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Research paper

Ionic polymeric micelles based on chitosan and fatty acids and intended for wound healing. Comparison of linoleic and oleic acid

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ABSTRACT

Chitosan is well known for its positive properties in wound healing. Also unsaturated fatty acids are described as able to accelerate tissue repairing mechanisms.

In this work hydrophobically modified chitosan was obtained by ionic interaction with either oleic or linoleic acid. In aqueous environment self-assembling into nanoparticles occurred. The presence of hydrophobic domains, similar to those present in polymeric micelles, was demonstrated by changes in pyrene spectra. Both oleate and linoleate derivatives showed mucoadhesion behaviour. Cytotoxicity tests on human dermal fibroblasts demonstrated good biocompatibility of especially oleate derivatives. Clarithromycin, a poorly soluble model drug proposed for use in infected wounds was successfully encapsulated in both oleic and linoleic based polymeric micelles. The ionic structure of the carriers is responsible for their loosening at neutral pH and in the presence of salts. This behaviour should impair parenteral administration of the systems, but can be useful for topical delivery where the micelle components, chitosan and fatty acid, can play a positive role in dermal regeneration and tissue repairing.

1. Introduction

Both chitosan and unsaturated free fatty acids are described in the literature for their interesting bioactive properties, useful to promote cell proliferation and tissue regeneration. A considerable amount of work has been reported on chitosan and its potential use in various bio-applications [1,2]. Chitosan is a natural product, derived from chitin, that is characterised by biodegradability, biocompatibility, mucoadhesion [3,4] and antimicrobial activity [2,5]. It is also effective in many steps of tissue regeneration process, as it activates complement, promotes migration of polymorphonuclear leucocytes (PMN), induces the release of inflammatory mediators (tumour necrosis factor and interleukins), improves granulation and reorganization of damaged tissue, therefore accelerating the repairing of hard to heal wounds and of burns [6–8].

Also unsaturated free fatty acids, especially linoleic and oleic acid, have shown a positive role in wound healing, thanks to their ability to modulate the inflammation and to enhance reparative response *in vivo* [9,10]. This effect has been confirmed by Pereira L.M. et al. in an *in vivo* study on a rat model, where both oleic and linoleic acids were able to increase the presence of neutrophils in the wound and to reduce the thickness of the necrotic tissue [11]. Moreover, after incubation of neutrophils with oleic and linoleic acids, a dose-dependent increase in the release of vascular endothelial growth factor-alpha (VEGF-α) and interleukin-1beta (IL-1β) was observed [10]. From these evidences it is possible to expect that the pro-inflammatory effect of oleic and linoleic acids is able to speed up the wound healing process, especially in case of chronic wounds, where the tissue regeneration is somehow impaired in one of its phases.

Hydrophobically modified chitosans are widely described in the literature to give polymeric micelles with a core in which hydrophobic poorly soluble molecules can be efficiently loaded [12–14]. In most cases the chitosan amino groups are covalently bound to carboxylic functions of the hydrophobic moieties, with the occurrence of amidic bonds. This derivatization has been described also to obtain oleic and linoleic chitosan derivatives [15,16]. In quite few cases nanoparticles are obtained by ionic interaction between chitosan and a hydrophobic or an amphiphilic counterion [17]. In the present study a carrier delivery system based on the ionic interaction between the amino groups of chitosan and the carboxylic groups of oleic or linoleic acids was prepared. This interaction imparts chitosan an amphiphilic behaviour, with hydrophobic domains represented by the fatty acids molecules that induce self-aggregation of the polymer. The ionic bonds are weaker than the covalent ones and more sensitive to medium pH and ionic strength. This impairs the employment of ionic polymeric micelles in parenteral delivery, where good stability also upon dilution is required. However, in the case of topical delivery, such as in application to wounds, micelles are likely to get looser

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structure and eventually to dissociate into their components, that are in this case chitosan and fatty acid, with a possible action favourable to wound repair. Aim of the present work was to characterise and evaluate biocompatibility of nanoparticles obtained by spontaneous assembling of chitosan hydrophobically modified by ionic interaction with linoleic and oleic acid. These nanoparticles were characterised for dimensions and resuspendability upon freeze drying. The presence in their structure of hydrophobic domains was evaluated by means of pyrene spectra. The maintenance of the mucoadhesion behaviour well described in the literature for chitosan was assessed. Biocompatibility was evaluated on a human fibroblast cell culture. Clarithromycin, a poorly soluble antibiotic proposed in the literature for the topical treatment of infected wounds was used as model drug and loaded in polymeric micelles [18].

2. Materials and methods

2.1. Materials

The following materials were used: Chitosan (CS) low molecular weight, deacetylation 98% and 250 kDa molecular weight [19] (Primex chitoclear TM 1504, Giusto Faravelli, Mi, I). Fatty acids (FFAs): linoleic acid (LA) (Sigma–Aldrich, Milan, I), oleic acid (OA) (Fluka, Milan, I), pyrene 99% (Fluka, Milan, I), mucin type II from porcine stomach (Sigma Aldrich, Milan, I), clarithromycin (Sigma Aldrich, Milan, I). Chloridric acid (HCl), sodium hydroxide (NaOH) and hydrogen peroxide (H_2O_2) were purchased from Carlo Erba reagents (Milan, I). For cell culture studies, where not specified, all the products were purchased from Sigma–Aldrich (Milan, I).

