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Self-assembly of hydrophobin and its co-assembly with hydrophobic nutraceuticals in aqueous solutions: Towards application as delivery systems

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ABSTRACT

Hydrophobins (Hyd) are small cysteine-rich, amphipathic proteins with molecular masses of about 10 kDa. They are present in large amounts in fungal cell walls, where they form part of the outermost layer ("rodlet layer"); some of them are secreted into the medium. Secreted Hyd have the ability to convert hydrophobic surfaces to hydrophilic ones and vice versa by self-assembly into an amphipathic protein membrane. Hyd are among the most surface-active molecules known. Due to their amphiphilic nature and self-assembly properties, hydrophobins have been suggested to serve as surfactants, emulsifiers and encapsulating agents for drugs, but their application in nanoencapsulation of nutraceuticals for food enrichment has apparently not yet been explored. Using pyrene fluorescence, we determined the critical micellization concentration (CMC) of a commercially available Hyd CMC=(0.041 ± 0.006) mg/mL. Using intrinsic fluorescence quenching we showed that both vitamin D_3 (VD_3) and Nile Red (NR) bind to Hyd: NR-Hyd $K_a = (8.2 \pm 0.5) \times 10^5\ M^{-1}$; VD_3-Hyd $K_a = (0.65 \pm 0.04) \times 10^5\ M^{-1}$. Using dynamic light scattering we showed that while VD_3 particles had a mean diameter of 235 nm, VD_3-Hyd complexes were much smaller (two subpopulations of particles were formed: 79% of the particles had a mean diameter of 30 nm, and 21% had a mean diameter of 100 nm). While VD_3 in phosphate buffer showed low stability, with ~70% loss in 21 days of storage, the VD_3–Hyd nano-particles system was colloidally and chemically very stable, without any loss of VD_3 during that time. Hyd were thus found to be promising nanovehicles of hydrophobic nutraceuticals for food and clear beverages enrichment.

1. Introduction

Enriching staple foods and beverages with nutraceuticals is a promising approach for promoting health of broad populations. Enrichment with hydrophobic nutraceuticals is highly challenging due to their low aqueous solubility, poor bioavailability and sensitivity to degradation during production, shelf life and digestion.

Protein- based delivery systems are relatively simple to prepare and are capable of delivering hydrophobic bioactives, while protecting them from degradation (Chen & Subirade, 2008; Livney, 2010; Livney, 2012; Matalanis, Jones, & McClements, 2011; Semo, Kesselman, Danino, & Livney, 2007).

Hydrophobins are small cysteine-rich, amphipathic proteins with 100 ± 25 amino acids and molecular masses of about 10 kDa. They are present in large amounts in fungal cell walls, where they form part of the outermost layer ("rodlet layer") (Penas et al., 1998) and are sometimes secreted into the surrounding medium (Wessels, de Vries, Ásgeirsdóttir, & Schuren, 1991). Different hydrophobins are associated with different developmental stages of a fungus, and their biological functions include protection of the hyphae against desiccation and attack by either bacterial or fungal parasites, hyphal adherence, and the promotion of aerial growth of the hyphae (Penas et al., 1998).

Secreted hydrophobins have the ability to convert hydrophobic surfaces to hydrophilic ones and hydrophilic surfaces to hydrophobic ones, by self-assembling into an amphipathic protein membrane. They are also among the most surface-active molecules known (Askolin, Nakari-Set, & Tenkanen, 2001; Linder, 2009).

Hydrophobins are divided into two classes: the members of class I form aggregates that are quite insoluble in aqueous solutions, whereas the members of class II form aggregates that are

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water soluble (Linder, 2009). Only class I hydrophobins have been found in edible mushrooms, which belong to the Basidiomycetes division (Linder, 2009).

As mentioned earlier, hydrophobins have been shown to be highly surface active. In terms of structure, this can be attributed to a hydrophobic patch on one side of the protein's surface, and a hydrophilic patch on the other side. The hydrophobic patch is made up of aliphatic side chains, and the presence of this patch is conserved among all hydrophobins. It is interesting to note that, in most globular proteins, thermodynamics favors a structure in which hydrophobic side chains are buried within the protein core. In hydrophobins, however, this patch is found on the protein surface, due to a series of eight conserved Cys residues, which are thought to hold the protein's structure in place via four disulfide bonds. Furthermore, the aliphatic side chains in the hydrophobic patch form a beta sheet secondary structure, which facilitates interactions with surfaces (Linder, 2009).

Hydrophobins can be very soluble in water. There are much data showing that also in clear solutions the hydrophobins are present in some self-assembled structures (Linder, 2009; Wang, 2004). While there are data suggesting lateral interactions between hydrophobins at the air–water interface, there are still not much data describing the molecular details of interactions of hydrophobins in aqueous solution (Linder, 2009).

There are several possible technological applications for hydrophobins, including the use of hydrophobin membranes to immobilize cells or proteins to surfaces (e.g. in biosensors), changing surface hydrophobicity in order to increase biocompatibility in tissue engineering, and acting as oil-dispersing agents in different branches of industry (Askolin et al., 2001; Sunde, Kwan, Templeton, Beever, & Mackay, 2008). Another interesting application is to use hydrophobins in the manufacture of aerated foods such as ice cream where hydrophobins could be used to stabilize the dispersed air bubbles, particularly in low fat ice cream (Crilly, Russell, Cox, & Cebula, 2008). A hydrophobin coating for stabilizing oil droplets could also be useful for drug delivery (Askolin et al., 2001; Wessels, 1996). Haas Jimoh Akanbi et al., have shown that the class I hydrophobin SC3 of *Schizophyllum commune* can be used to suspend water insoluble drugs in water (Haas Jimoh Akanbi et al., 2010), and Valo et al., introduced a method for preparing nanoparticles of low-solubility drugs using class II hydrophobin HFBII expressed in *Trichoderma reesei* (Valo et al., 2010).

