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Research paper

Development of chitosan oleate ionic micelles loaded with silver sulfadiazine to be associated with platelet lysate for application in wound healing

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ABSTRACT

In the treatment of chronic wounds, topical application of anti-infective drugs such as silver sulfadiazine (AgSD) is of primary importance to avoid infections and accelerate wound repair. AgSD is used in burns and chronic wounds for its wide antibacterial spectrum, but presents limitations due to poor solubility and cytotoxicity.

In the present work polymeric micelles obtained by self-assembling of chitosan ionically modified by interaction with oleic acid were developed as carriers for AgSD to overcome the drawbacks of the drug. The AgSD loaded micelles were intended to be associated in wound healing with platelet lysate (PL), a hemoderivative rich in growth factors. Unloaded micelles demonstrated good compatibility with both fibroblasts and PL. The relevance of chitosan concentration and of the ratio between chitosan and oleic acid to the drug loading and the particle size of nanoparticles was studied. A marked increase (up to 100 times with respect to saturated solution) of AgSD concentration in micelle dispersion was obtained. Moreover, the encapsulation reduced the cytotoxic effect of the drug towards fibroblasts and the drug incompatibility with PDGF-AB (platelet derived growth factor), chosen as representative of platelet growth factors.

1. Introduction

Chronic wounds represent an important disease of great socio-economic impact, often being subject to secondary infections that can be, in some cases, even life threatening [1]. The emergence of antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus*, increasingly limits the use of antibiotics in the treatment of chronic wounds. One suitable approach to this problem is represented by the topical delivery of anti-infectives loaded in nanoparticle carriers able to target them at higher concentrations to the site of infection. In particular, some of the nanocarriers described in literature, such as chitosan-containing nanoparticles and metal-containing nanoparticles, are showing promise in the reduction of resistance development thanks to the occurrence of different simultaneous mechanisms of action; another important aspect is the nanoparticle's ability to overcome low drug uptake, drug efflux and biofilm formation [2].

Among topical anti-infective agents there are silver derivatives such as silver sulfadiazine (AgSD), which is still largely used in burn and wound treatment for its broad activity spectrum [3]. AgSD is known for its cytotoxicity and for its impairment of proliferation effect, related to interference with replication of both bacterial as well as host cells. The association of AgSD with epithelial growth factors has been proposed to overcome this limitation: it has been observed that the co-administration of Epidermal Growth Factor restored the viability of a HaCaT cell culture, impaired by AgSD, in a dose-dependent way [4].

On the other hand, growth factors are widely used in promotion of wound healing in skin and other tissue lesions. The therapeutic

Abbreviations: AgSD, silver sulfadiazine; PL, platelet lysate; CS, chitosan; OA, oleic acid; PDGF-AB, platelet derived growth factor; GFs, growth factors; ELISA, enzyme-linked immunosorbent assay; PCS, photon correlation spectroscopy; DMEM, Dulbecco's Modified Eagle's Medium; MTT, (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; HBSS, Hank's Buffered Salt Solution; DMSO, dimethyl sulfoxide; FBS, foetal bovine serum; CFU, colony forming unit; MIC, minimum inhibitory concentration.

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use of hemocomponents such as platelet gel, platelet rich plasma [5–7] and platelet lysate [8–11] appears increasingly promising for the recognized positive actions of platelet growth factors (GFs) on tissue repair and regeneration, vascular remodelling and regulation of inflammatory and immune responses [5,12]. A reduction in the cytotoxic effect of AgSD on fibroblasts has been demonstrated after encapsulation in clay-chitosan nanocomposites [13], while the encapsulation in solid lipid nanoparticles has reduced the negative effect of the drug on platelet lysate stability [9].

Another drawback of AgSD is represented by its low solubility that limits the possibilities of application in hydrophilic formulations and in an aqueous environment.

Polymeric micelles have been proposed as suitable nanoparticle systems to enhance dissolution of poorly soluble drugs that are loaded in the hydrophobic core [14,15]. In literature, a large number of papers deal with structures obtained by the self-assembling of hydrophobically modified chitosan [16]. Chitosan is, in fact, a multifunctional polymer with well recognized properties such as mucoadhesion, absorption enhancement, antibacterial activity, immune-stimulating and haemostatic properties and promotion of wound healing [17–22]. The hydrophilic shell of chitosan based polymeric micelles is made of the polymer chains. In the case of chitosan they can maintain useful properties of interaction with the biological substrate, for example mucoadhesion behaviour or penetration enhancement [23,24]. Furthermore, oleic acid is widely described in literature as able to promote wound healing [25] and it has been demonstrated that derivatization of chitosan with oleic acid results in micelles with good antibacterial properties [26]. In a previous paper, it was shown that simple ionic association of chitosan and oleic acid results in nanoparticles with characteristics of polymeric micelles that are able to increase the aqueous concentration of clarithromycin, a poorly soluble antibiotic used in skin wounds. The peculiarity of these micelles consists in the relatively low stability of chitosan:oleic acid interaction that gets looser at the site of administration making polymer, oleic acid and encapsulated drugs available for their biological action [27].

