Interfacial biomimetic synthesis of silica nanocapsules using a recombinant catalytic modular protein

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KEYWORDS

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ABSTRACT

This paper reports interfacially-driven synthesis of oil-core silica-shell nanocapsules using a rationally-designed Recombinant Catalytic Modular Protein (ReCaMoP), in lieu of a conventional chemical surfactant. A 116-residue protein, D4S2, was designed by modularizing a surface-active protein module having four-helix bundle structure in bulk and a biosilicification-active peptide module rich in cationic residues. This modular combination design allowed the protein to be produced via the industrially-relevant cell factory *Escherichia coli* with simplified purification conferred by thermostability engineered in design. Dynamic interfacial tension and thin film pressure balance were used to gain an overview of the protein behavior at macroscopic interfaces. Functionalities of D4S2 to make silica nanocapsules were demonstrated by facilitating formation and stabilization of pharmaceutically-grade oil droplets through its surface-active module and then by directing nucleation and growth of a silica shell at the oil–water interface through its biosilicification-active module. Through these synergistic activities in D4S2, silica nanocapsules could be formed at near-neutral pH and ambient temperature without using any organic solvents that might have negative environmental and sustainability impacts. This work introduces parallelization of biomolecular, scale-up and interfacial catalytic design strategies for the ultimate development of sustainable and scalable production of a recombinant modular protein that is able to catalyze synthesis of oil-filled silica nanocapsules under environmentally-friendly conditions, suitable for use as controlled-release nanocarriers of various actives in biomedical and agricultural applications.

INTRODUCTION

Advances in interfacial chemistry have enabled synthesis of silica nanocapsules having core–shell architecture.^{1,2} The synthesis methods involve sol–gel reactions of aqueous silica precursor on colloidal core templates, i.e., gas bubbles,^{3,4} nanoparticles,⁵⁻¹¹ and emulsion droplets,¹²⁻²⁰ resulting in nucleation and growth of silica at the boundary of gas–liquid, solid–liquid, and liquid–liquid interfaces, respectively. With silica shells encapsulating the core materials, the nanocapsules combine properties of silica, i.e., biocompatibility, easy functionalization, mechanical stability, and optical transparency,^{1,21} and tunable functionalities of the core. Depending on the core’s type, silica nanocapsules have found diverse applications in fields of chemical, biomedical and agricultural applications, for example, as nanoreactors;⁵ as supports of catalytic metals,⁶ semiconductor,⁷ fluorescent⁸ and magnetic⁹ nanoparticles, and enzymes¹² for their long-term activity and/or dispersibility in aqueous environment; as adsorbents;^{13,14} and as nanocarriers of drugs¹⁵ and pesticides¹⁶ for their storage, protection, and release.

Surface chemistry of the colloidal core templates plays important roles for the synthesis of silica nanocapsules,^{1,2} thereby maintaining template stability and concurrently catalyzing silica formation preferentially at the interface. Therefore, various chemicals have been decorated surrounding the template surfaces via adsorption, including cationic surfactants (e.g., tetradecyltrimethylammonium bromide,⁶ cetyltrimethylammonium bromide^{3,18} and cetyltrimethylammonium chloride¹⁹), non-ionic surfactants (e.g., Brij 97,¹³ Tween 80¹⁴ and Triton X-100²⁰), and polymers (e.g., poly(acrylic acid),⁵ poly(vinylpyrrolidone)¹⁰ and poly(ethyleneoxide)-*b*-poly(propyleneoxide)-*b*-poly(ethyleneoxide)¹⁷). Despite great progress in the interfacial synthesis of silica nanocapsules, the aforementioned chemical methods^{12-15,17-20} variously incorporate toxic chemical catalysts and organic solvents that would pose adverse

environmental effects. An alternative way to construct silica nanocapsules using biocompatible components and benign conditions is still highly desired especially for biomedical and agricultural applications.

