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Apoferritin nanocage as streptomycin drug reservoir: Technological optimization of a new drug delivery system

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ABSTRACT

The aim of this study is to formulate and characterize streptomycin-loaded apoferritin nanoparticles (**ApoStrep NPs**) for their potential therapeutic use in bacterial resistant infections (i.e. tuberculosis). **ApoStrep NPs** were prepared by disassembly/reassembly process via pH method and changing apoferritin/drug molar ratio, purified by dialyses process also associated with gel filtration chromatography and characterized in their chemico-physical and technological parameters as yield, size distribution, polidispersivity, morphology, internal structure, zeta potential and loading efficacy. The results showed that spherical reproducible NPs could be obtained by using apoferritin/drug molar ratio lower than 1:25 and purification based on the combination of dialysis and gel filtration chromatography. Photon correlation spectroscopy, Uv–visible detection and electron microscopy showed the maintenance of the native apoferritin chemico-physical properties and structure. When formulated with apoferritin/drug 1:10 and 1:25 molar ratio, **ApoStrep NPs** showed remarkable encapsulation efficacy (35% and 28%, respectively) along with kinetic profile of drug delivery, approximately 15% at 37 °C in 72 h, as evidenced by “in vitro” release experiments.

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1. Introduction

Streptomycin is a broad spectrum aminoglycoside antibiotic typically used for the treatment of active tuberculosis, in combination with other antituberculosis agents. It is composed of a metabolic product of Streptomyces globisporus or another similar organism (Zumla et al., 2013). The major disadvantages of streptomycin consists of the inadequate penetration into the cells (due to its hydrophilicity) and rapid elimination from the blood stream (due to both efficient renal filtration and low level of association to plasma proteins) (Coessens et al., 1996). Moreover, different evidences describe a rapid bacterial resistance oocurring after Streptomycin treatment (75% of bacterial infectious including tubercoloses after one-four months of treatment). In most cases, this resistance has proven to be permanent. The importance of this problem is obvious, since further treatment with streptomycin were shown to be ineffective when the infecting organisms are drug-resistant (Pfuetze and Ashe, 1948). Above the development of novel antitubercolosis drugs, the creation of nanocarriers able to modulate drug delivery could be interestingly applied on drug-resistant epidemic. In fact, loading currently-used antitubercolosis drugs in nanocarriers-based formulations may shorten drug regimen duration, reduce frequency, and deliver medications more efficaciously. That way, a sustained release in both blood plasma as well as organ tissue, an increase in bioavailability, the reduction of patient default and the improvement of completion rates could be achieved. Taken together, all these advantages could lead to significant potential in the reduction of drug resistant tubercolosis cases (Smith, 2011; Asadi, 2014).

An exciting improvement in drug delivery systems is represented by the possibility to efficiently load drugs into apoferritin nanocage. Apoferritin is the demineralized ferritin, composed of 24 polypeptide subunits packed together to form a nanosphere of diameter ~12 nm with an internal cavity ~8 nm. This protein, as drug delivery system, was demonstrated to be a promising vehicle for targeted delivery of anticancer drugs (Arosio et al., 1991; Belletti et al., 2016). Generally, apoferritin may enhance drug selectivity for cell surfaces that express ferritin receptors, in particular ferritin-binding sites and endocytotic pathways, which are expressed in all body cells and mainly in cells in rapid proliferation (Burdo and Connor, 2003; Aisen 1991). Besides these aspects, apoferritin displays a number of advantages which could be exploited in the application toward

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drug-resistant tubercolosis epidemic. Ferritin is a major iron storage protein in humans; therefore its bio-compatibility and biosafety are assured. Moreover, compared with other drug carriers (i.e. polymeric nanoparticles and liposomes), apoferritin is much smaller in size, thus leading to a longer circulation half-life. Despite the rigidness under physiological conditions, the ferritin nanocages can be broken down into subunits in acidic environment by a reversible process. In fact, when the pH is tuned back to neutral, the subunits are reconstituted into nanocage structures, almost in an intact manner (Maham et al., 2009; Lin et al., 2011a). Another advantage of apoferritin consists of its chemical "reactivity" since, as polymeric nanoparticles or liposomes, its surface can be easily modified to selectively direct to target site (Lin et al., 2011b; Uchida et al., 2006; Dominguez-Vera 2004.

Regarding the drug to be loaded into apoferritin, streptomycin (and particularly sulfate salt used in this research) possesses chemico-physical properties favorable for its location into the apoferritin nanocage, as its stability both in acids and in bases which is needed for formulative protocols requiring *extreme* pH values. Streptomycin is featured by a pKa of about 8 due to its secondary amino group and pKa ranging between 10 and 12 due to its two guanidine groups (Nys et al., 1971), providing a net positive charge both in physiological enviroment (pH 7) and during the formulation conditions (acidic pH), which favors electrostatic interaction with the apoferritin. Moreover, the MW (723 Da) is suitable for a stable internalization into protein cage avoiding drug loss across protein pores.

In this paper, starting from preformulative evaluation on several parameters involved in disassembly/reassembly protocols (protein concentration, volume of reaction, pH during the process, ionic concentration in solution, temperature, stirring), we aimed to optimize the technological conditions needed for stable formulation of streptomicyn into apoferritin nanoparticles **(ApoStrep NPs)**. Particularly, we focused on the impact of the molar ratio between apoferritin and drug (1:10, 1:25, 1:50) and the purification process (by comparing dialyses and gel filtration chromatography) on drug loading, without changing the native structure of **ApoStrep NPs**. Then, we tried to consolidate the possibility of using apoferritin as drug delivery systems, not only for anticancer drugs (Belletti et al., 2016), but also for other kinds of drugs and active molecules.

