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Multivalent dendrimers presenting spatially controlled clusters of binding epitopes in thermoresponsive hyaluronan hydrogels

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ABSTRACT

The controlled presentation of biofunctionality is of key importance for hydrogel applications in cell-based regenerative medicine. Here, a versatile approach was demonstrated to present clustered binding epitopes in an injectable, thermoresponsive hydrogel. Well-defined multivalent dendrimers bearing four integrin binding sequences and an azido moiety were covalently grafted to propargylamine-derived hyaluronic acid (Hyal-pa) using copper-catalyzed alkyne–azide cycloaddition (CuAAC), and then combined with pN-modified hyaluronan (Hyal-pN). The dendrimers were prepared by synthesizing a bifunctional diethylenetriamine pentaacetic acid core with azido and NHBoc oligo(ethylene glycol) aminoethyl branches, then further conjugated with solid-phase synthesized RGDS and DGRS peptides. Azido terminated pN was synthesized by reversible addition-fragmentation chain transfer polymerization and reacted to Hyal-pa via CuAAC. Nuclear magnetic resonance (NMR), high performance liquid chromatography, size exclusion chromatography and mass spectroscopy proved that the dendrimers had well-defined size and were disubstituted. NMR and atomic absorption analysis confirmed the hyaluronan was affixed with dendrimers or pN. Rheological measurements demonstrated that dendrimers do not influence the elastic or viscous moduli of thermoresponsive hyaluronan compositions at a relevant biological concentration. Finally, human mesenchymal stromal cells were encapsulated in the biomaterial and cultured for 21 days, demonstrating the faculty of this dendrimer-modified hydrogel as a molecular toolbox for tailoring the biofunctionality of thermoresponsive hyaluronan carriers for biomedical applications.

1. Introduction

Engineered microenvironments can provide a better understanding of normal and pathologic cellular behavior and expand therapeutic tissue repair solutions [1]. Indeed, the ability to control in great detail the two-dimensional (2-D) and three-dimensional (3-D) microenvironments in which cells are encapsulated allows for deciphering the influence of chemical and mechanical cues in defined conditions, and potentially designing instructive mimicking matrices [2].

Recent advances in the field of bio-nanotechnology have allowed the development of small-molecule-based materials that are bioactive, biomimetic, multifunctional and biodegradable [3]. Among these are dendrimers. These molecules are branched polymer nanostructures with a globular shape and well-defined molecular weight imparting a high degree of spatial control over selected biofunctionalities as a result of their architecture. While the application of dendrimers to biomaterials has only recently been explored, these well-defined nanostructures have been

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widely used in a variety of therapeutic applications, such as agents for delivering drugs, DNA or other bioactive compounds [4]. For example, dendrimers synthesized from polyesters, polyester-polyamide or diethylenetriamine pentaacetic acid (DTPA) cores have limited immunogenicity and are biodegradable, with low protein denaturation and blood-clotting properties [5–9].

Recent works have also demonstrated their potential for incorporation within biomaterials, resulting in targeted biological effects [10] and for promoting osteogenic differentiation in vitro [11,12]. However, there are no reports on their use to tailor hydrogel microenvironments. Of particular interest, hyaluronic acid hydrogel compositions have been shown to form relevant developmental microenvironments for stimulating neotissue growth and differentiation of human embryonic stem cells [13], to allow for spatially controlled non-viral delivery of DNA in vivo [14], and to improve the release kinetics of bone morphogenetic protein 2 (BMP-2) compared with collagen sponges [15].

Hyaluronic acid, also commonly referred to as hyaluronan, is a shear thinning biopolymer with ubiquitous biological roles that has been modified to create chemically and physically crosslinkable hydrogels that may also serve as injectable cell carriers [16–18]. Photocrosslinking methacrylated hyaluronic acid has been shown to influence cellular retention and modulate the dispersion of extracellular matrix (ECM) produced by terminally differentiated human mesenchymal stem cells (hMSC) [19]. Thermoresponsive hydrogels based on hyaluronan were also recently proposed as carriers for cell delivery [20] and an encapsulation matrix for both in vitro 3-D cell culture [16] and organ culture systems [21,22]. The thermoresponsive hydrogel is composed of copolymers based on a hyaluronic acid backbone and poly(*N*-isopropylacrylamide) (pN) brushes. The latter component is synthesized by reversible addition-fragmentation chain-transfer (RAFT) polymerization and grafted onto hyaluronan-bearing alkyne functions via a copper catalyzed azide–alkyne cycloaddition reaction (CuAAC) [16] This copolymer system in solution forms thermoreversible hydrogels above a lower critical solution temperature (LCST). The hydrogel stiffness and swelling properties were controlled by the length and grafting density of the pN segment.

