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Organic solvent-free preparation of keratin nanoparticles as doxorubicin carriers for antitumour activity

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ABSTRACT

Doxorubicin is one of the most effective chemotherapeutic agents for the treatment of several neoplastic conditions, such as leukemia, neuroblastoma, soft tissue and bone sarcomas, breast cancer, ovarian cancer and others. However, its clinical application is limited by cardiotoxicity, such as cardiomyopathy, that once developed carries a poor prognosis and is frequently fatal. The controlled release of doxorubicin by means of a smart carrier is a strategy to overcome the aforementioned drawback. Herein, doxorubicin/keratin nanoparticles were prepared by loading the drug through ionic gelation and aggregation methods, without using cross linkers, organic solvents neither surfactants. Both methodologies afford nanoparticles with yields up to 100 wt%, depending on the loading amount of doxorubicin. Although aggregation yield smaller nanoparticles (≈100 nm), ionic gelation allows a higher drug loading (up to 30 wt%,). More importantly, nanoparticles obtained through this procedure display a pH-responsive release of the drug: indeed Peppas-Salhin model suggests that, the doxorubicin release mechanism is predominantly controlled by diffusion at pH 7.4 and by protein swelling at pH 5.

Moreover, nanoparticles prepared by ionic gelation resulted in more efficient cell killing of MDA-MB-231 and MCF-7 breast cancer cells than those prepared by aggregation. Based on the herein presented preliminary results, ionic gelation emerges as a promising approach for the preparation of keratin-based doxorubicin nanocarriers for cancer therapy, that is worth to further investigate.

1. Introduction

Doxorubicin (DOX) is one of the most effective chemotherapeutic drug for the treatment of a wide spectrum of tumours [1]. However, the clinical application of DOX is limited by its cardiotoxicity expressed into severe cardio myopathy or even congestive heart failure [2]. An established approach to reduce these side-effects is the use of nanoparticles-based drug carriers [3]. To this end, several kinds of nanoparticles such as liposomes [4], polymeric micelles [5], polysaccharides [6], as well as protein aggregates [7] have been developed. The main advantage of using nanocarriers as delivering vehicles, lies on the lower exposure of healthy tissues to the cytotoxic drug, thanks to the enhanced permeability and retention (EPR) effect [8], which favour the preferential accumulation and retention of nanozized objects, e.g. nanoparticles, within the large fenestrated tumour tissues as respect to the normally fenestrated heathy ones. For instance, Doxil™ (Johnson and Johnson) is PEGylated liposomal formulation of DOX used in clinical practice, with reduced cardiotoxicity and good antitumour activity [9].

Among biopolymers, albumin, the most abundant plasma protein, has been recognized as an ideal drug delivery carrier. In recent years, several methods for preparing albumin-based drug nanocarriers have been reported, including conventional desolvation followed by crosslinking method [10]. In this method, ethanol is generally used to force the aggregation of albumin molecules into unstable nanoparticles, and glutaraldehyde is used to crosslink the protein and stabilize the system. However, glutaraldehyde is toxic and may cause severe inflammation and central nervous system depression upon repeated i.v. administration [11].

The high-pressure homogenization approach has been successfully proposed as an alternative procedure for nanoparticles preparation, which avoids toxicity problems due to crosslinkers and solvents. Several hydrophobic drugs such as paclitaxel (Abraxane™), docetaxel and rapamycin have been efficiently encapsulated into albumin nanoparticles using this method [12]. However, all these drugs are highly

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hydrophobic and require to be dissolved in ethanol and chloroform for performing the high-pressure homogenization, which in turn results to be an unsuitable procedure for the production of water soluble DOX hydrochloride (DOX·HCl) protein nanoparticles.

In this view, Yuan and co-workers developed a molecular switch method to encapsulate both hydrophobic and hydrophilic drugs into albumin nanoparticles. This procedure is based on the reduction of protein disulphide bonds with β-mercaptoethanol to slack the compact structure of albumin, followed by reversible oxidation of thiol groups into disulfide bonds by removal of β-mercaptoethanol; the molecular switch allows to obtain albumin nanoparticles with a DOX loading of about 4.3 wt%, [13].

In the present work, wool keratin is proposed as a promising and innovative biomaterial for the production of biodegradable and biocompatible nanoparticles. Keratin is a cystine-rich and fibrous protein, highly abundant in nature being the major component of wool, feathers, hairs, horns and nails; however, despite its excellent biocompatibility and low-toxicity, little work has been done on drug carriers based on keratin. In our previous work, chlorin e6-conjugated keratin nanoparticles, prepared by self-assembling and desolvation methods, have proven to be very effective in the photodynamic treatment of osteosarcoma and glioblastoma tumour cells [14]. In addition, Yuan et al. described a two-step method for the preparation of DOX-loaded keratin nanoparticles, which includes desolvation and crosslinking followed by the electrostatic absorption of DOX onto the NPs shell [15].

In the present study, we describe the preparation stable keratin nanoparticles loaded with doxorubicin (DOX-KNPs), obtained by two different straightforward and high yielding procedures, e.g. ionic gelation and aggregation, which do not require the use of toxic substances, such as crosslinking agents and/or surfactants. DOX-KNPs characterization, including particles size, ζ-potential and morphology, is thoroughly described along with an in-depth infrared-spectroscopy study of the keratin/doxorubicin chemical interaction, as well as the protein folding induced by the two different methods.

