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Thermoresponsive chitosan–agarose hydrogel for skin regeneration

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ABSTRACT

Healing enhancement and pain control are critical issues on wound management. So far, different wound dressings have been developed. Among them, hydrogels are the most applied.

Herein, a thermoresponsive hydrogel was produced using chitosan (deacetylation degree 95%) and agarose. Hydrogel bactericidal activity, biocompatibility, morphology, porosity and wettability were characterized by confocal microscopy, MTS assay and SEM. The performance of the hydrogel in the wound healing process was evaluated through *in vivo* assays, during 21 days.

The attained results revealed that hydrogel has a pore size (90–400 μm) compatible with cellular internalization and proliferation. A bactericidal activity was observed for hydrogels containing more than 188 μg/mL of chitosan. The improved healing and the lack of a reactive or a granulomatous inflammatory reaction in skin lesions treated with hydrogel demonstrate its suitability to be used in a near future as a wound dressing.

1. Introduction

Burned patients may experience a wide number of potentially fatal complications including shock, infection, electrolyte imbalances and respiratory failure (Böttcher-Haberzeth, Biedermann, & Reichmann, 2010). Furthermore, they can also experience severe psychological and emotional distress due to long periods of hospitalization, scarring and deformity (Evers, Bhavsar, & Mailänder, 2010). Such, highlights the importance of developing new wound dressings that improve the healing process, making it less painful and, simultaneously, contribute for the reestablishment of skin structure and functions in a shorter period of time (Atiyeh & Hayek, 2005; Metcalfe & Ferguson, 2007).

Among the different wound dressers produced so far, hydrogels due to their intrinsic properties are the ones that better mimic the extracellular matrix (ECM) and have the potential to direct cell migration, adhesion and growth during tissue regeneration, events that are crucial for skin regeneration (Lakes, 2007; Nicodemus & Bryant, 2008; Yu & Ding, 2008). When applied at the wound site hydrogels promote a moist healing and cool the surface of the wound, which may lead to a relevant reduction in pain and therefore have high patient acceptability (Balakrishnan, Mohanty, Umashankar, & Jayakrishnan, 2005; Boateng, Matthews, Stevens, & Eccleston, 2008). Some hydrogels have the particularity of gelling within the desired tissue or body cavity as a result of polymer interactions. Such *in situ*-forming systems advantageously flow freely as injectable liquids before administration and gel under physiological conditions. Temperature-sensitive systems that gel at body temperature are especially attractive (Schuetz, Gurny, & Jordan, 2008).

Herein, the main goal of this study was to produce a new *in situ* thermoresponsive hydrogel composed by agarose and chitosan to be used as an injectable scaffold for tissue regeneration. As described above *in situ* formed hydrogels are mouldable, *i.e.*, are able to acquire the right shape at the wound site, without wrinkling or fluting and interacting with the damaged tissue.

Agarose is a biocompatible linear polysaccharide extracted from marine algae (Buckley, Thorpe, O'Brien, Robinson, & Kelly, 2009), consisting of 1,4-linked 3,6-anhydro-α-L-galactose and 1,3-linked β-D-galactose derivatives that forms thermorreversible gels with suitable properties for tissue engineering applications (Xu et al., 2005). The mechanical properties presented by agarose are similar to those of tissues and can be easily tailored by varying polymer concentration. When solubilized in water, it forms a gel with a rigid network, resulting on a three-dimensional porous structure providing a good environment for cell adhesion, spreading and proliferation (Cao, Gilbert, & He, 2009; Mano et al., 2007; Martin,

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Minner, Wiseman, Klank, & Gilbert, 2008; Trivedi, Rao, & Kumar, 2014).

Furthermore, agarose hydrogels may be polymerized *in situ* reducing invasiveness of the surgery and also allow the hydrogel to acquire the required shape (Varoni et al., 2012).

Chitosan, the partially acetylated cationic (1–4)-2-amino-2-deoxy-β-D-glucan, is industrially produced from marine chitin. Its regenerative properties have been amply recognized (Busilacchi, Gigante, Mattioli-Belmonte, Manzotti, & Muzzarelli, 2013). In acidic aqueous solutions the protonated free amino groups of glucosamine promote the solubility of this polymer. Its hydrophilic surface promotes cell adhesion, proliferation and differentiation (Francis Suh & Matthew, 2000; Hutmacher, Goh, & Teoh, 2001; Muzzarelli, 2009). Chitosan derivatization allows an extensive adjustment of mechanical and biological properties, contributing for its anticholesterolemic and antimicrobial activity, biocompatibility, biodegradability, hemostasis and capacity to stimulate the healing process (Felt, Carrel, Baehni, Buri, & Gurny, 2000; Hirano & Noishiki, 2004; Li, Ramay, Hauch, Xiao, & Zhang, 2005; Muzzarelli, 1997).

The interaction of chitosan and agarose allows the production of hydrogels capable of gelling within the desired site, as a result of polymer interactions.

In this study, deacetylated chitosan was combined with agarose in order to explore the polymeric interactions and the thermosensitive character of agarose for producing a hydrogel. The produced hydrogel was characterized through *in vitro* and *in vivo* assays, in order to evaluate its suitability for being used as a wound dressing.