2. Materials and methods

2.1. Chemicals

Apoferritin from equine spleen (solution ~40 mg/mL in 0.15 M sodium chloride) and streptomycin sulfate (MW = 728.69, water solubility = 50 mg/mL) were purchased from Sigma Aldrich (Milan, Italy). Acetate cellulose dialysis membranes were purchased from CelluSepT1 (MWCO:3500, Membrane Filtration Products, Seguin, TX, USA). Sepharose CL–2 B resin was purchased from Sigma Aldrich (Milan, Italy). Micro BCA protein assay kit composed of reagent A (alkaline tartrate-carbonate buffer), reagent B (bicinchonic acid solution) and reagent C (copper sulfate solution) was purcheased from Thermo Fisher Scientific Inc (Milan, Italy). All the solvents were of analytical grade; all other chemicals were commercially obtained and used without further purification. A MilliQ water system (Millipore, Bedford, MA, USA), supplied with distilled water, provided high-purity water (18 MΩ).

2.2. The encapsulation of streptomicine in apoferritin via pH changing method

Streptomicyin was formulated in apoferritin NPs by disassembly/reassembly protocols accordingly with others adopted for different types of drugs (Xing et al., 2009a; Cutrin et al., 2013; Dominguez-Vera 2004; Blazkova et al., 2013; Liu et al., 2006; Kilic et al., 2012). For all preparations, apoferritin solution (15 mg/mL, 0.031 μmoles) was obtained by diluting apoferritin stock solution with water (370 μL diluted to 1 mL). After gently pipetting, apoferritin solution, monitored in term of size and surface charge (by means of PCS using a Zetasizer Nano ZS, Malvern UK as explained in paragraph 4a) and morphology (by means of trasmission electron microscopy (TEM) analysis as explained in paragraph 4b), was acidified to pH 2 (by addition of 800 μL of 0.1 N HCl), thus providing the disassociation of the apoferritin subunits. The sample was mixed by using magnetic stirring for 20 min at 20 °C. Then, different amounts of streptomycin sulfate (0.23, 0.57, 1.14 mg corresponding to 0.31, 0.78 and 1.56 μmoli, respectivelly) solubilized in water (200 μL) and acidified to the pH 2 (addition of HCl 0.1 N) was added dropwise to the protein solution to finally have 1:10, 1:25, 1:50 apoferritin/drug molar ratios. As control sample, un-loaded apoferritin NPs were obtained following the same protocol previously exposed. Briefly, 200 μL of water, previously acidified to pH 2 (by addition of 0.1N HCl), was added to apoferritin solution (pH 2), simulating the same increase in volume of the case of addition of drug solution.

In order to recover the native structure of the protein, the pH value of the solution was adjusted to pH 8 by adding NaOH 0.1 N (about 700 uL, under magnetic stirring). The final volume was adjusted to 3 mL by adding water (basified to pH 8). The resulting solution was then stirred over 2 h at 25 °C to allow the complete structural reassembly of the protein.

The following formulations were obtained: **CNTR** (unloaded), **ApoStrep 1:10**, **ApoStrep 1:25** and **ApoStrep 1:50** (streptomicyn loaded NPs).

2.3. Purification of samples

2.3.1. Dialysis procedure

A membrane diffusion method was used to purify free streptomycin from streptomycin loaded NPs. Each sample (1 mL of suspension) was placed in the dialysis tube (MWCO 3500 Da), permeable to drug alone, but not to NPs. Tube was closed at both ends using closure devices (Spectrum Medical Industries, Houston, TX, USA), and placed in a solution at pH 8.0 (50 mL, 0.05 M KH_2PO_4), gently shaken with a magnetic stirrer (100 rpm at 20 ± 1 °C for 4 h). Free drug diffused out of samples and through the membrane to the receiver phase. After this time, volume of receiving phase was then adjusted to 1 mL by using rotatory evaporation, filtered by an acetate cellulose (AC) membrane with 0.2 μm porosity (Sartorious, Göttingen, Germany).

Samples purified by dialysis were recovered and named: **CNTR-D, ApoStrep 1:10-D, ApoStrep 1:25-D and ApoStrep 1:50-D**.

2.3.2. Gel filtration chromatography

The apoferritin samples, recovered after dialysis purification and adjusted to 1 mL as final volume, was eluted through the Sepharose CL–2 B resin (Sigma Aldrich), packed in 17.5 × 1.5 cm column, by using 0.05 M KH_2PO_4 solution (pH 8.0) as mobile phase. The separation efficiency was achieved by working in "flash mode", exerting a pressure of about 9 bar by means of an N_2 flow. 40 fractions were collected, one of each minute starting from the 3rd min after deposition. Fractions were analyzed by UV spectrophotometry (Jasco V-530 UV–vis Spectrophotometer, JASCO Europe, Cremella, Italy) by means of spectral scanning acquisition mode over the wavelength range 200–400 nm, with 5 min measurement intervals. The collected fractions containing apoferritin (identified by typical peak at 280 nm-UV absorbance) were submitted to test by PCS, which highlighted the elution of apoferritin NPs within the interval 8–15 min (data not shown). These fractions were mixed and the volume adjusted to 3 mL (by rotary evaporation). Purified

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