Nature's ability to form silica using biomolecules as in cell walls of diatoms²² and spicules of sponges²³ provides an example for "green" synthesis of silica-based nanomaterials. The biomolecules play important roles in biosilicification by inducing nucleation and growth of silica species in aqueous solution under physiological conditions.²⁴ To mimic the naturally occurring biomolecules, many studies have used short peptides for biosilicification in bulk aqueous solutions,²⁵⁻²⁸ but very few studies report biosilicification at biphasic interfaces.^{11,16} Graf *et al.* used two tripeptides (CKK) connected with a disulphide bridge to facilitate formation of silica shell encapsulating a silver nanoparticle core.¹¹ We recently reported synthesis of oil-core silica-shell nanocapsules via a novel emulsion and biomimetic dual-templating approach¹⁶ using bifunctional SurSi peptide (Ac-MKQLAHS VSRLEHA RKKRKKRKKRKKGGGY-CONH₂) that modularized a surface-active sequence (Sur module, MKQLAHS VSRLEHA) capable of stabilizing pharmaceutically-grade oil droplets with a sequence for catalyzing biosilicification at the oil–water interfaces (Si module, RKKRKKRKKRKKGGGY).

Synthesis of oil-core silica-shell nanocapsules using biomolecular engineering¹⁶ instead of chemical surfactant approaches¹²⁻²⁰ may contribute substantially to the development of an environmentally-friendly approach to produce biocompatible nanocarriers having controlled-release properties. Various hydrophobic actives could potentially be encapsulated in the nanocapsules' core with high encapsulation efficiency, thus broadening opportunities to wide range of applications especially in biomedical and agricultural fields. In turn, sustainable and scalable processes of making such nanocapsules would be highly demanded. Peptide surfactant,

a key ingredient in the synthesis of silica nanocapsules using this new approach,¹⁶ is currently produced based on solid-phase chemistry²⁹ which is useful for research but limiting for large-scale production.³⁰ Recombinant peptide production offers the potential of process scale-up using established bioprocess unit operations,³¹ for example by expression of recombinant DNA encoding expression of the peptide via the low-cost “cell factory” *Escherichia coli*.³² Significant challenges may exist during peptide expression as peptides may self-associate forming insoluble aggregated materials³³ and undergo intracellular degradation.³⁴ To counter these problems, peptides are commonly fused to carrier proteins to mask the self-assembly characteristic and improve the intracellular stability.³⁵ Design of fusion protein combining the peptide with a carrier protein should enable its expression in *E. coli*, allowing peptide cleavage from the carrier protein if necessary, to facilitate simplified purification.

In this study, we designed a 116 residue protein, D4S2, by fusing the Si module of the SurSi peptide, known for its biosilicification activity,¹⁶ with a helical DAMP4 variant module as carrier protein derived from recombinant DAMP4 protein which has known surface activity.³⁶ This modular combination design would enable expression of D4S2 in *E. coli* at high solubility while potentially allowing easy process-scale manufacture as demonstrated for DAMP4.³⁷ We hypothesized that this strategy would allow the purified D4S2 to be directly used for making silica nanocapsules without the need for peptide cleavage from the carrier protein. In addition, the recombinant D4S2 would be expected capable of (1) facilitating formation and stabilization of nanoemulsions through its surface-active module, and (2) catalyzing biosilicification at the oil–water interfaces through its biosilicification-active module, forming oil-core silica-shell nanocapsules at room temperature and nearly neutral pH in the absence of any toxic organic solvents. To the best of our knowledge, this is the first report of parallel biomolecular, scale-up

and interfacial catalytic design strategies for the development of a sustainable and scalable production route to a recombinant catalytic modular protein (ReCaMoP) that is able to catalyze interfacial synthesis of silica nanocapsules under environmentally-friendly conditions.

