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Halloysite and chitosan oligosaccharide nanocomposite for wound healing

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ABSTRACT

Halloysite is a natural nanotubular clay mineral (HNTs, Halloysite Nano Tubes) chemically identical to kaolinite and, due to its good biocompatibility, is an attractive nanomaterial for a vast range of biological applications.

Chitosan oligosaccharides are homo- or heterooligomers of N-acetylglucosamine and D-glucosamine, that accelerate wound healing by enhancing the functions of inflammatory and repairing cells.

The aim of the work was the development of a nanocomposite based on HNTs and chitosan oligosaccharides, to be used as pour powder to enhance healing in the treatment of chronic wounds.

A 1:0.05 wt ratio HTNs/chitosan oligosaccharide nanocomposite was obtained by simply stirring the HTNs powder in a 1% w/w aqueous chitosan oligosaccharide solution and was formed by spontaneous ionic interaction resulting in 98.6% w/w HTNs and 1.4% w/w chitosan oligosaccharide composition. Advanced electron microscopy techniques were considered to confirm the structure of the hybrid nanotubes.

Both HTNs and HTNs/chitosan oligosaccharide nanocomposite showed good in vitro biocompatibility with normal human dermal fibroblasts up to 300 μg/ml concentration and enhanced in vitro fibroblast motility, promoting both proliferation and migration. The HTNs/chitosan oligosaccharide nanocomposite and the two components separately were tested for healing capacity in a murine (rat) model. HTNs/chitosan oligosaccharide allowed better skin reepithelization and reorganization than HNTs or chitosan oligosaccharide separately. The results suggest to develop the nanocomposite as a medical device for wound healing.

Statement of Significance

The present work is focused on the development of halloysite and chitosan oligosaccharide nanocomposite for wound healing. It considers a therapeutic option for difficult to heal skin lesions and burns.
The significance of the research considers two fundamental aspects: the first one is related to the development of a self-assembled nanocomposite, formed by spontaneous ionic interaction, while the second one is related to the possibility to find an effective treatment for cutaneous non healing lesions.
The characterization of this hybrid system involves a multidisciplinary approach considering integrated techniques of solid state investigation and advanced electron microscopies, and in vitro/in vivo models to understand biocompatibility and proliferation properties (enhancement of in vitro fibroblast motility, proliferation and migration, and of in vivo burn healing), to understand safety and effectiveness of the developed nanocomposite.

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1. Introduction

Cutaneous wounds represent a major issue in medical care, with approximately 300 million chronic and 100 million traumatic wound patients worldwide. The incidence of chronic wounds is likely to dramatically increase as long as the population ages due to the rising prevalence of type 2 diabetes, peripheral vascular disease and metabolic syndrome. This will have a huge impact on financial burden of health-care systems worldwide [1]. Whereas the treatment strategies adopted for acute and limited area traumatic wounds are effective, the problems arise in the long-term care of patients with large area burns, infected and severe chronic wounds.

Skin serves as protective barriers against the outside world, therefore any break in this barrier must be mended rapidly. Wound healing process is based on various phases, typically hemostasis, inflammation, proliferation and remodelling, and depends on many factors, involving multiple cell populations and extracellular matrix (ECM) bioactive molecules as soluble mediators [2]. During inflammation, inflammatory cells (neutrophils, activated monocytes and macrophages), are recruited at the site of injury and release proinflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukins IL-1 and IL-6, and growth factors, such as transforming growth factor β (TGFβ) and insulin like growth factor (IGF) [3,4]. These bioactive molecules coordinate the transition from inflammatory to proliferating phase. The length and the severity of the inflammation phase ultimately dictates the quality of repaired tissue. During the proliferative phase, the granulation tissue is deposited and fibroblasts and endothelial cells migrated into the wound. Since fibroblasts produce collagen based extracellular matrix (ECM) reepithelialisation of the wound occurs and keratinocytes migrate into the wound from wound margins covering the neoformed tissue. After that the remodelling occurs and disorganized collagen III is substituted by collagen I bundles. The objective of wound managements is to heal the wound in the shortest time to prevent infection and to minimize pain, discomfort and scarring. Traditional wound management relied on simple materials such as gauze according to the principle "to cover and conceal" [5]. A more recent approach is to promote healing by using smart dressings able to enhance the formation of viable tissue in vivo [5].

