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Nanoparticles based on quaternary ammonium–chitosan conjugate: A vehicle for oral administration of antioxidants contained in red grapes

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ABSTRACT

The purpose of this study was to demonstrate that nanoparticles (NP), obtained by ionotropic cross-linking of reduced-MW chitosan (rCh) derivatives with reduced-MW hyaluronan (rHA), could be profitable vehicles for oral administration of beneficial doses of antioxidant constituents of red grape seeds extracts (GSE). NP have previously shown aptitude for internalisation by endothelial progenitor cells. Here they were seen by a fluorescence microscope migrating across excised rat intestine from mucosal to serosal side and permeating from the donor to the acceptor compartment of an Ussing chamber while carrying significant (15–20%) steadily associated fractions of initial antioxidant load. Freshly prepared NP dispersions were stabilised by lyophilisation. The NP dispersions were regenerated from the lyophilised products by adding water under gentle stirring. Lyophilisation-regeneration caused a significant NP size increase yet particles did not grow beyond the upper size limit of particles able to cross the intestinal barrier (500 nm).

1. Introduction

It has been reported that the incidence of coronary artery disease is comparatively low with populations that consume large amounts of red wine [1] thanks to the antioxidant properties of polyphenols, contained in red wine, and increased nitric oxide bioavailability [2,3]. These factors exert their beneficial effects on endothelial progenitor cells (EPC), i.e., bone marrow-derived cells that are mobilized to the peripheral circulation when vascular repair and neovascularization are required [4]. In fact, a decreased EPC number is found in patients with coronary artery disease and cerebrovascular disease [5–7]. Unfortunately the amount of red wine needed to provide a level of phenolic compounds in the organism adequate to produce a significant antioxidant effect is too high to allow one to disregard the harmful consequences of alcohol [8], such as increased incidence of cardiomyopathies, hypertension and ictus. In a recent study it was shown that the antioxidant products extracted from Italian red grape skin and seeds (essentially polyphenols) can exert the same beneficial effects on EPC as red wine, in the absence of alcohol [9]. Aiming at designing an adequate pharmaceutical system for the administration of red grape seed extract (GSE), nanoparticle dispersions were prepared by ionotropic gelation of thiolated quaternary ammonium–chitosan conjugates with hyaluronic acid [10]. To control the particle size to the nano-scale the MWs of polymers were reduced by controlled de-polymerization. GSE could be entrapped into the nanoparticle system up to around 90%. Nanoparticles were prepared from FITC-labelled chitosan derivative, then their uptake into EPC was observed under a fluorescence microscope. Internalisation of GSE-loaded nanoparticles by EPC resulted in cell protection from oxidative stress [10]. For storage the nanoparticle dispersion was lyophilised. Since the dispersion could be regenerated from the lyophilised product by mild agitation in water, it was felt that the lyophilised GSE-loaded nanoparticles could result in a convenient pharmaceutical system for oral administration of natural antioxidants [10]. In fact, free, non-

List of abbreviations: NP, nanoparticles; EE, encapsulation efficiency; EPC, endothelial progenitor cells; FITC, fluorescein isothiocyanate; GSE, extract of red grape seeds; NAC, N-acetylcysteine; PB, phosphate buffer pH 7.4; QA-rCh60, quaternary ammonium–chitosan conjugate synthesised at 60 °C from a reduced-MW chitosan; QA-rCh60-SH, thiolated QA-rCh60; rCh, reduced-MW chitosan; rHA, reduced-MW hyaluronan.

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encapsulated GSE is barely bioavailable due to poor absorption and rapid oxidation in physiological fluids. Nevertheless, for being accepted as an effective vehicle for GSE oral administration, the nanoparticle dispersion should meet the following requirements: (1) the particles should be able to penetrate across the intestinal epithelium into the bloodstream still preserving their supramolecular aggregation state and size; (2) the particles should retain a significant load of antioxidant material for at least the time needed for their intestinal absorption and uptake by the target EPC. Demonstrating these properties of GSE-loaded nanoparticulate systems, obtained by ionotropic crosslinking of either quaternary ammonium–chitosan conjugate or its thiolated derivative with hyaluronan, has been the purpose and, at the same time, the major novelty of the present work. Point (1) was addressed by bringing the FITC-labelled nanoparticle dispersion into contact with the mucosal side of excised rat intestine in an Ussing type chamber for a measured time, after which the fluorescence of the acceptor medium was measured, and gut slices were cut and observed under a fluorescence microscope. Concerning point (2), GSE release from the nanoparticles was studied by lyophilising the nanoparticle dispersion immediately after preparation, regenerating the dispersion from the lyophilised product in stirred water at time zero, withdrawing aliquots of dispersion at measured intervals, centrifuging them, and analysing each supernatant for its GSE content by the Folin-Ciocalteu reagent.

