Acta Biomaterialia xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Engineering cell-adhesive gellan gum spongy-like hydrogels for regenerative medicine purposes

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ARTICLE INFO

Article history:
Received 31 March 2014
Received in revised form 4 July 2014
Accepted 9 July 2014
Available online xxxx

Keywords:
Tissue engineering
Cell adhesion
Hydrogel
Gellan gum

ABSTRACT

The similarity between the extracellular matrix of soft tissue and hydrogels, characterized by high-water-content viscoelastic polymeric networks, has been sustaining the advancement of hydrogels for tissue engineering and regenerative medicine (TERM) purposes. Current research on hydrogels has focused on introducing cell-adhesive peptides to promote cell adhesion and spreading, a critical applicability limitation. Here we report the development of gellan gum (GG) spongy-like hydrogels with ameliorated mechanical performance and flexibility in relation to hydrogels, using a simple and cost-effective method. Most importantly, these materials allow the entrapment of different cell types representing mesenchymal, epidermal and osteoblastic phenotypes that spread within the three-dimensional microstructure. This effect was associated with microstructural rearrangements characterized by pore wall thickening and pore size augmentation, and lower water content than precursor hydrogels. These properties significantly affected protein adsorption once cell adhesion was inhibited in the absence of serum. Spongy-like hydrogels are not adhesive for endothelial cells; however, this issue was surpassed by a pre-incubation with a cell-adhesive protein, as demonstrated for other substrates but not for traditional hydrogels. The proposed cell-compatible GG-based structures avoid time-consuming and expensive strategies that have been used to include cell-adhesive features in traditional hydrogels. This, associated with their off-the-shelf availability in an intermediary dried state, represents unique and highly relevant features for diverse TERM applications.

1. Introduction

Over the last few years one of the challenges in designing hydrogels has been modeling hydrogel properties to form cell-compatible structures characterized by their ability to control cell–material interactions to further direct cell responses [1]. In the context of tissue engineering and regenerative medicine (TERM), cell-compatible hydrogels have been engineered to recreate cell microenvironments to construct bioartificial stem cell niches, as well as three-dimensional (3-D) tissue-like structures [2,3]. However, cell adhesion and migration, crucial to attain successful results for both strategies and mediated by receptor–ligand interactions at the cell–material interface, are features that are not naturally demonstrated by traditional hydrogels. This behavior has been attributed to hydrogel's extreme hydrophilicity, which per se allows water molecules to bind to the polymer backbone [4,5], as well as to negatively charged polymers [6,7] that repulse cells, both limiting the adsorption of cell-adhesive proteins prior to cell attachment [8].

A common strategy to overcome the absence of cell adhesion within non-adhesive polymer hydrogels relies on the combination with extracellular matrix (ECM) glycoproteins [9–11], or the covalent bond of glycoprotein peptide sequences [12–15], capable of binding to cellular membrane receptors. Furthermore, modification of hydrogel's physicochemical properties, either by the incorporation of functional groups [16] or by means of different processing techniques [17], has also been shown to affect several cellular processes, presumably by influencing protein adsorption prior to initial cell attachment [16,18,19]. Both cryogels [20] and superporous hydrogels [21] are examples of hydrogels formed by macro- and microstructure rearrangement. While cryogels were shown to support the attachment and biosynthetic activity of bovine chondrocytes [22,23], proteins or peptide sequences are still frequently used in the preparation of cryogels to allow the adhesion and proliferation of other cell types such as mesenchymal stem cells, fibroblasts and endothelial cells [24,25]. Similarly, cell

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http://dx.doi.org/10.1016/j.actbio.2014.07.009
1742-7061/

compatibility has only been observed for superporous hydrogels after the incorporation of RGD [18] and proteins [26].

Gellan gum (GG), a bacterial exopolysaccharide (Fig. S.1) that resembles ECM glycosaminoglycan composition, forms hydrogels after transition from a coiled form at high temperatures, to a double-helix structure at room temperature (RT). These hydrogels can be then crosslinked by the addition of counterions, thus introducing the possibility of tuning the GG crosslinking degree and consequent mechanical properties. Together with the in situ gelation ability of GG hydrogels, those properties have been supporting their exploitation for different regenerative medicine applications. Either acellular or cellular strategies were successfully proposed for drug delivery [27], cartilage [28] and invertebral disc repair [29,30]. Nevertheless, cell adhesion features to GG hydrogels have been provided only by the combination with gelatin [31] and GG modification with a GRGDS peptide sequence [32].

Considering that the incorporation of either proteins or peptide sequences renders cell-adhesive hydrogel production more complex and the final systems more expensive, we propose GG spongy-like hydrogels with tunable cell-adhesive character by a simple and cost-effective method, distinct from cryogels and superporous hydrogels. We hypothesized that during the subsequent freezing, freeze-drying and rehydration steps of the processing method, specific parameters, such as crosslinking solution, stabilization time, freezing temperature and time, along the consecutive stages of preparation, affected the physical/mechanical properties of GG spongy-like hydrogels. By varying those specificities in the sequential but integrated processing stages of hydrogel formation into dried polymeric networks, we were able to tune spongy-like hydrogel properties. Moreover, the stability of GG spongy-like hydrogel precursors, dried polymeric networks, was assessed after 1 year of storage in order to determine their off-the-shelf availability. By retaining a major feature of hydrogels – their bio-friendly environment characterized by high water content – GG spongy-like hydrogels with tailored cell adhesive and improved physical properties represent a major advance in the use of hydrogels for TERM.

