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Harnessing proteins to control crystal size and morphology, for improved delivery performance of hydrophobic bioactives, using genistein as a model

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ABSTRACT

Protein-surface and protein-crystal interactions are important in many areas of technology including drug and nutraceutical delivery, as many bioactives are highly hydrophobic and tend to crystallize, resulting in poor bioavailability. The improved ability to control lipophilic bioactive nanocrystal formation and dispersibility can increase colloidal stability, and open new ways to control the release of incorporated bioactives and their bioavailability.

Herein we compared three model proteins: β-casein, hydrophobin, and β-lactoglobulin, representing different structural groups of proteins, and assessed their functionality in preventing crystal growth, using genistein as a model hydrophobic crystallizing bioactive.

Dynamic light scattering, polarized light microscopy and cryo-TEM showed that β-lactoglobulin, hydrophobin and β-casein, respectively inhibit genistein crystal growth in aqueous solution in increasing order of efficacy. Protein structure determines the mechanism and the efficacy by which it affects crystal growth and morphology: β-lactoglobulin, a rigid globular protein with an inward facing hydrophobic domain, indirectly suppresses crystallization by binding and reducing concentration of free hydrophobic compound molecules. Hydrophobin, a rigid globular protein with a flat external hydrophobic domain, adheres to the surface of certain crystal faces, limiting growth in the perpendicular directions. β-casein, a rheomorphic protein with an external hydrophobic domain, adheres to different crystal faces nonspecifically, thereby blocking growth in all directions. Consequently, an inverse correlation was observed between nanocrystal size and *in vitro* bioavailability. Based on this study, amphiphilic proteins can be more effectively selected and applied to control crystal growth and morphology of hydrophobic bioactives to improve their delivery and bioavailability in food and drug systems.

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1. Introduction

Many nutraceuticals and pharmaceuticals are highly hydrophobic, resulting in low water-solubility and poor bioavailability (Li, Zheng, Xiao, & McClements, 2012). A common approach to address this problem is to disperse a lipophilic phase into droplets within an aqueous phase, using emulsifiers.

It is possible to disperse an insoluble compound using a lipid-based carrier such as nanoemulsions (Walker, Decker, & McClements, 2015), solid lipid nanoparticles (Salminen, Helgason, Kristinsson, Kristbergsson, & Weiss, 2013) or nanostructured lipid carriers (Salminen et al., 2013), using various fats or oils, and lipid surfactants, as has been recently reviewed (Livney, 2015). In the case of bioactives, nanoemulsions (whose droplets are below 100 nm) may form clear systems, and improve bioavailability. It has been shown that, in general, the smaller the droplet containing the bioactive compound, the greater the bioavailability (Li et al., 2012), but chemical stability is often limited. A different method is to create nanocrystals, consisting of 100% bioactive, (i.e. not dissolved in a carrier triglyceride). Crystalline materials are generally more chemically stable, and their compact structure may increase

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loading of nanoparticles. In this approach, the insoluble drug aggregates are either broken into sufficiently tiny particles by top-down techniques such as pearl milling or high pressure homogenization and become stabilized as a suspension, or, alternatively, nanocrystals can be produced by bottom-up techniques such as precipitation (Livney, 2015; Müller, Gohla, & Keck, 2011). Nanocrystals of crystalline compounds dissolve more rapidly with decreasing particle size (Peltonen & Hirvonen, 2010), and their melting temperatures and melting enthalpies generally decrease as well (Liu, Yang, & Jiang, 2007), both of which may facilitate their improved absorption.

Once nanocrystals are formed, colloidally stabilizing them over time is another challenge. Different kinds of materials, such as polymers, surfactants and lipids, have been tested for their ability to stabilize various nano-crystalline drugs (Douroumis & Fahr, 2006; Lindfors, Forssén, Westergren, & Olsson, 2008; Helgason et al., 2009; Peltonen & Hirvonen, 2010).

Certain food proteins, such as hydrophobin (Hyd), β-casein (β-CN) and β-lactoglobulin (β-LG), may serve as surfactants, emulsifiers and nanoencapsulating agents in food systems and in oral drug delivery. Such proteins have the ability to control crystal nucleation and growth, and this ability has been utilized in the field of drug delivery. Valo et al. showed that adsorption of the amphiphilic protein hydrophobin HFBII onto the crystal-water interface limited crystal growth of the lipophilic drug beclomethasone dipropionate. The physical adsorption is based on interactions between hydrophobic domains of the protein and the drug crystal. Hydrophobin adsorption at solid-liquid interfaces is known to be strong, depending on the hydrophobicity of the surface area. Thus, hydrophobins may be effectively used to provide stabilization for hydrophobic drugs and in the production of drug nanoparticles (Valo et al., 2010).

Recent research from our lab showed that the adsorption of β-CN to the lipophilic drug paclitaxel suppressed paclitaxel crystal growth, and the resulting crystals were about 100–200 nm, compared to more than 10,000 nm (10μm) in diameter, in the absence of the protein. Moreover, without β-CN, paclitaxel displayed a needle-like crystal morphology, whereas in the presence of β-CN more uniform polygonal structures were observed (Shapira, Davidson, Avni, Assaraf, & Livney, 2012).

