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Silk fibroin nanoparticles: Efficient vehicles for the natural antioxidant quercetin

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ABSTRACT

This article describes how silk fibroin nanoparticles (SFNs) are capable of adsorbing and releasing quercetin (Q) and how its integrity is highly preserved, as confirmed by antioxidant activity assays. Q loading onto SFNs was optimized in terms of the Q/SFN ratio (w/w), time of adsorption and solvent mixture. Quercetin-loaded silk fibroin nanoparticles (QSFNs) were characterized using the dynamic light scattering technique to measure the diameter (Z-Average) and Z-potential (ζ). Loaded particles were slightly bigger than the SFNs, while their ζ was less negative. The antioxidant activity against DPPH• showed that the Q loaded in QSFNs not only retains the antioxidant activity but also has a synergistic scavenging activity due the intrinsic antioxidant activity of the SF. The drug loading content (DLC) and the encapsulation efficiency (EE) varied with the relation between Q and SFN in the loading solution. The sustained release of Q occurred throughout the experiment both in phosphate buffer saline (pH 7.4) and simulated intestinal fluid (pH 6.8). The results point to SFNs as promising candidates for Q loading, transport and gastrointestinal delivery with potential applications in nanomedicine, while retaining their nano-size and their antioxidant properties.

1. Introduction

In the last few decades several epidemiological reviews have associated a flavonoid-rich diet with an increase in average life in the Mediterranean area, a reduction in the frequency of cardiovascular diseases (Formica and Regelson, 1995; Erlund, 2004), and protection against agents that cause DNA damage through a reduction in oxidative stress, modulation of the enzymes responsible for the bioactivation of genotoxic agents and detoxification of their reactive metabolites (Luca et al., 2016). Flavonoids can be categorized as anthocyanidins, flavanols, flavanones, flavones, flavonols and isoflavones, all of which are widespread in the vegetable kingdom. Quercetin (Q) (IUPAC name: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) is the most common flavonoid and the most frequently studied, due its specific biological effects on the cardiovascular system: it reduces inflammatory responses (modifying the biosynthesis of eicosanoids), decreases the oxidation of low-density lipoproteins (preventing the formation of atherosclerotic plaque), obstructs the aggregation of platelets (avoiding the formation of thrombi in the bloodstream), and relaxes smooth muscles (reducing hypertension and heart arrhythmias). Apart from that, flavonoids also have antiviral and antitumoral properties (Formica and Regelson, 1995). In addition to the above mentioned specific effects, the flavonoids can modulate the activity of important enzymes such as calmodulin, ornithine carboxylase or protein kinases, that can be considered targets for developing drug delivery programmes (Gonçalves et al., 2015).

Flavonoids contains several hydroxyl groups with free radical scavenging activity through an electron donating process (Murota and Terao, 2003). Their catechol structure (*o*-dihydroxyl moiety), with two adjacent hydroxyl groups, merits special mention due their enhanced electron donating ability. The Q, as other flavonoids containing the catechol structure, presents important antiradical activity (Rice-Evans et al., 1996). Quercetin is a common

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component of raw fruits and vegetables, like red onions, strawberries, grapes, as well as juices or beverages, such as red wines and teas (Souza et al., 2013).

It has been estimated that the total amount of flavonoids we obtain from the diet is several hundred milligrams each day (Ross and Kasum, 2002). However, it is well known that dietary flavonoids have low solubility in digestive fluids, which results in poor intestinal absorption. Indeed, these compounds are transformed chemically by intestinal microorganisms. In the case of Q, its low water solubility (Srinivas et al., 2010), combined with its gastrointestinal degradation, limits its biological effects *in vivo* (Souza et al., 2013). Thus, the consumption of supplements or Q-enriched foods may not be sufficient to achieve the plasma concentration of Q required for it to be pharmacologically active due to the poor bioavailability of this flavonoid (Russo et al., 2012). There are two major approaches to increase Q bioavailability – chemical modification or its inclusion in suitable quercetin delivery systems (Barras et al., 2009).

It is well known that Q interacts with different food components, primarily with biomacromolecules (Bordenave et al., 2014). It has been found that an increase in the pH or temperature of the aqueous medium accelerates the degradation of Q (Wang and Zhao, 2016). In contrast, proteins protect against the degradation of flavonoids. Wang and co-workers observed that hydrophobic interactions with proteins were responsible for stabilization of flavonoids (Wang and Zhao, 2016).

A bioactive compound encapsulated in a biopolymer can be efficiently protected from harmful environmental agents like light, oxygen or water (Desai and Park, 2005). Thus, encapsulation is one of the strategies used to increase the stability and shelf life of food ingredients. Encapsulation not only permits the phenolic compounds from raw vegetables or fruits to be stored, but also to be recovered under specific conditions (Bakowska-Barczak and Kolodziejczyk, 2011; Saénz et al., 2009; Ferreira et al., 2007; Laine et al., 2008; Lozano-Pérez et al., 2014).

