



DEGRADATION BEHAVIOUR OF POLYMER-PEPTIDE HYBRID MATRICES IN THE PRESENCE OF HYALURONIDASE

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INTRODUCTION

Skin, the largest organ in the body, is a very heterogeneous layered system. Skin primary function is to serve as protective barrier against the external environment, but it also plays a crucial role in maintaining the stability of the body's internal milieu (Metcalf & Ferguson 2007). The ability of the skin to repair itself after a minor wound is remarkable, although when the damage is severe and the dermis is affected, such as in conditions of severe burn victims and diabetic ulcers, a proper and immediate coverage of the wound surface with an adequate dressing is needed to protect and accelerate wound healing. In the last years, several strategies have been proposed for developing skin substitutes, which resulted in clear improvements in the field of skin regeneration. However, none of them fulfil the criteria for fully functional skin, presenting some limitations, such as lack of engraftment resultant from deficient vascularization (Supp & Boyce 2005, Bottcher-Haberzeth, S. et al. 2010, Priya et al. 2008). Therefore, bioactive matrices that can regulate cell activities (adhesion, proliferation and differentiation) would be greatly beneficial in skin regeneration strategies.

Hyaluronan (HA) is one of a group of polysaccharides typically found in the connective tissues of vertebrates and is one of the major ECM components in skin (Sakai et al. 2000). It is an extremely large polymer made up of disaccharide repeating unit of N-acetylglucosamine and glucuronic acid. HA offers many unique advantages as a starting material to obtain medical products for skin regeneration applications, as it appears at times of cell migration during embryogenesis, morphogenesis, and wound repair (Clark et al. 2007). Another advantage of using this natural polymer over synthetic ones is that degradation of natural ECM can occur concomitant with cell invasion, as it happens during normal granulation tissue formation.

Recently, the design of molecular and supramolecular materials for regeneration of tissues is becoming of great interest in advanced medicine. The application of self-assembling materials that can interact with cells and

trigger the differentiation of human stem cells is of major interest in this field.

Recently, a remarkable discovery was made on self-assembly of macromolecules and small molecules (Capito et al. 2008). Under specific conditions, instant self-assembly between HA and peptide molecules of opposite charge occurs at the liquid-liquid interface and can result in the formation of self-sealing sacs or 2D matrices. The self-assembling matrices have a number of features that make them ideal candidates to investigate new directions in wound healing. First, the biomimetic nature of self-assembling matrices, composed of HA (a glycosaminoglycan found in skin) and peptide molecules, resembles the natural extracellular environment of skin. Second, it allows the incorporation of bioactive ligands directly into the structure for cell signaling. The third important characteristic of the system lies on the potential of the matrix to encapsulate drugs or cells during the process of self-assembly. A fourth important feature of these matrices is their biodegradability. In the proposed strategy, biodegradation is a part of the material design since the artificial matrix will degrade overtime into amino acids and natural sugars which are nontoxic and easily cleared in the body.

It has been shown that the degradation rate of the supportive matrix had a strong influence on cell migration, proliferation, differentiation and morphology of the newly formed tissue. The aim of this study is, therefore, to evaluate the degradation behavior of 2D membranes, obtained by self-assembly between a peptide amphiphile and hyaluronan, in the presence of hyaluronidase.

MATERIALS AND METHODS

Synthesis and characterization of peptide amphiphile (PA)

The chemical structure of the PA used in this study contains 3 valine (V), 3 alanine (A) and 3 lysine (K) residues, followed by an alkyl tail of 16 carbons (C₁₆V₃A₃K₃). The PA was synthesized on a 2 mmol scale using an automated peptide synthesizer and following standard solid phase Fmoc chemistry. After cleavage of PA from the resin, the crude product was isolated by precipitation with cold diethyl ether and

further purified by reverse phase high performance liquid chromatography (RP-HPLC).

Peptides were characterized by electrospray ionization mass spectrometry (ESI-MS), transmission electron microscopy (TEM) and circular dichroism (CD) spectroscopy.

Membrane production and characterization

Membranes were prepared by casting 1 wt% HA and 2 wt% PA aqueous solutions onto individual wells of a 24-well plate, followed by incubation at 60 °C for 4 h. The microstructure of the membranes was investigated by scanning electron microscopy (SEM).

Enzymatic degradation studies

The degradation behavior of the membranes was studied in the presence of hyaluronidase enzyme (HAase) which has the ability to hydrolyze $\beta(1,4)$ glycosidic bonds on HA chain, producing fragments with a N-acetyl-D-glucosamine at the reducing end. The degradation can thus be monitored by quantification of these reducing ends. Studies were carried out by incubating membranes, with and without HAase, using three different conditions: (i) PBS; (ii) 2.6 U/mL HAase to simulate physiological conditions in human plasma and (iii) 50 U/mL HAase. Samples were incubated at 37 °C for 14 days. At predetermined time points, the solution was collected for further quantification of N-acetylamino sugars using the fluorimetric Morgan-Elson assay (Elson & Morgan, 1933) method and identification of degradation products by mass spectrometry. The morphology of the membranes after degradation was analyzed by SEM.

RESULTS

Peptide characterization by mass spectrometry confirmed that the synthesized peptide presented the expected mass, $1150 \text{ g}\cdot\text{mol}^{-1}$ ($1151: \text{M}+\text{H}^+$, $576: \text{M}+2\text{H}^{2+}$). Previous studies have shown that the formation of stable beta-sheet is very important and necessary for peptide self-assembly (Wang et al. 2008). Circular dichroism spectra showed that depending on the pH, the peptide can acquire a different conformation. At pH 7, a typical spectrum for beta-sheet structure is presented with a minimum at 215 nm and a maximum at 200 nm.

We have further tested the ability of the peptide to form 2D membranes by self-assembly in the presence of the polymer hyaluronan. Membrane degradation behavior was studied in three different conditions, PBS and PBS containing 2.6 U/mL and 50 U/mL Hase. Degradation was followed by the quantification of N-acetylglucosamine in solution and by SEM analysis. Incubation in PBS did not show the presence of N-acetylamino sugars in the supernatant up to 14 days, which indicates that the membranes are relatively stable in buffer solutions. As expected, the degradation is more evident when higher enzyme concentration is used (50 U/mL) than in the solution resembling physiological

conditions (2.6 U/mL). In the latter case, the degradation of the membranes is progressive along the time without extensive degradation. SEM analysis of the membranes collected at different time points corroborates these results, showing the appearance of pores on the membrane surface and cross-section as a result of HA degradation by HAase.

CONCLUSIONS

These studies showed that the 2D matrices, formed by self-assembling between HA and a positively charged PA, are susceptible to enzymatic degradation by hyaluronidase and the degradation is faster with higher enzyme concentration. In the presence of hyaluronidase at physiological concentration, the membranes slowly degrade overtime, this may present an advantage over other systems, since the slow degradation will also induce the slow migration of cells and/or the release of bioactive molecules that may be incorporated on the matrices. The matrix degradation also provides space that is essential for new tissue formation. We expect that the proposed biodegradable hybrid matrices could offer significant potential in skin regeneration strategies as a bioactive supportive matrix for wound healing.

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