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Peptide-decorated chitosan derivatives enhance fibroblast adhesion and proliferation in wound healing

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ABSTRACT

RGD peptide sequences are known to regulate cellular activities by interacting with $\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrin, which contributes to the wound healing process. In this study, RGDC peptide was immobilized onto chitosan derivative 1,6-diaminohexane-O-carboxymethyl-N,N,N-trimethyl chitosan (DAH-CMTMC) to display RGDC-promoting adhesion for enhanced wound healing. The efficiency of N-methylation, O-carboxymethylation and spacer grafting was quantitatively and qualitatively analyzed by ^{1}H NMR and FTIR, yielding 0.38 degree of substitution for N-methylation and >0.85 for O-carboxymethylation. The glass transition temperatures for chitosan derivatives were also studied. Peptide immobilization was achieved through sulfhydryl groups using sulfosuccinimidyl (4-iodoacetyl)amino-benzoate (sulfo-SIAB method).

RGDC immobilized peptide onto DAH-CMTMC was found to be about 15.3 μg/mg of chitosan derivative by amino acid analysis (AAA). The significant increase of human dermal fibroblast (HDF) viability *in vitro* over 7 days suggests that RGDC-functionalized chitosan may lead to enhanced wound healing (viability >140%). Moreover, bio-adhesion and proliferation assays confirmed that coatings of RGDC-functionalized chitosan derivatives exhibit *in vitro* wound healing properties by enhancing fibroblast proliferation and adhesion. These results showed that RGDC peptide-functionalized chitosan provides an optimal environment for fibroblast adhesion and proliferation.

1. Introduction

Biopolymers may serve as carriers of biological signal molecules, an advantageous property for the application in cutaneous wound healing. Wound healing is a very complex process, organized in a cascade of cellular and molecular events. These consist of the initial inflammatory phase, followed by proliferation and migration of dermal cells and the remodeling phase. The latter phase also includes angiogenesis and formation of granulation tissue (Boateng, Matthews, Stevens, & Eccleston, 2008; Rossi et al., 2013). Each of these healing phases is controlled and regulated by growth factors. Growth factors can diffuse through the wound if it is sufficiently moist. In large wounds, dressings are applied regularly to keep the wound clean and to enhance wound healing (Muzzarelli, 2009). Therefore, there is a major interest to develop biopolymer matrices used as wound dressings that could enhance the healing process. It should be noted that dressings also act as physical barriers, remain permeable for moisture and oxygen, and protect the wound against microorganisms and foreign materials (Lloyd, Kennedy, Methacanon, Paterson, & Knill, 1998; Zhang et al., 2015). In this perspective, chitosan is a suitable candidate to be used as a matrix for wound healing. Chitosan is obtained through the deacetylation of chitin (Van der Lubben, Verhoef, Van Aelst, Borchard, & Junginger, 2001) and can be successfully applied as a matrix to cover the wound (Huang & Fu, 2010). Chitosan is known to promote wound healing when functionalized with RGD peptide sequences able to bind $\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins, which mediate signals between the extracellular matrix (ECM) and

Abbreviations: CMTMC, O-carboxymethyl-N,N,N-trimethyl chitosan; DAH-CMTMC, 1,6-diaminohexane-O-carboxymethyl-N,N,N-trimethyl chitosan; RGDC-DAH-CMTMC, Arg-Gly-Asp-Cys-1,6-diaminohexane-O-carboxymethyl-N,N,N-trimethyl chitosan.

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cytoskeleton (Muzzarelli, 2009). Additionally, chitosan has high antibacterial activity, is biocompatible and biodegradable (Boateng, Burgos-Amador, Okeke, & Pawar, 2015; Croisier & Jérôme, 2013).

However, chitosan remains insoluble at pH values exceeding 6.5 (Qin et al., 2006). Trimethylation of the amine function or other chemical modifications was chosen as a preferred strategy to overcome the low solubility of chitosan close to the physiological pH of 7.4 (Boateng et al., 2008). N,N,N-trimethylated chitosan (TMC) was obtained from N-methylation at the C-2 position of the chitosan backbone. As a result, TMC is permanently positively charged due to the quaternized amines (Amidi et al., 2006). Furthermore, O-carboxymethyl-N,N,N-trimethyl chitosan (CMTMC) can be obtained from TMC by O-carboxymethylation at the C-6 position. The advantage of this chitosan derivative is that CMTMC can act as a polyelectrolyte, carrying positive charges of TMC and negative charges of the carboxyl groups. Previous studies revealed the absence of CMTMC cytotoxicity *in vitro* (Patrulea, Applegate, Ostafe, Jordan, & Borchard, 2015). Therefore, CMTMC may be suitable to serve as a scaffold-carrier for peptides like RGD to induce cell adhesion and migration (Ruoslahti & Pierschbacher, 1987). Further addition of a diaminohexane (DAH) 4-carbon long spacer between CMTMC and RGD peptides is expected to improve the display and bioactivity of the peptide (Xu, Li, Jin, Bai, & Liu, 2014). In addition, DAH spacer allows a control over the peptide binding, so that each amino group crosslinked to CMTMC will bind one RGDC peptide. This is, to our knowledge, the first application of this strategy to better control peptide grafting.

In this regard, the present study focused on the: (i) preparation of RGDC-functionalized chitosan derivatives; (ii) characterization of new derivatized polymers; and (iii) evaluation of their ability to promote fibroblast adhesion and proliferation.

