Materials Science and Engineering C 75 (2017) 408–417

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Natural polymeric microspheres for modulated drug delivery

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ARTICLE INFO

Article history:
Received 30 July 2016
Received in revised form 16 December 2016
Accepted 14 February 2017
Available online 16 February 2017

Keywords:
Gelatin
Chitosan
Microspheres
Drug delivery
Angiogenesis

ABSTRACT

Microspheres can be regarded as a suitable platform for the development of ad hoc drug delivery systems, since the targeted release of a therapeutic agent can effectively contribute to support and improve a pharmacological protocol. However, several crucial factors related to the selection of materials, drugs and fabrication techniques should be critically analyzed in order to enhance the expected performance. Dealing with highly compatible materials, e.g. naturally-derived polymers and "green" reagents, can be a valid approach. For this aim, gelatin, chitosan and blend microspheres were produced by emulsion technique simply using distilled water and olive oil. Necessarily, due to the intrinsic instability of the selected materials in aqueous environment, microspheres were cross-linked with genipin, an extremely low cytotoxic agent, at three different concentration (i.e., 0.1, 0.5, 1% w/v). Collected microspheres were then loaded with methylene blue (MB), as drug model. Morphological analysis revealed homogeneous microspheres characterized by an average diameter comprised in the range 42–54 μm. In vitro MB temporal delivery was assessed until complete release, which occurred in about 3 days for gelatin and 30 days for chitosan microspheres. Nanoindentation analysis was performed to evaluate how polymers and genipin influenced the mechanical properties of microspheres. Finally, the effect of released MB was investigated by means of chicken embryo chorioallantoic membrane assay, highlighting anti-angiogenic properties for gelatin differently from chitosan and blend microspheres.

1. Introduction

The development of a controlled and targeted drug delivery system represents one of the main challenges for the definition of an effective pharmacological therapy. To obtain the maximum efficacy, the selected agent should be delivered to the target tissue by optimizing both the dose and the release period, thus causing minimal side effects. Most pharmaceutical formulations are systemically administered and, therefore, not specifically directed to the target organ or tissue. This implies that several biological barriers have to be crossed, such as organs, cells and even intracellular compartments, often causing undesirable side-reactions, and concurring to partially inactivate the drug [1]. It is also well established that the conventional therapeutic approach is limited by, e.g., fluctuating drug levels and poor efficacy [2]. In addition, it is not possible to control drug concentration and bioavailability, thus limiting the expected therapeutic outcome. In this regard, the development of ad hoc drug delivery systems can support and improve alternative clinical treatments, providing an effective release over time. In fact, systems with reproducible and predictable release kinetics do not necessitate multiple administrations and assure a higher drug concentration at the target site. For this aim, microspheres are generally regarded as a valuable option, especially when made up of bioresorbable polymers. Both synthetic and natural polymers can be considered, though naturally-derived ones can offer an enhanced response owing to an intrinsic biological affinity. Here, gelatin and chitosan were selected as two representative polymers to be used for a microsphere-based therapeutic strategy. Gelatin, derived from collagen denaturation, is non-immunogenic, bioresorbable, non-cytotoxic, and available at relatively low cost [3]. In addition, it has been found to have cell-affinitive and enzyme-cleavable domains [4], so it can be broken down by cellular action through the secretion of specific matrix metalloproteinases [5]. Chitosan is a natural linear biopolyaminosaccharide and is obtained by alkaline deacetylation of chitin, which is the second most abundant polysaccharide after cellulose [6]. Its degradation leads to

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http://dx.doi.org/10.1016/j.msec.2017.02.051
0928-4931/

the release of amino sugars, which can be incorporated into glycosaminoglycan and glycoprotein metabolic pathways or excreted [7]. In vivo tests have proven that chitosan-based biomaterials do not have any remarkable inflammatory or allergic reaction following implantation, injection, topical application, or ingestion in the human body [8]. However, both polymers are not stable in aqueous environment and this limitation can impair their mid-, long-term efficacy, deeply affecting the releasing characteristics. In order to overcome this drawback, cross-linking represents a possible solution, which implies, however, a critical selection of the chemicals to be used in order to minimize toxic side-effects. In this study, genipin was considered as a cross-linking agent, because of its properties: (*i*) it is naturally derived from the fruits of *Gardenia jasminoides* Ellis, (*ii*) it is about 10,000 times less toxic than glutaraldehyde, and (*iii*) it elicits a moderate in vivo inflammatory response [9–11].

To assess the suitability of this proposal, methylene blue (MB), a hydrophilic tricyclic phenothiazine drug, was selected, which is the very first fully synthetic compound ever used for different clinical applications, including photodynamic therapy and antimicrobial treatment [12–16]. A specific role for MB can be also highlighted in angiogenesis, a key-process that should be strictly evaluated, being implicated, e.g., in both normal development, tumor growth and metastasis, inflammation and wound repair, and intra-abdominal adhesions after surgical procedures [17]. In this regard, the potential of MB has been already assessed, inducing a significant reduction on histopathologically determined adhesion markers and affecting angiogenesis through platelet-derived growth factor [18]. This means that MB could represent a suitable pharmaceutical option, especially if topically delivered, when a tailored strategy to control new blood vessels formation is needed.