2. Materials and methods

2.1. Materials

Agarose (low melting point-ultrapure grade) was acquired from Nzytech (Lisboa, Portugal). Amphotericin B, Bovine serum albumin (BSA), Chitosan (medium molecular weight (MMW) (degree of deacetylation: 83.35% ± 0.23); Dulbecco's modified Eagle's medium (DMEM-F12), Ethylenediaminetetraacetic acid (EDTA), LB Broth, Kanamycin, *N*-acetyl-D-glucosamine, phosphate-buffered saline solution (PBS), resazurin (7-hydroxy-3*H*-phenoxazin-3-one-10-oxide), streptomycin and trypsin were purchased from Sigma-Aldrich (Sintra, Portugal). Acetic acid and sodium hydroxide were bought to Pronalab (Barcelona, Spain). Normal human dermal fibroblasts adult (NHDF), criopreserved cells were purchased from PromoCell (Labclinics, S.A.; Barcelona, Spain). *Staphylococcus aureus* (*S. aureus*) ATCC 25923 was used to evaluate antimicrobial properties of hydrogel. Fetal bovine serum (FBS) (free from any antibiotic and heat inactivated) was acquired from Biochrom AG (Berlin, Germany). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) was purchased from Promega (Canada, USA). Tris Base was obtained from Fisher Scientific (Portugal).

2.2. Synthesis and characterization of deacetylated chitosan

Chitosan was deacetylated by adapting a method previously described in literature (Gaspar, Sousa, Queiroz, & Correia, 2010) and then purified. The recovered chitosan was dissolved in 1 M acetic acid solution filtered with a 0.22 μm filter to remove traces of any solid particles. Afterwards, the pH was adjusted to 7 with 1 M NaOH, which resulted in the precipitation of the chitosan material. The product was then centrifuged at 4500 rpm (Sigma 3K18C centrifuge), this procedure was repeated three times and finally the recovered pellet was lyophilized for one day. The degree of deacetylation was measured by using the first derivative UV-spectroscopy (1DUVS) method (Muzzarelli & Rocchetti, 1985). UV–vis chitosan spectra were obtained using a Shimadzu 1700 UV–vis spectrophotometer. Table S1 shows the degree of deacetylation for the commercial and deacetylated chitosan samples. The chitosan used for hydrogel production had a deacetylation degree of 95.08% ± 0.48, meaning that almost all of the primary amine groups of the chitosan polymer chain are positively charged.

2.3. Production of chitosan–agarose hydrogel

For chitosan–agarose hydrogel (CAH) production, different percentages of deacetylated chitosan (from 0.75% to 2.5%), previously filtered with a 0.22 μm filter, were initially dissolved in 1% acetic acid solution. Then, agarose powder was added to the chitosan solution, under stirring at 50 °C, until reaching a final concentration of 3%, w/v (please see Table S2 for further details).

2.4. Determination of contact angle of CAH

Contact angles of CAH (3% agarose–0.75% chitosan and 3% agarose–1.5% chitosan) were determined using a data physics contact angle system OCAH 200 apparatus, operating in static mode. For each sample, water drops were placed at various locations of the materials surface, at room temperature (RT). The reported contact angles are the average of at least three measurements.

2.5. Study of water uptake ability (swelling)

To evaluate CAH (3% agarose–1.5% chitosan) water uptake ability, a known weight (W_0) of the hydrogel was immersed in 1 mL Tris buffer, at pH 5 and 37 °C ($n = 5$). At predetermined intervals the swollen CAH was removed from the solution, the excess of water removed with filter paper, and subsequently weighted (W_t) (Zhang, Guo, Peng, & Jin, 2004). The swelling ratio was evaluated by using Eq. (1):

$$\text{swelling ratio } (\%) = \frac{W_t - W_0}{W_0} \times 100$$

where W_t is the final weight and W_0 is the initial weight of CAH.

2.6. Proliferation of human fibroblast cells in the presence of CAH

To evaluate NHDF growth in the presence of CAH, cells were seeded in 96-well plates, containing the hydrogel, at a density of 2×10^4 cells/cm^2 per well, and incubated at 37 °C in a 5% CO_2 humidified atmosphere, for 24 and 72 h. Cell growth was monitored by using an Olympus CX41 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera.

2.7. Characterization of the cytotoxic profile of CAH

To evaluate NHDF cell viability in the presence of CAH an MTS assay was used. First, 2×10^4 cells per well were seeded on CAH surface. After 24 and 72 h of incubation at 37 °C, the culture medium was removed and replaced by a mixture of 100 μL of fresh culture medium and 20 μL of MTS/PMS (phenazine methosulfate) reagent solution. Then, cells were incubated for 4 h, at 37 °C, under a 5% CO_2 humidified atmosphere. Subsequently the absorbance was measured at 492 nm using a microplate reader (Sanofi, Diagnostics Pauster). Ethanol 96% was added to cells to be used as positive controls (K^+), whereas cells without biomaterials were used as negative controls (K^-) (Bhat & Kumar, 2012; Marques, Gaspar, Costa, Paquete, & Correia, 2014; Palmeira-de-Oliveira et al., 2010; Ribeiro et al., 2009).