In the present work, ionic polymeric micelles based on chitosan hydrophobically modified by electrostatic interaction with oleic acid (CS:OA), have been loaded with AgSD with the aim of improving drug solubilization in aqueous environment and of reaching higher and more effective concentrations at the site of action. At the same time, encapsulation would protect human cells against the drug's cytotoxic effects. Chitosan oleate (CS:OA) polymeric micelles were evaluated in terms of proliferation activity on fibroblast cell cultures and compared to chitosan and oleic acid alone. To assess the ability of CS:OA micelles to load AgSD and to improve its solubilization, a full factorial design was used in which the effects of polymer concentration and of ratio between polymer and fatty acid on drug loading and particle size were studied. The protective effect of AgSD encapsulation in terms of cytotoxicity was evaluated in fibroblast cell culture. Finally, platelet derived growth factor PDGF-AB was quantified in PL and in its mixtures with unloaded and AgSD loaded micelles. It was chosen as representative of platelet GFs because it is present in relatively high amounts in platelets [28].

2. Materials and methods

2.1. Materials

Chitosan low molecular weight (TM 1504, about 250 KDa, 98% deacetylated) was purchased from Giusto Faravelli (Milan, Italy). Oleic acid (OA) and silver sulfadiazine (AgSD) were obtained from Sigma–Aldrich (Milan, Italy). HCl 1 N, H_2O_2 30% v/v and NaOH pellets were provided by Carlo Erba Reagents (Milan, Italy). For cell culture studies, where not specified, all the products used were purchased from Sigma–Aldrich (Milan, Italy). Platelet lysate (PL) was prepared by the Apheresis Service of Immunohaematology and Transfusion Service Center for transplant immunology of I.R.C.S.S. Policlinico San Matteo (Pavia). Aliquots of hyperconcentrated platelets (high platelet concentration in small plasma volume and minimal leucocyte contamination) were obtained using a sterile connection technique from apheresis, carried out on regular blood donors. The platelet pool was frozen at −80 °C for 5 h, unfrozen in a sterile water bath at 37 °C, divided into aliquots and maintained at −80 °C so that the same pool was used during the entire study. An automated platelet count was performed after saline dilution before the freezing–thawing process to confirm a platelet number/ml in accordance with physiological values (in the range 150–450 $10^3/\mu l$).

In the present study PL was used diluted 40 times. After dilution, it was characterized by means of ELISA test, revealing a PDGF AB concentration of 472 pg/ml.

2.2. Methods

2.2.1. Chitosan depolymerization

Chitosan was depolymerized as described by Tian et al. [29] to obtain a molecular weight of about 100 KDa, previously found suitable to prepare chitosan micelles [27]. Briefly, chitosan was dissolved in bidistilled water at 2% (w/v) and equimolar HCl was added, followed by 0.5 M H_2O_2, to start the depolymerizing reaction. After 3 h, 0.5 M NaOH was added up to pH 7.0 to stop depolymerization and induce chitosan precipitation. Chitosan was separated by centrifugating at 815*g* for 30 min (ALC 4218 Centrifuge, ALC International, Cologno Monzese, Milan, Italy) and then resuspended by adding 0.5 N HCl, until complete dissolution. Chitosan HCl (CS-HCl) solution was dialyzed in bidistilled water for 24 h to remove the excess HCl and then freeze-dried (Heto Dryer, Analitica De Mori, Milan, Italy).

2.2.2. Preparation of polymeric micelles

Unloaded micelles were prepared by drop-to-drop addition of 1% w/v oleic acid in acetone, under magnetic stirring, to the chitosan solution previously prepared by dissolving chitosan hydrochloride (CS-HCl) 0.5 mg/ml in bidistilled filtered water (cellulose acetate, 0,22 μm, Whatman® Schleicer & Schuell, Whatman GmbH, Dassel, Germany). The amount of fatty acid added was calculated to obtain a final stoichiometric mole/mole ratio between the amino groups of deacetylated chitosan and the carboxylic groups of oleic acid of either 1:1 or 1:0.5. Acetone was finally evaporated under nitrogen flux at room temperature. Micelles were sonicated (Elmasonic S 80 H, Elma Hans Scmidbauer GmbH and Co, Singen, Germany) for 15 min to reduce particle aggregation [27]. To prepare loaded micelles, silver sulfadiazine (AgSD) was added at 2 mg/ml concentration to the organic phase containing oleic acid. After the addition of this phase to the chitosan solution, as described for the unloaded micelles, acetone was evaporated by nitrogen flux and the colloidal solution was then centrifuged at 3460*g* for 30 min (ALC 4206 Centrifugette, ALC International, Cologno Monzese, Milan, Italy), to induce the precipitation of the drug not loaded into micelles. The supernatant containing the micelles was collected. A full factorial experimental design was performed. As factors, chitosan concentration and CS:OA ratio were considered at two different levels: 0.5 mg/ml and 1.5 mg/ml for chitosan concentration, 1:0.5 and 1:1 for the molar ratio between chitosan and oleic acid. Hence, four types of micelles were obtained, as indicated in Table 1. The influence of the factors was evaluated on the concentration of loaded AgSD and on the particle size, as response variables. Statistical analysis of data was performed using a