2. Materials and methods

2.1. Materials

The GSE used in this study was taken from the batch obtained previously [10] from Sangiovese pre-veraison red grape seeds, from Montecucco DOC area (Tuscany, Italy), following the extraction procedure described by Di Stefano and Cravero [11]. Reduced–MW chitosan (rCh, viscometric MW 32 kDa) and hyaluronic acid (rHA, viscometric MW 470 kDa) were prepared by Zambito et al. [12]. The quaternary ammonium–chitosan conjugate was synthesised at 60 °C from rCh (QA-rCh60) and thiolated (QA-rCh60-SH) by Zambito et al. [12]. Both QA-rCh60 and QA-rCh60-SH were FITC-labelled according to a previously described procedure [13–15]. All aqueous solutions/dispersions were prepared with freshly distilled water.

2.2. Identification of GSE constituents

The GSE constituents were separated and identified by HPLC–PDA–ESI-MS/MS analysis using Surveyor LC pump, a Surveyor autosampler, coupled with a Surveyor PDA detector, and a LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with Xcalibur 3.1 software. Analyses were performed using a Synergi 4 μ Fusion-RP 80 A, 150 × 4.60 mm column (Phenomenex, Bologna, IT). The eluent was a mixture of 0.1% acetonitrile solution of CH_3COOH (solvent A) and a 0.1% aqueous solution of CH_3COOH (solvent B). The solvent gradient was as follows: 0–30 min, 5–30% A, 30–31 min, 30–70% A, 31–36 min, 70–100% A. Elution was performed at a flow rate of 0.8 mL/min with a splitting system of 2:8 to MS detector (160 μL/min) and PDA detector (640 μL/min), respectively. The volume of the injected acetonitrile GSE solution (1 mg/mL) was 10 μL. Analyses were carried out with an ESI interface in the negative mode. The ionization conditions were optimised, and the parameters were as follows: capillary temperature, 320 °C; capillary voltage, −10.00 V; tube lens offset, −35.00 V; sheath gas flow rate, 40.00 arbitrary units; auxiliary gas flow rate, 15.00 arbitrary units; spray voltage, 4.50 kV; scan range of *m/z* 150.00–2000. PDA data were recorded over the 210–600 nm range with preferential channel 280 nm as the detection wavelength.

2.3. Quantitative determination of whole antioxidant competence of GSE

The antioxidant competence of GSE constituents as a whole was correlated with concentration by reacting standards, of known GSE concentrations in phosphate buffer pH 7.4 (PB) 0.13 M (isosmotic), with the Folin-Ciocalteu reagent (Sigma), and measuring the resulting absorbance at 750 nm, as previously described [9,16]. Samples and standards were stabilised for at least 5 h by adding 0.05 mg/ml N-acetylcysteine (NAC). A linear absorbance–concentration correlation was found ($r^2 = 0.996$; $n = 4$) which allowed determination of unknown GSE sample concentration by the following equation:

$$\text{Concentration} = (\text{Absorbance} - 0.022)/0.0978$$

2.4. Preparation of nanoparticles

To prepare GSE-loaded nanoparticles (NP) based on QA-rCh60, QA-rCh60-SH, or these polymers labelled with FITC, a 400 μl volume of PB, 0.13 M (isosmotic) containing 0.022 mg/ml rHA and 3.1 mg/ml GSE was added portionwise (100 μl) to 5 ml of 2 mg/ml rCh derivative solution in PB of different strength (0.13 M (isosmotic), 0.026 M, 0.013 M or 0.007 M), under stirring. The resulting particles were analysed for size by light scattering (Nano Z690 Malvern). Blank NP were prepared by the same procedure, except that the rHA solution contained no GSE. Following preparation, each GSE loaded and blank NP dispersion was centrifuged (19,500 rpm, 1 h) and the supernatant analysed by the Folin–Ciocalteu reagent for total content of antioxidant material, as described in Section 2.3. To obtain the GSE amount in the supernatant (GSEsup) the absorbance for the blank NP was subtracted from that for the GSE loaded NP. The NP entrapment efficiency (EE) was calculated by the following equation:

$$EE = (GSEtot - GSEsup)/GSEtot$$

where GSEtot represents the total GSE mass used for the NP preparation.

To obtain stable systems the GSE-loaded NP dispersions were lyophilised (VirTis adVantage, thermal treatment step: −35 °C for 180 min; drying cycle steps: −30 °C for 360 min; −10 °C for 360 min; +10 °C for 240 min; +25 °C for 180 min). The NP dispersions could be regenerated from the respective lyophilised products by adding water under gentle stirring. In each case a water volume was added apt to generate an isosmotic dispersion, namely, 5, 1, 0.5, 0.3 ml water was added to lyophilised products obtained from NP dispersions in 0.13, 0.026, 0.013, 0.007 M PB, respectively. By this procedure isosmotic pH 7.4 dispersions of different NP concentrations could be obtained.

2.5. Study of lyophilised GSE-loaded NP ability to retain antioxidant material after regeneration

Three lyophilised QA-rCh60-or QA-rCh60-SH-based GSE-loaded NP preparations in PB 0.026 M were pooled and regenerated with 3 ml water containing 0.05 mg/ml NAC for GSE stabilisation, as described in Section 2.3. The regenerated dispersions were stirred at 37 °C. At times 0, 1 and 4 h 1 ml of each regenerated NP dispersion was withdrawn, centrifuged (19,500 rpm, 1 h), and the supernatant analysed for GSEsup as described in