Recently clay minerals have been proposed as substrates and matrices to enhance stem and progenitor cell proliferation and differentiation, thus representing an innovative platform for tissue regeneration and biomaterial design [6,7]. Moreover, cells could interact with clays suggesting new opportunities for functionalization of surfaces for enhanced reparative response. The combination of polymers/oligomers with clay minerals to obtain nanocomposites provides a further opportunity to develop new functional materials. Recently, biomedical applications of nanoclay-polymer composites have been rapidly increasing as antimicrobial coatings and bone healing implants [8].

Halloysite is a tubular aluminosilicate with hollow structure: it is formed by 10–15 aluminosilicate layers of 0.7 nm thick kaolin sheet rolled to form a hollow cylinder. It has a negatively charged outer surface and a positively charged inner lumen with an outer overall diameter of 40–70 nm with a length of 0.4 to 1.5 μm. Halloysite can adopt different forms including elongated tubes, short tubes, spheroidal squat cylinders and plates: the nanotubular and the spheroidal forms are the most common ones [9]. Halloysite nanotubes (HNTs) are a safe and biocompatible material [8], apparently able to stimulate processes related to cell growth and proliferation [10,11]: the shorter dimension of HNTs (2 μm in length) in comparison to asbestos fibers (several tens of microns) allows their removal by macrophages [12]. HNTs based nanocomposites have been mainly described in literature as materials with improved mechanical and thermal properties [12] and carriers of drugs including biomacromolecules such as proteins and DNA [12,13]. Moreover tube end capped systems have been developed to optimize the release rate of bioactive molecules [12,14]. In particular Dzamukova et al. have proposed an enzyme activated system for the intracellular drug delivery [15]. HNTs nanocomposites have been studied by using different microscopy techniques: SEM, TEM and AFM which also allow to visualize the rolling aluminosilicate sheets [14,16–18].

Chitosan oligosaccharides are hetero- or homo-oligomers of D- acetylglucosamine and D-glucosamine. They are able to accelerate wound healing by enhancing the functions of inflammatory and repairing cells having anti-inflammatory and immunostimulating activities [19]. Moreover chitosan oligosaccharides possess antioxidant activity involving free radical scavenging and the induction of antioxidative enzyme expression [20].

Given these premises, the aim of the work was the development of a nanocomposite based on HTNs nanotubes and chitosan oligosaccharide, to be used as pour powder to enhance healing and to prevent infections in the treatment of chronic wounds. The HTNs/chitosan oligosaccharide nanocomposite was tested for in vitro biocompatibility towards fibroblasts and in vitro wound healing properties to evidence fibroblast proliferation and migration. Moreover the HTNs/chitosan oligosaccharide wound healing efficacy was tested in a murine (rat) model.

2. Experimental part

2.1. Materials

The nanocomposites has been prepared by using:

- clay mineral: halloysite nano tubes (HTNs) premium grade (New Zealand China Clays Ltd. Auckland, NZ);
- chitosan oligosaccharide (average molar mass = 1000 Da, with 75.4% deacetylation degree) (Heppe Medical Chitosan GmbH, Halle, Germany).

2.2. Methods

2.2.1. HTNs/chitosan oligosaccharide nanocomposites preparation (NC)

HTNs/chitosan oligosaccharide nanocomposites were prepared by simple solid liquid interactions [21–23]. Known amounts of clay mineral powder (0.1–2 g) were dispersed in 10 ml chitosan oligosaccharide aqueous solution (1% w/w). The resulting dispersions were shaken at 150 rpm for 24 h in a water bath (Falc Instruments, I) at room temperature and the solid phases were subsequently recovered by centrifugation at 22,000 rpm for 30 min (Jouan K22i, Italia), frozen at −20 °C for 24 h and freeze-dried for 24 h (Heto 15, Analitica De Mori, I). Table 1 reports the HTNs/chitosan oligosaccharide ratio (R), and the theoretical composition of the prepared nanocomposites obtained with HTNs/chitosan oligosaccharide. Actual composition is also given (see Section 2.2.2.1)

2.2.2. Nanocomposite characterization

2.2.2.1. Composition. Chitosan oligosaccharide was quantified by means of a ninhydrin assay modified from Leane et al. [24] and Sandri et al. [23]. A calibration curve was prepared by using chitosan oligosaccharide aqueous solutions at the following concentrations: 0.75, 0.5, 0.25 and 0.1 w/w. Each sample was diluted 1:1 v/v with 2 ml of the ninhydrin reagent (ninhydrin 2% w/v, hydrindantin 6.8 mg/l in 3:1 v/v DMSO:lithium acetate buffer 4 M, pH 5.2; Sigma-Aldrich, I) under nitrogen blanket. Each sample was placed in a shaking bath at 100 °C for 8 min. The vials were

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