2. Experimental

2.1. Hydrogel and spongy-like hydrogel preparation

GG hydrogels were prepared as previously described [33] with modifications. Briefly, gelzan powder (Sigma, USA) was dissolved in deionized water (0.75% and 1.25% (w/v)), under stirring and at 90 °C. After dissolution, the solution was cast into the desired molds and rapidly mixed with the crosslinking solution containing mono and/or divalent cations such as $CaCl_2$ (0.03% w/v) (Merck Millipore, Germany), phosphate-buffered saline solution (0.16×) (PBS, Sigma, USA) or MEM alpha medium (Life Technologies, Scotland). The hydrogel was progressively formed until room temperature was reached. Spongy-like hydrogels were prepared from these hydrogels following successive steps. Hydrogels were stabilized in PBS solution (2 h or 48 h), then frozen (−196 °C in liquid nitrogen (N_2), or −20 °C or −80 °C in the freezer) for 18–20 h or 42–44 h, and then freeze-dried (LyoAlfa 10/15, Telstar, Spain) for 3 days to obtain GG dried polymeric networks. Spongy-like hydrogels were formed after rehydration of the dried polymeric networks.

2.2. Microscopic analysis

The macroscopic appearance of hydrogels, dried polymeric networks and spongy-like hydrogels was analyzed using a stereo microscope Stemi 1000 (Zeiss, Germany). A JEOL JSM 6301F/Oxford INCA Energy 350/Gatan Alto 2500 microscope was used to analyze the microstructure of hydrogels and spongy-like hydrogels. Samples were placed between miniature rivets on a vacuum transfer rod, slam-frozen to −210 °C in N_2 slush and transferred to the cryostat chamber at −130 °C. The top rivet was flicked off to produce a fractured surface, sublimed at −90 °C for 2 min and coated with gold/palladium.

2.3. Micro-computed tomography (μ-CT)

Dried polymeric network's microarchitecture was analyzed using a high-resolution X-ray microtomography system Skyscan 1072 scanner (Skyscan, Belgium). Samples were scanned in a high-resolution mode using a pixel size of 11.31 μm and integration time of 1.7 s. The X-ray source was set at 35 keV of energy and 215 μA of current. Representative data sets of 150 slices were transformed into a binary picture using a dynamic threshold of 45e255 (gray values) to distinguish polymer material from pore voids. These data were used for morphometric analysis (CT Analyzer v1.5.1.5, SkyScan, Belgium), which included quantification of the pore wall thickness, structure porosity and pore size. 3-D virtual models of representative regions in the bulk of the structures were also created, visualized and registered using the image processing software (CT-Vox and Data Viewer, both from SkyScan, Belgium).

2.4. Compressive testing

The mechanical behavior of hydrogels and spongy-like hydrogels (hydrated in PBS for 24 h, at RT) was tested, under static compression using an INSTRON 5543 (Instron Int. Ltd, USA). The samples were submitted to a pre-load of 0.1 N and tested up to 60% of strain, at a loading rate of 2 mm min^{-1}.

2.5. Recovery from deformation

The recovery capacity of spongy-like hydrogels was determined by measuring, using a caliper, the structure height before (Hi), and after each time point (Ht), after being submitted to 60% of strain, according to Eqs. (1) and (2) below. For calculations, height deformation after 60% of strain (HDef) was set as 0% of recovery.

$$\mathrm{HDef} = 0.4 \times \mathrm{Hi} \quad (1)$$

$$\mathrm{Recovery}\ (\%) = (\mathrm{Ht} - \mathrm{HDef})/\mathrm{HDef} \times 100 \quad (2)$$

2.6. Mass loss

Hydrogels were immersed in PBS up to 48 h at 37 °C, to determine the mass loss profile during stabilization period. Samples were weighted prior (Wi) and after each time point (Wf) and the percentage of mass loss along the time was calculated using Eq. (3):

$$\mathrm{Mass\ loss}\ (\%) = (\mathrm{Wf} - \mathrm{Wi})/\mathrm{Wi} \times 100 \quad (3)$$

2.7. Water uptake and water content quantification

Dried polymeric networks were immersed in PBS up to 7 days at 37 °C, to determine the water uptake profile. Samples were weighted prior (Wd) and after each time point (Ww) and the percentage of water uptake along the time was calculated using Eq. (4) below. The water content of hydrogels and spongy-like hydrogels was determined using the weight of the materials in the wet state (Ww) and in the dried state (Wd):

$$\mathrm{Water\ uptake/content}\ (\%) = (\mathrm{Ww} - \mathrm{Wd})/\mathrm{Wd} \times 100 \quad (4)$$

2.8. Cell isolation and culture

A set of different anchorage-dependent cells relevant for distinct approaches within the tissue engineering context was

Please cite this article in press as: da Silva LP et al. Engineering cell-adhesive gellan gum spongy-like hydrogels for regenerative medicine purposes. Acta Biomater (2014), http://dx.doi.org/10.1016/j.actbio.2014.07.009