's Medium (IMDM), Fetal Bovine Serum (FBS), Large Vessel Endothelial Supplements (LVES), Penicillin-Streptomycin and Alamar Blue™ Cell Viability Reagent were purchased from Invitrogen (USA).

2.2. Preparation of DFO loaded PLGA nanoparticles (DFO NPs)

The DFO NPs were prepared using the double emulsion solvent diffusion (DES-D) method [20] (Fig. 1A). Briefly, 1 ml of 2.8% PVA containing 20 mg of DFO was prepared in water and emulsified with 3 ml of ethyl acetate containing 3% PLGA using a probe sonicator at 25% amplitude for 120s. The resulting primary emulsion was added to 10 ml sucrose solution (20%w/v) and was again sonicated for 120 s at 50% amplitude. Both the emulsions were prepared on an ice bath and EtAc was eliminated by stirring the final emulsion overnight. Bare NPs were prepared by the same procedure without addition of the drug. The NPs formed were centrifuged at 9000*g* for 10 min and lyophilized with addition of trehalose as a cryoprotectant.

2.3. Physicochemical characterization of DFO NPs

Nanoparticle size and other physical parameters were determined by Zetasizer Nano-ZS (Malvern), which uses the principle of dynamic light scattering (DLS) and was confirmed with SEM (JEOL JSM-6490LA ANALYTICAL SEM). FTIR (Shimadzu IRAffinity-1S) was performed to confirm the presence of functional groups and their interaction within the nanoparticles.

2.4. Encapsulation efficiency and in vitro DFO release from DFO NPs

The encapsulation efficiency was calculated by dissolving known quantity of lyophilized particles in dimethyl sulfoxide (DMSO), a common solvent for both PLGA and DFO, to ensure the complete dissolution of particles. The supernatant retrieved during the nanoparticle preparation was also stored and analyzed for indirect evaluation of loading. The concentration of DFO was evaluated spectrophotometrically by reacting it with ferric ions from 3 mM aq. solution of $FeCl_3$ [21]. For the reaction, equal volumes of DFO solution (either in water or in DMSO) and 3 mM aqueous solution of ferric chloride were mixed together and allowed to react for 30 min. This was followed by absorbance measurement using UV/Vis spectrophotometer. Two standard calibration curves were prepared, and the absorbance of solutions (with the absorbance maxima of 451 and 471 nm for aqueous and DMSO DFO solutions respectively) was found to be linearly dependent on the concentration of DFO (20 and 500 μM). The *in vitro* DFO release profile of DFO NPs was carried out under sink conditions in phosphate buffered saline (PBS) with 1% FBS at 37° C. After specific time intervals the nanoparticles were pelleted and supernatant was analyzed for the cumulative drug release percentage.

2.5. Preparation of chitosan-hyaluronic acid hydrogel (CS-HA)

Chitosan (2 g) (CS) was added to 100 ml of 1% acetic acid solution and was allowed to dissolve overnight. To induce gelation, the pH of the CS solution was increased to a desired range by adding 1N NaOH drop wise. The CS hydrogel thus obtained, was centrifuged at 6000 rpm and washed thrice with water in order to remove the excess NaOH. 3% w/w ratio of HA was added to the hydrogel and blended using stirrer to obtain CS-HA gel (Fig. 1B).

2.6. Preparation of DFO and DFO NPs loaded CS-HA hydrogel and in vitro release profile

To prepare free DFO and DFO NPs loaded composite hydrogels, 250 μg (0.025% w/w) free DFO or its equivalent nanoparticles was added to 1 g CS-HA hydrogel system and manually blended to obtain homogenous composite hydrogel (Fig. 1C). To study the drug release from composite hydrogels, the DFO NPs were loaded into the CS-HA hydrogels and a predefined quantity of composite hydrogels was taken in microcentrifuge tubes and PBS supplemented with 1% FBS was added. At specific time intervals, the tubes were spinned down at 10000 *g* for 10mins and the supernatant was replaced with fresh PBS supplemented with 1% FBS. The aspirated supernatant was evaluated for the amount of drug present and the drug release profile was plotted.

2.7. Injectability and inversion studies

To test the injectable nature of the gels, manual shear force was applied to a gel loaded 2 ml syringe. The gel flow and stability under the effect of gravity was analyzed by performing an inversion test. The composite and control gels of equal volumes were placed in vials, inverted and left undisturbed for 24 h.

2.8. Rheological analysis

The viscoelastic properties of the gel were tested using Malvern Kinexus pro rheometer (U.K.). The parameters were same as