Another research from our lab showed that β-LG solubilized the flavonoid naringenin up to 3 times its solubility limit and prevented crystal formation in the aqueous medium (Shpigelman, Shoham, Israeli-Lev, & Livney, 2014).

In the present study, we used genistein as a model hydrophobic bioactive. Genistein (Gen) is an abundant isoflavone in soybeans, with a molecular weight of 270.24 gr/mol. Gen is a hydrophobic substance (logP of 3.1 ± 1.1, logD of 1.53 at pH 7 (Lehane & Saliba, 2008; Wang, Komolpis, Kaufman, Malakul, & Shotipruk, 2001)) with poor solubility in water (at 25 °C): 0.0053 mM according to (Wu, Ge, Zhang, Yu, & Zhang, 2010), and 0.0011 mM according to (Shimoda, Kobayashi, Akagi, Hamada, & Hamada, 2008). It is crystalline at room temperature with a melting point of 298–299 °C (pH 7, 25 °C) (SciFinder, 2015).

Gen has many health benefits: it acts as a phytoestrogen, an antioxidant, an anti-cancer agent; it decreases the risks of cardiovascular diseases in general and of osteoporosis in women, and reduces postmenopausal symptoms (Dixon & Ferreira, 2002).

Calculation of the Gen dose required to exert its beneficial health effects is based on the consumption of soy products in Asian countries, giving a recommended isoflavone dose of 40–50 mg daily (Dixon & Ferreira, 2002; Setchell & Cole, 2003). Most people in western countries do not consume this amount, so it would be highly advantageous to add Gen to food products. However, Gen has a bitter taste and low water solubility, limiting its applicability in the food industry. Moreover, the low aqueous solubility, low water/oil partition coefficient and consequent poor absorption strongly limit the bioavailability of Gen (Zhou et al., 2008; Rusin et al., 2010).

The aim of this work was to study the relationship between protein structure and its efficacy in controlling nanocrystal size and bioavailability. To achieve that, we compared three model proteins: hydrophobin (Hyd), β-casein (β-CN) and β-lactoglobulin (β-LG), representing different structural groups (see Fig. 1 and Table 1), thus to improve the colloidal stability and bioavailability of crystallizing hydrophobic bioactives, such as Gen.

2. Materials and methods

2.1. Materials

Genistein (Gen, CAS registry number 446-72-0) was purchased from Wuxi Gorunjie Natural-Pharma Co, Ltd. (Wuxi-Jiangsu, China). The powder was stored at −20 °C until use.

Hydrophobin H STAR protein B (Hyd, 95% purity by supplier specification, originally from *Aspergillus nidulans*, a fusion protein (19 kDa) overexpressed in *Escherichia coli* (Wohlleben et al., 2010)) was kindly donated by BASF SE (Ludwigshafen, Germany), and was dialyzed against de-ionized water by Spectra/Por molecular porous membrane tubing (M.W.C.O 6–8000). The purity of the protein was also evaluated at the Technion Smoler Proteomics Center using LC-MS, and found to be about 90%.

Bovine β-casein (β-CN, C6905, 90% purity) for the initial characterization studies was purchased from Sigma-Aldrich Israel Ltd. (Rehovot, Israel). As larger quantities were required for the simulated digestion and the *in-vitro* bioavailability experiments, bovine β-CN from an industrial source (lot number JC2-013-05, total protein 88%, of which 76% is β-CN) was kindly donated by Arla Foods (Denmark). β-CN was further purified (to >85% purity, on protein basis by SDS-PAGE): the sample was dissolved in a pH 6.8 phosphate buffer (PB); pH was adjusted to 4.9, and the sample was centrifuged at 1000*g* for 10 min at 4 °C. The pellet was dissolved in a pH 7.5 PB and heated for 20 min at 80 °C, then centrifuged at 11000*g* for 10 min at 4 °C. The supernatant was dialyzed against de-

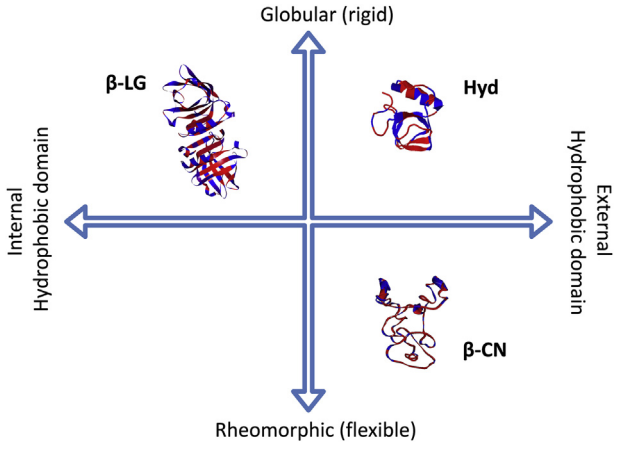

Fig. 1. Model proteins: Hyd, β-CN and β-LG, representing different structural groups. Structure models of Hyd and β-LG were created using Jmol software, based on Hakanpää, Linder, Popov, Schmidt, and Rouvinen (2006) and Brownlow et al. (1997), respectively. β-CN model was created using Accelrys' Discovery Studio 3.5 software, based on Kumosinski, Brown, and Farrell (2003). Blue color represents hydrophilic residues while red color represents hydrophobic residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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