2. Materials and methods

2.1. Materials

Chitosan (81% degree of deacetylation, ChitoClear Cg10, 8 mPa s, Primex, Siglufjordur, Iceland). Chloroacetic acid, methyl iodide (99%), N-methyl-2-pyrrolidone (NMP; 99%) and 1,6-diaminohexane (DAH) were purchased from Sigma–Aldrich (Buchs, Switzerland). Sodium hydroxide (98.5%) and N-hydroxysulfosuccinimide sodium salt (95%; sulfo-NHS) were acquired from Acros Organics (Geel, Belgium). Sulfo-N-succinimidyl (4-iodoacetyl)aminobenzoate (sulfo-SIAB) was obtained from Brunschwig (Basel, Switzerland) and RGDC peptide from Bachem (Switzerland). Dulbecco's modified Eagle medium (DMEM) and all other biological reagents were purchased from Life Technologies (Switzerland). Amicon Ultra-15 Centrifugal Filter Units with a cut-off of 10 kDa were obtained from Merck Millipore (Darmstadt, Germany).

2.2. TMC synthesis

TMC was synthesized in two steps: dimethyl chitosan (DMC) synthesis followed by TMC production (Patrulea et al., 2015). In summary, 2 g of chitosan were dissolved in 6 mL of formic acid, 8 mL of formaldehyde and 36 mL of distilled water. The reaction was kept for 118 h at 70 °C under reflux (Liebig condenser), followed by the solvent evaporation (Rotavapor R-210, Büchi, Switzerland). After adjusting the pH to 12, the precipitate was collected, washed and filtrated. Next, the obtained DMC was diluted to a concentration of 2% (w/v) and pH 4.0, followed by dialysis for 3 days against water and lyophilization.

Dried DMC was then dissolved in water (160 mL to 1 g DMC) and the pH was adjusted to 11. The newly formed gel was washed twice in water and three times in acetone. Dried precipitate was treated with NMP (50 mL) at 70 °C and the mixture was left under reflux conditions until it dissolved completely. Methyl iodide was added to the dissolved DMC (molar ratio of 1:25) and allowed to react for 140 min. Product precipitation was done in a mixture of ethanol:diethylether (4:1 V/V) followed by centrifugation (4000 rpm × 15 min). The final product was solubilized in 10% NaCl. Dialysis was done on the first day in 1% NaCl and then, over the last 2 days in water (Spectra/Por 4, cut-off of 12–14 kDa; changing the buffer twice daily) before lyophilization and analysis.

2.3. TMC carboxymethylation

CMTMC was synthesized following the protocol described previously (Patrulea et al., 2015). Briefly, TMC and isopropanol (1 g TMC in 100 mL) were left to mix overnight at 45 °C. Then, we added 50% NaOH (0.5 mL) over a 20 min period, and the whole mixture was left stirring for 45 min. After adding mono-chloroacetate (molar ratio of 1:3) to TMC during 25 min, the reaction mixture was stirred for another 3 h at 60 °C under nitrogen atmosphere. Next, filtration and washing with 70% (V/V) ethanol initially, and later with pure ethanol was done after adjusting pH to neutral. The resulting O-carboxymethyl-N,N,N-trimethyl-chitosan (CMTMC) was then dried, and later prepared for dialysis. Dialysis was done over 3 days against water (changing water twice a day) by dissolving CMTMC in Milli-Q water. Dialyzed CMTMC was lyophilized and submitted to further analysis.

2.4. RGDC immobilization onto CMTMC

The chemical cross-linking of CMTMC was done by adding a spacer-arm (1,6-hexanediamine (DAH)). First, CMTMC (10 mg) powder was dissolved in TRIS buffer (1 M; pH 9.0) at high temperature until boiling point, in order to break down hydrogen bonds. The reaction solution was let to cool down and water-soluble EDC (0.8 M; 1 mL) and sulfo-NHS (0.2 M; 1 mL), both dissolved in 1 M TRIS buffer, were added to activate carboxyl groups in CMTMC. The carbodiimide reaction was left stirring for 2 h at room temperature. Thereafter, the unreacted reagents were removed by washing with TRIS buffer. Subsequently, DAH solution (0.4 M; 9 mL dissolved in TRIS) was added to the reaction mixture. Then, the whole reaction was left stirring for 2 days at 40 °C. The aminated mixture DAH-CMTMC (1,6-diaminohexane-O-carboxymethyl-N,N,N-trimethyl-chitosan) was purified using dialysis tubes (Amicon® Ultra-15, cut-off 10 kDa) and then washed with Milli-Q water. The final product was lyophilized and the chemical structure analyzed.

The immobilization reaction was achieved using a modified sulfo-SIAB method (Mallik, Wa, & Hage, 2007) in order to prepare iodoacetyl-activated DAH-CMTMC functionalized with RGDC, as shown in Fig. 1. First, 10 mg DAH-CMTMC polymer was washed with 3 mL borate buffer, pH 8.0 containing 0.05 M EDTA. Then, sulfo-SIAB crosslinker (1.7 mg mL^{-1} dissolved in borate buffer, pH 8.0) was added at a molar ratio of 1:5 to amino groups of DAH-CMTMC. The reaction was left stirring in the dark for 1 h at 40 °C. The crosslinked product was separated from the non-reacted crosslinker by dialysis and solubilized in borate buffer (pH 8.0). RGDC peptide (5.1×10^{-5} M) was added to the reaction mixture and left stirring in the dark for another 24 h at 40 °C. Before being freeze-dried for storage, the polymer solution was purified and washed by dialysis in order to remove any unreacted reagents. The degree of grafting was measured by amino acid analysis (AAA).