EXPERIMENTAL SECTION

Materials. Miglyol[®] 812 purchased from AXO Industry S.A. (Wavre, Belgium) was passed through heat-activated silica gel (Sigma-Aldrich) prior to use. Water with >18.2 M Ω cm resistivity was obtained from a Milli-Q system with a 0.22 μ m filter (Millipore, Australia). Other chemicals were of analytical grade purchased from either Sigma-Aldrich or Merck and used as received unless otherwise stated.

Protein expression, purification, and characterization. Plasmid pET-48b(+) composed of nucleotide sequence encoding for D4S2 protein (Protein Expression Facility, The University of Queensland) was transformed into chemically-competent *E. coli* BL21(DE3) (Novagen, Merck KGaA, Germany). Production of D4S2 protein was through expression of recombinant DNA in *E. coli* controlled by the T7 promoter. Purification was based on the reported precipitation process for DAMP4 protein³⁷ (Figures S1 and S2) and chromatography techniques (Figure S3) to obtain high purity of D4S2. Detailed transformation, expression, and purification can be found in the Supporting Information.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Qualitative analysis of protein samples was performed using NuPAGE[®] 4–12% Bis-Tris gels (Life Technologies, Australia) mounted in a Bio-Rad XCell 3 system (Bio-Rad, CA, USA). Procedures for sample preparation, gel staining and destaining, and gel imaging were provided in the Supporting Information.

Reversed-phase high-performance liquid chromatography (RP-HPLC): To determine concentration of D4S2 protein, RP-HPLC equipped with a Jupiter C₁₈ column (5 μ m, 300 Å, 150 mm $\times$ 4.6 mm) (Phenomenex, CA, USA) and connected to an LC-10AVP series HPLC system (Shimadzu, Japan) was used. Buffer A was 0.1 v/v% trifluoroacetic acid (TFA) in water, and buffer B was 90 v/v% acetonitrile and 0.1 v/v% TFA in water. Gradient conditions applied in the analysis were from 30 to 65% of buffer B in 35 min at a flow rate of 1 mL/min and a set detection wavelength of 214 nm.

Liquid chromatography–Mass spectrometry (LC-MS): LC-MS was performed using a Waters Alliance HPLC system (Waters, MA, USA) linked with a Quattro Micro API Quadrupole system to determine molecular mass of D4S2. The LC was equipped with a Kinetex C₁₈ column (2.6 μ m, 100 Å, 100 mm $\times$ 4.6 mm) (Phenomenex, CA, USA) with a mobile phase A, 0.01 v/v% TFA in water, and mobile phase B, 90 v/v% acetonitrile, 0.01 v/v% TFA in water. An applied gradient from 30 to 65% B in 35 min at a flow rate of 0.6 mL/min were used. For mass analysis, an electrospray mass spectrometry in positive-ion mode was performed with capillary, cone, extractor, and a radio-frequency lens voltages set at 3 kV, 24 V, 3 V, and 0 V, respectively.

Circular dichroism (CD): To determine the secondary structure of D4S2, CD spectroscopy (Jasco 810, Easton, MD) was performed on 0.025 mg/mL D4S2 filled in a 1 cm-path-length quartz cell (Starna, Australia) at 20 and 90 °C. Water was used to dissolve D4S2 in order to eliminate the effect of buffer salts on the CD signal. Far-UV CD spectra were recorded from 260 to 190 nm using 0.1 nm data pitch, 50 nm/s scan speed, 2 s response time, 1 nm bandwidth, and 10 accumulations. The obtained raw ellipticity was converted to molar ellipticity by following equation:³⁸

$$[\theta] = \frac{\theta \times M_{\text{mrm}}}{10 \times c \times l}$$

where $[\theta]$ is molar ellipticity (deg cm²/dmol), θ is raw ellipticity (mdeg), c is protein concentration (mg/mL), l is cell path length (cm), and M_{mrm} is mean residue molecular mass i.e., protein molecular mass/number of residues.