Due to their amphiphilic nature and self-assembly properties, hydrophobins show promise as highly effective surfactants, emulsifiers and nanoencapsulating agents in food systems. Not much is known about the molecular mechanism of hydrophobins self-assembly and co-assembly with hydrophobic substances in aqueous solution. Studying these interactions can pave the way for many important applications, such as entrapment and protection of bioactives, particularly nutraceuticals in food applications, as this application has apparently not yet been reported in the literature.

The aim of this work was to study a model co-assembled nanoparticle system based on a hydrophobin for the protected delivery of a model sensitive hydrophobic nutraceutical compound, Vitamin D_3. In particular, we wished to examine the possibility to enrich clear beverages, such as mineral water and related products, using this new delivery system.

2. Materials and methods

2.1. Materials

Hydrophobin H STAR PROTEIN B (class I (Vacano et al., 2011) Hyd, 95% purity, originally from *Aspergillus nidulans*, overexpressed in *Escherichia coli* (Guzmann, Eck, & Baus, 2009; Meyer, 2011; Vacano et al., 2011)) was kindly donated by BASF SE (Ludwigshafen, Germany), and was dialyzed against de-ionized water by Spectra/Por membrane tubing (M.W.C.O 6000–8000 Da).

Vitamin D_3 (VD_3, Cholecalciferol), Pyrene and Nile red (NR), were obtained from Sigma–Aldrich (Rehovot, Israel). Other reagents were of analytical grade, and were used as received.

2.2. Methods

2.2.1. Critical micellization concentration (CMC)

CMC was determined based on the increase in pyrene fluorescence intensity ratio I3:I1 during its entrapment in hydrophobic domains forming upon micellization (Liu & Guo, 2008).

A series of decimal dilutions of Hyd ($1.2*10^{-7}$ up to 1.2 mg/mL) was prepared using phosphate buffer solution (PB, pH 7.2, 30 mM). Pyrene dissolved in ethanol was added to each Hyd solution to a final pyrene concentration of 1.02 μM. Pyrene fluorescence was measured using a Fluorolog 3-22 spectrofluorometer (Horiba, Jobin Yvon, Longjumeau, France) at a right-angle mode at 23 °C. Emission spectra were recorded from 350 to 400 nm with an excitation wavelength of 338 nm, with a slit width of 1 nm in both excitation and emission. The statistical analysis and non- linear curve fitting were performed using Origin 8 (OriginLab, Northampton, USA).

2.2.2. Binding studies by spectrofluorometry

Quenching of protein fluorescence due to energy transfer from the Hyd Tyrosine (Tyr) residue to a bound ligand served to determine the binding affinity. The interaction between Hyd and either NR or VD_3 above the CMC of pure Hyd was studied by monitoring the changes in the Tyr fluorescence emission of Hyd solution (10.1 μM in 2 mL of 30 mM PB, pH 7.15. These conditions were chosen to resemble mineral water.) before and after the addition of various amounts of each hydrophobic substance, dissolved in ethanol. Tyr fluorescence was determined using an excitation at 275 nm, and emission was collected at 350 nm, with slit width of 5 nm, using the Fluorolog 3-22 spectrofluorometer. All fluorescence measurements were performed at 23 °C.

2.2.3. Binding studies by UV absorbance spectra measurement

NR – Hyd and VD_3 – Hyd co-assemblies were prepared by adding the hydrophobic ligand dissolved in ethanol to Hyd solution (0.19 mg/mL (10.1 μM) in 1 mL 30 mM PB, pH 7.15) at room temperature (22–25 °C). NR final concentration was 12.6 μM. Final VD_3 concentration was 19.8 μM. Appropriate controls of Hyd without ligand and of ligand without Hyd at the same concentrations and pH were tested as well. UV–vis spectra were measured by UV spectrophotometer (Ultrospec 3000, GE Healthcare). Measurements were made in duplicate at 23 °C.

2.2.4. Particle size distribution analysis by DLS

10 μL of VD_3 (2 mg/mL and 4 mg/mL, in ethanol) were added to Hyd solution (0.49 mg/mL, in 0.99 mL of 30 mM PB, pH 7.15), while vortexing. The final VD_3 concentrations were 52 μM and 104 μM, and in both cases the vitamin was supersaturated in the aqueous environment; VD_3: Hyd molar ratios were 2:1 and 4:1 respectively. Appropriate controls of Hyd without VD_3 and of VD_3 without Hyd at the same concentrations and pH were tested as well. Samples were prepared at room temperature.

Particle size distribution was determined by a dynamic light scattering (DLS) analyzer (NICOMP™ 380, Particle Sizing Systems, Inc., Santa Barbara, CA, USA) equipped with an Avalanche Photo Diode (APD) detector, used at a fixed angle of 90°. The 90 mW laser wavelength was 658 nm. Mono- bi- or tri-modal distributions were calculated from the scattered light intensity fluctuations, by Nicomp™ cumulants analysis of the autocorrelation function (the