Moreover, doxorubicin release profiles under different pH conditions have been evaluated, in order to assess nanoparticles behaviour in both physiological (pH 7.4) and slight acidic tumour microenvironment (pH 4.5); mathematical models were applied to the experimental data, with the aim of understanding the release mechanism of DOX.

Finally, we report preliminary in vitro analyses that compare the ability of DOX delivered by keratin nanoparticles, obtained by either gelation or aggregation, to enter MCF-7 and MDA-MB-231 breast cancer cells and to inhibit cell proliferation, as compared to free DOX.

2. Materials and methods

2.1. Materials

Australian Merino wool (21 μm) was kindly supplied by Cariaggi Fine Yarns S.p.A., doxorubicin hydrochloride sodium salts was provided by TCI Europe N.V. All other chemicals were purchased from Sigma-Aldrich. The human breast adenocarcinoma estrogen receptor-positive, non-metastatic cell line MCF-7 and the estrogen receptor-negative, metastatic MDA-MB-231 cell line were obtained from ATCC (Rockville, MD, USA) and maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Milan, Italy) at 37 °C, in an atmosphere of 5% CO_2.

Australian Merino wool (21 μm fineness) was kindly supplied by Cariaggi Fine Yarns, S.p.A.

2.2. Preparation of keratin nanoparticles loaded with doxorubicin by ionic gelation

Keratin powder (extracted from wool as described in the supporting information, section I) and doxorubicin hydrocloride (DOX) were separately dissolved in deionised water mQ at a final concentration of 1 mg/mL and 2 mg/mL, respectively. Then, the keratin aqueous solution was filtered using a 450 nm filter cut-off and maintained under stirring (730 rpm) during the dropwise addition of different amounts of doxorubicin solution. The amount of added doxorubicin with respect to keratin ranged from 5 to 45 wt%,. The mixture was maintained under stirring for 60 min and stored at 4 °C overnight. Afterward, the mixture was dialyzed with a cellulose membrane (cut off 12–14 kDa) against deionised water for 24 h in order to remove free doxorubicin and then centrifuged at 4000 rpm for 10 min in order to remove particle aggregate. Finally, a known volume of the supernatant was freeze-dried, and the resulting powder was weighted in order to determine the yield of keratin nanoparticles (DOX-KNPs_IG), by using the following equation:

$$\text{Yiels}\ (\%) = \frac{\text{weight of liophilized nanoparticles (mg)}}{\text{weight of starting keratin and added drug (mg)}} \times 100 \quad (1)$$

2.3. Preparation of doxorubicin loaded KNPs by aggregation

Keratin was dissolved in water carbonate buffer (pH 9) at a concentration of 4 mg/mL and filtered using a 450 nm filter cut-off. Different amounts of doxorubicin dissolved in water (1 mg/mL) were added dropwise to the keratin solution, under stirring at 730 rpm, in order to induce aggregation of keratin into nanoparticles (DOX-KNPs_Agg). The amount of added doxorubicin, with respect to keratin, ranged from 10 to 40 wt%,.

2.4. Characterization

The hydrodynamic diameter of the nanoparticles in aqueous solution (0.1 mg/mL), as well as their ζ-potential, were determined by dynamic light scattering (DSL), at 25 °C and using a NanoBrook Omni Particle Size Analyser (Brookhaven Instruments Corporation, USA), equipped with a 35 mW red diode laser (nominal 640 nm wavelength).

Doxorubicin loading onto keratin nanoparticles was evaluated by UV–Visible adsorption acquisition of nanoparticles suspension (0.1 mg/mL) using a UV–Vis spectrophotometer Cary 100 (Agilent Technologies). For the quantification, a calibration curve of doxorubicin dissolved in keratin solutions, in the 0–30 μg/mL concentration range, was obtained. The drug loading content (LC) and the encapsulation efficiency (EE) were calculated according to the following equations:

$$\text{LC}\ (\%) = \frac{\text{drug amount (mg)}}{\text{weight of nanoparticles (mg)}} \times 100 \quad (2)$$

$$\text{EE}\ (\%) = \frac{\text{drug amount in the nanoparticles (mg)}}{\text{initially added drug (mg)}} \times 100 \quad (3)$$

Scanning electron microscopy (SEM) images of dried nanoparticles were obtained with a Zeiss EVO LS LaB6 scanning electron microscope, using an acceleration voltage of 5 kV and a working distance of 5 mm. Samples for SEM analysis were prepared by spin coating of nanoparticles solution (0.1 mg/mL) onto SEM specimen mount at room temperature, followed by gold spattering. The FT-IR spectra were acquired using a Perkin-Elmer Spectrum BX FT-IR system. Before the acquisition, samples were mixed with KBr and pressed into pellets. 100 scans were taken in the 4000–500 cm^{-1} range and 8.0 gain. After the acquisition, spectra were baseline-corrected and smoothed with 5 points Savitzky-Golay function. For the protein secondary structure, the amide I and amide II bands were resolved in Gaussian shape bands related to different protein secondary structures. The second derivative was used as mathematical methods to resolve the amides into the individual bands corresponding to specific secondary structures. The curve-fitting was carried out with ORIGIN 8.1® software (OriginLab Corporation, MA, USA).