Interfacial tension. Oil–water interfacial tension studies employed droplet tensiometry with a Krüss Drop Shape Analysis System DSA-10 (Krüss GmbH, Germany). Protein solution of either D4S2 or DAMP4 (8 mL, 0.1 mg/mL) in HEPES buffer (25 mM, pH 7.5) was filled in an 8-mL quartz cuvette (Hellma, Germany), and an oil droplet was formed manually via a glass syringe connected to a U-shaped stainless steel capillary of known diameter submerged in the protein solution. Measurements of the interfacial tension were made automatically over 1000 s following initial oil droplet formation. Prior to measurement, the interfacial tension of oil in the buffer was measured at approximately 30 mN/m and remained constant for at least 10 min to ensure correct functioning, focusing, and cleanliness of the instrument.

Thin film experiment. The thin film pressure balance technique³⁹ was used to determine the surface forces in a liquid film stabilized by either D4S2 or DAMP4 (1.53 mg/mL) in HEPES buffer (25 mM, pH 7.5) under controlled capillary pressure. A liquid film was formed using a microfabricated bike-wheel microcell, designed for uniform radial drainage,⁴⁰ with diameter of the central hole inside the microcell of 0.75 mm. A vapor-saturated chamber inserted with the film holder was placed on an inverted microscopic stage (Olympus IX51). Temperature was maintained at 22 ± 0.1 °C using a water-jacket, and the air pressure inside the chamber was controlled by using a 100 mL gas-tight syringe (SGE, Australia) driven by a micropositioner

(M4454, Parker) as described by Wang and Yoon.⁴¹ To record images of the films, a digital video camera was used at a rate of 30 frames per second.

Synthesis and characterization of oil-core silica-shell nanocapsules. Lyophilized D4S2 protein was dissolved in HEPES buffer (25 mM, pH 7.5) to prepare D4S2 solutions from 0.47 to 2.5 mg/mL. Miglyol[®] 812 (10 v/v%) was added to the D4S2 solutions (1 mL), and homogenized using a Branson Sonifier 450 ultrasonicator for four 30 s bursts at 20 W and interspersed in an ice bath for 60 s. The nanoemulsion aliquot (400 μ L) was transferred into a 4-mL glass vial containing weighed tetraethoxysilane (TEOS, 32 μ mol). Interfacially-catalyzed reaction with TEOS was conducted under magnetic stirring at room temperature for 20 h.

Dynamic Light Scattering (DLS): Size distribution and zeta potential were determined by DLS using Malvern Zetasizer Nano ZS (Malvern Instrument, UK) at a scattering angle of 173° and a temperature of 25 °C. Samples were diluted by a factor of 100 prior to measurement to avoid multiple scattering effects.

Transmission Electron Microscopy (TEM): Morphology of the silica nanocapsules was visualized by TEM using a JEOL 1010 (JEOL, Japan) operated at 100 kV. Samples (2 μ L) were deposited onto Formvar-coated copper grids (ProSciTech, Australia). Size of the nanocapsules was analyzed by using iTEM software (version 3.2, Soft Imaging System GmbH).

RESULTS AND DISCUSSION

Protein design. The aim of the reported research was to synthesize oil-core silica-shell nanocapsules based on protein supramolecular chemistry allowing sustainable and scalable protein production via expression in the cell factory *E. coli*. Design of the protein followed the

concept of our recently-reported bifunctional SurSi peptide thereby modularizing a surface-active module with a module for biosilicification.¹⁶ Synergistic combination of both modules was considered essential to stabilize nanoemulsions and concurrently induce nucleation and growth of silica forming silica nanocapsules. This was confirmed by the fact that AM1 peptide (Ac-MKQLADS LHQLARQ VSRLEHA-CONH₂)—known for its surface activity⁴² and ability to stabilize nanoemulsions⁴³—was unable to effectively produce silica nanocapsules as the silica was largely form in bulk solution rather than at oil–water interfaces.¹⁶