Interestingly, a report describing hyaluronic acid modified with Arg-Gly-Asp-Ser (RGDS) integrin binding epitope functionalized pN was used to deliver human retinal pigment epithelial cells into mice subcutaneous implants. However, the lack of a reported cellular response caused by the biomaterial was probably due to the positioning of the peptide on the pN segment, which is responsible for the mechanism of the gelation of the biomaterial [20]. However, chemically crosslinked hyaluronan hydrogels loaded with a clustered RGDS peptides at concentrations of 0.01, 0.1 and 1 mm did in fact illicit a strong influence in murine MSC spreading [23]. The latter demonstrated that a clustering effect of the RGDS increased the cellular response in the hyaluronan hydrogel, which lacked reversibility of crosslinks and a decoupled control over the mechanics and functionality of the macromolecular network. Based on these previous studies, the present authors concluded that, within their system, (a) it is important that the RGDS peptide is grafted to the hyaluronic acid backbone to optimize the cellular recognition of the binding epitope, and (b) the optimal arrangement of lower concentrations of RGDS (~10 μm) is a clustered presentation. Hence, the present authors considered that the use of RGDS functionalized dendrimers as reported herein was a suitable test bed for the concept of controlled clustering via dendrimeric macromolecules to be compared with previous published reports.

This study reports on the development of a disubstituted dendrimer nanostructure with a singular molecular weight, which is employed in an injectable, hyaluronan matrix to present spatially controlled clusters of bioactive binding epitopes for stem cell therapies. These dendrimers are true highly branched macromolecules that display a precise architecture and can present several designed functionalities at their terminal ends [9,24]. It was hypothesized that, by augmenting the thermoreversible hydrogel microenvironment with the multivalent RGDS dendrimers onto hyaluronic acid, the clusters of RGDS would, in concert with soluble osteogenic induction factors, boost the hMSC response towards an osteogenic lineage in the bicomponent hydrogel system. Furthermore, the dendrimers could effectively create microenvironments featuring clusters of binding epitopes which, when combined with thermoreversible hyaluronan brushes, yield a bicomponent polymer system with the capacity to deliver cells, form a hydrogel on an increase in temperature, and localize the milieu to the delivery site (Fig. 1). By rheological measurement, it was tested and demonstrated that the temperature-induced gelation and moduli were not affected by a biologically relevant amount of dendrimer conjugates. Bone marrow-derived hMSC were encapsulated into bicomponent hydrogel beads containing (or not) dendrimers and incubated in either osteogenic or basal culture media for a 21-day period. Cell viability, morphology and gene expression were assessed, demonstrating the ability to tailor the biofunctionality of this 3-D thermoreversible hyaluronic acid-based microenvironment. The present authors believe this is the first report that describes the novelty of using multivalent dendrimers to tailor the biofunctionality of a hyaluronan hydrogel.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt from *Streptococcus equi* sp. (HyalNa) was purchased from Contipro Biotech s.r.o. (Czech Republic) (Mw = 1506 kDa and PD = 1.53). Tetrabutylammonium fluoride trihydrate, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride, *N*-hydroxysuccinimide, propargylamine (PPA), *N*-dimethylsulfoxide, sodium chloride, sodium azide, ascorbic acid sodium salt (AscNa), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), ethylenediaminetetraacetic acid disodium salt (EDTA), *N*-ethylpiperidine hypophosphite, *N*-isopropylacrylamide, azobisisobutyronitrile, *N*-dimethylformamide (DMF), sodium nitrate, Dowex 50X8 cation exchange resin (H Type), *N*,*N*′-diisopropylcarbodiimide (DIPCDI), trifluoroacetic acid (TFA), *N*,*N*-diisopropylethylamine (DIEA), triisopropylsilane (TIS), di-*tert*-butyl dianhydride, zinc, 10 wt.% Pd/C, dexamethasone–water soluble, β-glycerophosphate disodium salt hydrate, sodium citrate, phosphate buffered saline, 1-bromo-3-chloro-propane, diethyl pyrocarbonate (DEPC), the QuantiPro™ BCA Assay Kit, and the PKH26 red fluorescent cell linker kit were purchased from Sigma-Aldrich and were of the purest grade. Spectra/Por regenerated cellulose dialysis tubing (MWCO = 12–14 kDa) was purchased from Spectrum Laboratories. L(+)-AscNa, sodium chloride, calcium chloride, isopentane, xylene, toluidine blue and Eukitt®quick-hardening mounting medium were purchased from Fluka and were of the purest grade. Buffered 4% formalin was purchased from Formafix. NH_4Cl was purchased from Pancreac. O-(2-Aminoethyl)-O′-(2-azidoethyl)pentaethylene glycol (OEG), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, arginine (Arg), serine (Ser), aspartic acid (Asp) and glycine (Gly) were purchased from Iris Biotech. Dichloromethane (DCM) and methanol (MeOH) were purchased from Solvents Documentation Synthesis. Ethyl(hydroxyimino)cyanoacetate (OxymaPure) was purchased from Luxembourg Industries. Alpha modified Eagle's medium (α-MEM), Dulbecco's modified Eagle medium (DMEM, 4.5 g l^{-1} glucose), human MSC qualified fetal bovine serum (FBS-Hyclone), fetal calf serum (FCS), penicillin/streptomycin (P/S), non-essential amino acids (NEAA), trypsin-EDTA, SuperScript® VILO™ cDNA synthesis kit, TaqMan® Universal PCR