2.2. Methods

2.2.1. Chitosan depolymerisation with hydrogen peroxide

Chitosan was depolymerised as described by Tian et al. [20]. Briefly, chitosan was dissolved in bidistilled water at 2% (w/v), equimolar to HCl 1 N (Carlo Erba reagents, Rodano, Milan, I). 5 ml of H_2O_2 0.5 M (H_2O_2 30% w/w, Carlo Erba reagents, Rodano, Milan, Italy) was added under magnetic stirring to start the depolymerising reaction. After exactly 3 h, NaOH 0.5 M (Carlo Erba reagents, Milan, I) was added until pH 7.0 to stop the depolymerisation and induce chitosan precipitation. Chitosan was separated by centrifugation at 815*g* (ALC 4218 Centrifuge, ALC International, Cologno Monzese, Milan, I) for 30 min, and then resuspended adding HCl 0.5 N, until complete dissolution. Chitosan–HCl solution was dialysed in bidistilled water for 24 h and then freeze-dried (HetoDrywinner, Analitica de Mori, Milan, I). The molecular weight, determined by viscometry [19,21], resulted 100 kDa.

2.2.2. Preparation of polymeric micelles

1% (w/v) solution of either oleic or linoleic acid (Sigma–Aldrich, Milan, I) in acetone was prepared and added drop-wise under magnetic stirring to 0.5 mg/ml chitosan HCl solution in filtered distilled water (acetate cellulose, 0.22 μm). The amount of FFA acetone solution to be added was calculated taking into account the stoichiometric mole/mole ratio between the amino groups of deacetylated chitosan and the carboxylic groups of FFA molecules. Systems with 1:1 molar ratios, named CS:OA 1:1 and CS:LA 1:1, in which for a concentration of chitosan 0.5 mg/ml the FFA concentration resulted 0.86 mg/ml were prepared. Systems with 1:0.5 stoichiometric ratio, named CS:OA 1:0.5 and CS:LA 1:0.5 were also prepared, in which for a 0.5 mg/ml chitosan concentration the FFA concentration resulted 0.43 mg/ml. Acetone was finally evaporated under nitrogen flux at room temperature. Nanoparticles were sonicated in an ultrasonic bath (Elmasonic S 80 H, Elma Hans Schmidbauer GmbH & Co, Singen, G) for 15 min to reduce aggregation.

2.2.3. Dimensional analysis

The particle size and the Polydispersity Index (PI) were determined by Photon Correlation Spectroscopy (PCS) (N5 Submicron Particle Size Analyser Beckman Coulter, IL, Milan, Italy). Samples were diluted in filtered bidistilled water and analysed at 25 °C, at 90° detection angle. PI indicates the width of the size distribution ranging between 0 (monodispersity) and 1.

To verify the resuspendability after freeze drying, all the samples, in distilled water and diluted with a trehalose solution to final concentration 2% w/v were freeze dried (HetoDrywinner, Analitica de Mori, Milan, I). PCS analysis was performed on these samples before and after freeze drying, redispersing in this last case the powder to the initial volume with distilled water.

PCS analysis was performed also on samples diluted, immediately before the analysis, in phosphate buffers (final concentration 0.1 M) at different pH values.

2.2.4. Loading of pyrene fluorescent probe

Samples were prepared keeping them away from light. Pyrene was dissolved in acetone at a final concentration of 1.2×10^{-5} M. Nanoparticles were prepared adding this pyrene solution to 3 ml of chitosan–HCl solution 0.5 mg/ml, in order to obtain a final concentration of pyrene equal to 1×10^{-7}, or 2×10^{-7} M. Oleic and linoleic acid were then added to the chitosan–HCl, as previously described for nanoparticle preparation. When the acetone was evaporated, samples were sonicated in ultrasonic bath for 15 min and were analysed by spectrofluorimeter (Perkin Elmer, LS 50B, Milan, I) at λ_{exc} = 336 nm. The emission spectrum was considered between 350 and 500 nm and the ratio of the intensity at λ_{em} = 383 nm and λ_{em} = 372 nm was calculated according to what described by Kalyanasundaram and Thomas [22].

Pyrene spectra were obtained also on samples diluted, immediately before the analysis, in phosphate buffer pH 7.4 (final concentration 0.1 M) and in NaCl (final concentration 0.9% w/v).

2.2.5. Turbidimetric assay

Mucoadhesive properties were evaluated by means of a turbidimetric assay described by He et al. [23]. Micelles were diluted 1:1 with different concentrations of mucin (0.1%, 0.2%, 0.5%, 0.75% and 1% w/v) and were incubated at room temperature for 30 min. The absorbance of the mixtures was then measured by a spectrophotometer at a wavelength of 500 nm (Perkin Elmer Instrument Lambda 25 UV/VIS Spectrometer). The effective absorbance ($A_{muc+mic}$) was given by the mixture of mucin and micelles. The theoretical absorbance (A_{theor}) was calculated as the sum of A_{muc} and A_{mic}. The difference in absorption (ΔA) between $A_{muc+mic}$ and A_{theor} is the measure of the mucoadhesive interaction, that is positive for values of $\Delta A > 0$.

2.2.6. Cell culture and growth conditions

Normal Human Dermal Fibroblasts (NHDF, Promocell GmbH, Heidelberg, G) were grown in Dulbecco's Modified Eagle's Medium (Lonza, Milan, I) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Euroclone, Milan, I) and 1% v/v Penicillin-Streptomycin 100× and 1% (v/v) Amphotericin (PBI International, Milan, I). Fibroblasts were maintained in a humidified 5% CO_2 and 95% RH atmosphere at 37 °C and routinely splitted every 6 days using Trypsin-EDTA solution 0.25%. Cells were used between the 5th and the 15th passage.

2.2.7. In vitro cytotoxicity test

Fibroblasts were seeded at a 10^5 cell/cm^2 density in a 96-well plate and were incubated in standard growth conditions for 24 h.