D4S2 protein (M *DPS* MKQLADSLHQLARQVSRLEHA *DPS* MKQLADSLHQLARQVSRLEHA *DPS* MKQLADSLHQLARQVSRLEHA *DPS* MKQLADSLHQLARQVSRLEHA EPS RKKRKKRKKRKKGGGY), having theoretical molecular mass 13.3 kDa and *pI* 10.9, was designed by modularizing DAMP4 variant module (M *DPS* MKQLADSLHQLARQVSRLEHA *DPS* MKQLADSLHQLARQVSRLEHA *DPS* MKQLADSLHQLARQVSRLEHA *DPS* MKQLADSLHQLARQVSRLEHA EPS) with Si peptide module (RKKRKKRKKRKKGGGY) (Figure 1). Si peptide rich in cationic amino acid side chains known for its biosilicification activity was derived from SurSi peptide.¹⁶ DAMP4 variant module was derived from four-helix bundle DAMP4 protein (MD(PS MKQLADS LHQLARQ VSRLEHAD)₄) known for its surface activity,³⁶ except that the aspartic acid (D) residue at C-terminal DAMP4 was replaced with glutamic acid (E) residue followed by addition of proline (P) and serine (S) residues in order to provide molecular flexibility to the Si module projecting toward the bulk aqueous phase (Figure 1c).

DAMP4, designed by connecting four surface-active AM1 peptide⁴² via Asp-Pro-Ser (DPS) linkers, has been shown to have surface activity similar to the AM1 peptide,³⁶ was used as a basis for the design of surface-active module of D4S2. Several other reasons were also

considered when selected DAMP4. First, DAMP4 has been proved to be successfully produced in *E. coli* at high levels and solubility.^{36,37} Second, DAMP4 has demonstrated stability at high temperature (above 90 °C) and Na₂SO₄ concentrations (up to 1 M),³⁷ due to its four-helix bundle structure in bulk (Figure 1b), useful for disruption of the cell walls releasing the intracellular proteins and precipitation of the contaminant proteins while maintaining DAMP4 solubility, respectively. These properties allow simple separation of DAMP4 from contaminant proteins without losing its surface activity.³⁷ Finally, DAMP4 could be cleaved, if necessary or desired, at its DP sequence via acid hydrolysis,⁴⁴ and the resulting individual DAMP1 peptide (PS MKQLADS LHQLARQ VSRLEHA D) has been demonstrated to have surface activity as well.⁴⁴ In the case where peptide cleavage from the carrier protein is required for D4S2, acid-digestion of D4S2 would cleave the DP sequence while the EP sequence would be more stable against the acid, thus resulting in a sequence (PS MKQLADS LHQLARQ VSRLEHA EPS RKKRKKRKKRKKGGGY) comprising DAMP1 variant peptide, expected to have surface activity close to DAMP1 peptide,⁴⁴ and biosilicification-active Si peptide.¹⁶

Based on these considerations, incorporation of DAMP4 variant module in D4S2 would be expected to form four-helix bundle protein structure and impart surface activity, while the Si module would provide biosilicification activity for D4S2 (Figure 1). We hypothesized that the surface-active module of D4S2 would undergo interfacial adsorption to the freshly formed droplet (Miglyol[®] 812 oil) surfaces upon sonication, during which the four-helix bundle of D4S2 unfolds at the interface causing the hydrophobic residues to face the oil phase while the hydrophilic residues face toward bulk aqueous phase and stabilize the droplets. This hypothesis is based on extrapolation of the known physical chemistry of DAMP4.⁴⁵ Upon addition of silica precursor tetraethoxysilane (TEOS) molecules, the biosilicification module of D4S2 oriented

within the sub-interfacial region toward bulk solution would act as a catalytic site for nucleation and growth of silica layer surrounding the droplets forming oil-core silica-shell nanocapsules at near neutral pH, room temperature and without using any toxic organic solvents.