Mukhopadhyay and Prajapati recently have reviewed over the last few decades the most efficient biopolymeric carrier systems used to improve the bioavailability of quercetin (Mukhopadhyay and Prajapati, 2015). In recent years, multiple formulations with different encapsulation methods and carriers for Q have been described. For example, Q was efficiently encapsulated into poly-D, L-lactic acid (PLA) nanoparticles by means of a solvent evaporation technique (Kumari et al., 2010), through a co-precipitation with Eudragit® E and PVA (Wu et al., 2008), or encapsulation using a supercritical anti-solvent method employing Pluronic F127 as carrier (Fraile et al., 2014).

Other approaches used to improve the stability and bioavailability of flavonoids were based on the use of cyclodextrins (Calabrò et al., 2004; Lucas-Abellán et al., 2008), lipid nanocapsules (Russo et al., 2012), liposomes (Mignet et al., 2013; Priprem et al., 2008) or inorganic materials, such as silica microspheres (Young Ho et al., 2015).

In addition, nano-delivery systems have shown to be more effective than conventional formulations, because their size leads to more effective targeting, higher bioavailability at damaged tissues and a reduced systemic negative effects. For example, nano-delivery systems present similar or better therapeutic efficacy compared to conventional formulations, even at low drug concentrations, in the treatment of the inflammatory bowel disease (IBD) (Hua et al., 2015).

As is widely known, silk fibroin (SF) is a fibrous protein obtained from *Bombyx mori* cocoons. It presents excellent biomechanical properties, including non-toxicity, slow biodegradation and exceptional biocompatibility, making it an outstanding biomaterial for use in a wide range of therapies (Omenetto and Kaplan, 2010). Their extensive hydrogen bonding, their hydrophobic nature and high degree of crystallinity contribute to the stability of silk biomaterials (Altman et al., 2003). Formulated as particles, SF is used in medicine for its capacity to act as a reversible carrier of bioactive molecules (Hofmann et al., 2006; Liu et al., 2015; Mottaghitalab et al., 2015; Zhao et al., 2015).

The side chains of the amino acidic residues of SF can interact with small molecules, such as antioxidants, antibiotics, pigments, and chemotherapeutics. As a results of this interaction the stability of the molecules increases (Pritchard et al., 2012). For example, pigment like chlorophyll a, β-carotene, and astaxanthin adsorbed onto silk fibroin are more stable to visible and UV light than fibroin-free pigments, which are rapidly decolorized by UV light irradiation (Ishii et al., 1995). The components from crude olive leaf extract were immobilized on silk fibroin in order to purify olive leaf antioxidants and to obtain a stable silk protein-phytochemical conjugate that acted as an antioxidant, anti-inflammatory and antimicrobial agent. The resulting silk fibroin-conjugate exhibited good antioxidant and antimicrobial activity (Bayçın et al., 2007).

It has been demonstrated that simple adsorption is one of the best silk-based immobilization strategies for improving the stability of compounds or proteins (Pritchard et al., 2012). Adsorption onto silk nanoparticles produces an external envelope of compounds or proteins that may be of interest (Vepari and Kaplan, 2007). The technique has the advantage of being a mild, relatively straightforward and cheap, although the extent of compound loading will be influenced by the nature of the interactions between the compounds and the silk surface and must be investigated in each particular case. Finally, the overall purpose of this work is to demonstrate the efficiency of SFNs as carriers that adsorb and deliver Q, while improving its stability and bioavailability.

2. Material and methods

2.1. Materials

The processed white silk cocoons were selected from *B. mori* silkworms, reared at IMIDA (Murcia, Spain) and fed on *M. alba* L. fresh leaves. Two to seven days after spinning, the intact pupae were extracted from the cocoons by cutting open the cocoons, in order to avoid the SF contamination that occurs when the pupae are baked in the closed cocoons during the sericin removal step. All reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Purified water (18.2 MΩ cm at 25 °C from Direct-Q1 ultra-pure water system, Millipore, Billerica, MA). All other chemicals and solvents were of analytical grade and were used without further purification.

2.2. Preparation of liquid silk fibroin (SF)

Prior to SF dissolution, the silk cocoons were boiled twice in a 0.05 M Na_2CO_3 aqueous solution for 45 min to remove the sericin. The remaining SF fibres were rinsed thoroughly with ultrapure water and left to dry at room temperature overnight. Then, SF was dissolved at 10% (w/v) in *Ajisawa*'s reagent, composed of a mixture of $CaCl_2$/ethanol/H_2O (1:2:8 in molar ratio), and left for 3 h at 70 °C (Ajisawa, 1998). To remove $CaCl_2$, pigments, small peptides and other impurities the hydro-alcoholic silk solution was dialyzed for 48 h against ultra-pure water using a cellulose semi-permeable membrane (cut-off 3.5 KD). This gave a final SF concentration of 2 wt.% in water.

2.3. Preparation of silk fibroin nanoparticles (SFNs)

SFNs were prepared according to an adapted version of the method described by Zhang et al. (2007). Briefly, the freshly