Moreover, and related to the need to finely control the delivery of a specific therapeutic agent, the influence of (*i*) a selected material on drug release was here investigated, and (*ii*) a straightforward approach was proposed in order to modulate MB release by simply modifying gelatin/chitosan ratio in the blend formulation.

Morphological, thermal and chemical characteristics of gelatin, chitosan and blend microspheres were preliminarily evaluated. A mechanical characterization of the microspheres was also provided by applying nanoindentation. Finally, drug delivery and angiogenic assays were performed.

2. Materials and methods

2.1. Materials

Gelatin (type A, from porcine skin), chitosan (molecular weight: 50,000–190,000 Da) and MB ($C_{16}H_{18}ClN_3S \cdot 3H_2O$) were supplied by Sigma-Aldrich. Acetic acid was supplied by Carlo Erba Reagenti. Phosphate buffer saline (PBS) tablets were supplied by Gibco, Invitrogen Corporation. Genipin was supplied by Wako. Gelatin sponges (Gelfoam®) were supplied by Upjohn (Kalamazoo Mich, USA). All materials and reagents were used as received.

2.2. Microspheres preparation

Microspheres were prepared by water-in-oil emulsion. Gelatin powder was added to distilled water, heated at 80 °C and gently stirred until complete dissolution, to produce a 10% w/v polymeric solution. In order to cross-link gelatin, genipin at three different concentrations (0.1%, 0.5% or 1% w/v), was added to the solution and stirred at 750 rpm for 1 min at 80 °C. The obtained solution was then poured into a test tube and mixed with a vortex mixer for 30 s at 2500 rpm.

Using a syringe fitted with a 22 G needle, the resulting solution was injected into 200 ml of olive oil, which had been preheated at 80 °C. The resulting emulsion was stirred, at the same temperature, for 1 h at 800 rpm. Microspheres were collected onto a 22 μm mesh nylon filter in vacuum condition, and washed with acetone to remove oil residuals. Finally, the microspheres were air dried.

Chitosan microspheres were prepared using the same protocol as reported above, with the exception that the polymer was dissolved in aqueous acetic acid solution (1% v/v) to produce a 2% w/v polymeric solution.

Gelatin-chitosan microspheres were produced according to the same method of fabrication, but this time different volumes of the above prepared solutions were mixed to obtain two different gelatin/chitosan blend systems, labelled blend 5/1 and blend 5/3, respectively.

2.3. Morphological analysis

The morphological characterization of microspheres was performed by means of scanning electron microscopy (SEM; Leo Supra 35). Samples were sprinkled onto metal stubs using a double sided carbon tape and sputter coated with gold. The average microsphere diameter and size distribution were calculated from SEM micrographs by measuring about 100 microspheres randomly selected (ImageJ, NIH).

SEM investigation was also carried out to morphologically assess possible microsphere modifications during prolonged incubation period. For this aim, microspheres were soaked into PBS and after 10 and 30 days were recovered, washed with distilled water, dried and then observed according to the same procedure above reported.

2.4. Fourier transform infrared spectroscopy analysis

The chemical structure of microspheres was investigated by means of Fourier transform infrared spectroscopy (FTIR), by using a Perkin Elmer Spectrum 100. The samples were ground with KBr to prepare pellets by means of a hydraulic press. FTIR spectra were collected in the range 4000–400 cm^{-1} at a resolution of 4 cm^{-1}.

2.5. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was carried out to investigate the thermal properties of microspheres. The samples were placed in aluminium pans and heated at 10 °C/min by means of a differential scanning calorimeter equipped with a thermal analysis data system (Nestch DSC 200 PC). DSC measurements were performed, under nitrogen atmosphere, within a specific heating range, i.e., 30–250 °C for gelatin microspheres and 30–350 °C for chitosan and chitosan-gelatin microspheres, respectively. An empty aluminium pan was used as reference.

2.6. Mechanical characterization of microspheres through micro-compression testing

Nanoindentation was performed to estimate the mechanical properties of gelatin, chitosan, and blend 5/1 microspheres. In detail, the Nanoindenter XP (Agilent/MTS company), characterized by a theoretical force resolution of 50 nN and a theoretical displacement resolution lower than 0.01 nm, was adopted, and a specific protocol based on micro-compression tests was developed for the characterization of the mechanical behaviour of microspheres. For this aim, a flat end punch with a diameter of 500 μm was selected for indentation. Remembering that in nanoindentation, when the contact between specimen and indenter tip is detected, the displacement is measured as the load is applied, here the applied compression test was characterized by three steps: loading, hold and unloading.

During the loading and unloading phases the velocity of the indenter punch was set at a constant value of 10 nm s^{-1}. The hold phase consisted of a stabilization period along which the maximum value of the load was maintained for a period of 5 s. The maximum value of the load was obtained, for all microspheres, in correspondence of an indentation depth equal to the 5% of their initial diameter.