Figure 1. Design of recombinant catalytic modular protein (ReCaMoP), D4S2. (a) Schematic amino acid sequence of DAMP4, DAMP4 variant and D4S2 proteins. The proteins consist of four AM1 peptides⁴² (dark blue rectangle) comprising three heptads with repeating motif abcdefg in each, in which the alternate three and four spacing residues (a and d) have hydrophobic side chains (mainly methionine (M), leucine (L) and valine (V)), while the remaining sites are predominantly hydrophilic allowing formation of α -helix structure. (b) End-on view helical wheel projection of DAMP4 variant module in D4S2 with its four- α -helical structure

interlocking of hydrophobic residues at the core that might be expected to give rise to the protein stability. (c) Molecular D4S2 modularizing DAMP4 variant module (dark blue) forming four-helix bundle in bulk and Si module (light blue) projecting toward bulk aqueous phase.

Protein production and characterization. To recover D4S2 from *E. coli* cell pellet, a purification process was developed based on the bake-to-break and precipitate method of the DAMP4 purification process,³⁷ thereby heating the re-suspended cell pellet in a buffer containing 1 M Na₂SO₄ at 90 °C for 30 min (Figure S1). Under such high temperature, the cell walls disrupted and the intracellular proteins including D4S2 released into the bulk phase. In the presence of Na₂SO₄, most proteins precipitated (Figure 2, lane 2), whereas D4S2 remained soluble (Figure 2, lane 3) as concentrated Na₂SO₄ salted-in the four-helix bundle structure³⁷ of D4S2. The protein solution contained high levels DNA (Table S1) which derived from either the expression construct or the genomic DNA.⁴⁶ Protein and DNA may form non-covalent complexes via electrostatic interactions between anionic phosphate groups on the DNA and cationic functional groups on protein at physiological pH.^{47,48} Binding of DNA to protein could alter protein properties (e.g., charge and size) and may cause increased viscosity of the protein solution. To separate D4S2 from DNA, the solution was diluted with water to lower Na₂SO₄ concentration and adjusted to pH 3.5 (i.e., dilute precipitation process), which is well below the isoelectric points of both D4S2 and DNA,⁴⁹ thus inducing charge-charge repulsion between D4S2 and DNA. Under this condition, D4S2 precipitated from the solution as the Na₂SO₄ concentration was insufficient to salt-in D4S2, while most DNA separated from the precipitate and remained in the aqueous phase. At this stage, DNA concentration of the solubilized D4S2 (Figure 1, lane 4) reduced by approximately 95% (Table S1). Prior to the functionality test of D4S2, the protein solution was filtered and passed through desalting chromatography. Then,

purified D4S2 was used to form nanoemulsions via sonication in HEPES buffer (25 mM, pH 7.5). Although D4S2 is rich in cationic amino acids (theoretical pI 10.9), the resulting nanoemulsions unexpectedly had negative zeta potentials at pH 7.5. This is probably because the anionic DNA residues were still present in an amount sufficient to cover the cationic protein side chains at the oil–water interfaces. As a consequence, the nanoemulsions were unable to react with silica precursor tetraethoxysilane (TEOS) molecules and form a silica shell at pH 7.5 as both nanoemulsion surfaces and silica species⁵⁰ bore negative charges.

Figure 2. SDS-PAGE analysis of D4S2 protein after heating/cell lysis, contaminant precipitation and dilute precipitation steps, and IMAC purification step. (Lane 1) Total protein; (Lane 2) Re-suspended precipitate and (Lane 3) supernatant after heating/cell lysis and contaminant precipitation step; (Lane 4) Solubilized precipitate after dilute precipitation step; (Lane 5) Passed-through fraction, (Lane 6) washed fraction and (Lane 7) eluted fraction after IMAC process; (Lane 8) Novex® sharp pre-stain protein standard.

To further remove DNA from D4S2, immobilized-metal affinity chromatography (IMAC) was used applying high salt concentration (1.2 M NaCl) (Figure S4a) followed by desalting chromatography (Figure S4b). D4S2 bound to the IMAC column through its high intrinsic histidine content by forming coordination bonds with Ni^{2+} ions immobilized on the column. DNA dissociated from the bound D4S2 and passed through the column as the electrostatic interactions between D4S2 and DNA were screened under the high salt concentration. D4S2 could then be eluted from the column using imidazole (Figure 2, lane 7) and passed through desalting chromatography to obtain high-purity D4S2 protein (Figure 3a), with a reduction of DNA concentration by approximately 97% as compared to the sample before load to the IMAC column. The calculated molecular mass of D4S2 protein based on the mass spectrum is 13308.2 Da (Figure 3b), which is very close to its theoretical molecular mass i.e., 13299.3 Da confirming the correct protein sequence. The far-UV circular dichroism (CD) measurements for D4S2 at 20 and 90 °C showed nearly identical spectra (Figure 3c) dominated by a double minimum at 208 and 222 nm and a maximum near 195 nm, typical of α -helical conformation.⁵¹ The ability of D4S2 to maintain the helical structure at a temperature up to 90 °C suggests that D4S2 has thermal-stability properties likely due to the four-helix bundle structure that formed through association of four amphiphilic α -helices interlocking the hydrophobic core within D4S2 (Figure 1b). Proteins having four-helix bundle structure have been recognized for their high molecular stability including to temperature⁵² and chemical denaturants,⁵³ and their design and utility have been an area of intense and expanding research.⁵⁴

Figure 3. Characterization of D4S2. (a) Reversed-phase high-performance liquid chromatography showing high purity of D4S2. (b) Mass spectrum determining D4S2 molecular mass of 13308.2 Da. (c) Circular dichroism spectra showing secondary structure of D4S2 in water (0.025 mg/mL) at 20 and 90 °C.

Surface activity. Initial surface-active studies focused on the interfacial tension of D4S2 solution against an Miglyol® 812 oil droplet and in comparison with DAMP4. Oil–water dynamic interfacial tension data were recorded at the low weight concentration of 0.1 mg/mL in HEPES buffer (25 mM) at pH 7.5, corresponding to the pH value for biosilicification reaction,¹⁶ to follow adsorption events at an earlier period (Figure 4). It is clear that D4S2 is surface-active as it spontaneously adsorbed to the oil–water interface and lowered the interfacial tension (γ), despite the high temperature and high salt concentrations applied for its purification. The surface activity of D4S2 was likely due to α -helical structure of D4S2 (Figure 3c) giving rise to its amphiphilic character that allowed D4S2 to unfold upon interfacial adsorption, thus, oriented the hydrophobic residues toward the oil phase, while the hydrophilic sites faced toward the bulk aqueous phase. The interfacial tension of D4S2 approached equilibrium at 18.6 ± 0.3 mN/m, while DAMP4 showed increased interfacial adsorption as the equilibrium interfacial tension reached 16.5 ± 0.4 mN/m. D4S2 showed approximately 81% of the decrease in interfacial tension occurred after 50 s, while DAMP4 gave more rapid adsorption, with 93% of the decrease in interfacial tension seen in the first 50 s. The slower interfacial adsorption indicates existence of energy barriers to protein adsorption that can be affected by molecular mass⁴⁵ and net electrical charge of the protein.⁵⁵ D4S2 has higher molecular mass (M 13308.2 Da) (Figure 3b) than DAMP4 (M 11116.5 Da)³⁶ that results in a lower bulk diffusion coefficient (D) of D4S2, since $D \sim M^{-1/3}$,^{45,56} hence lowering the relative adsorption rate. With the D4S2 theoretical isoelectric point being 10.9 in bulk solution, D4S2 would be expected to carry more positive charges than DAMP4 (theoretical pI 6.7) at pH 7.5, thus yielded an increased charge-charge repulsion causing slower interfacial saturation and conformational changes at the oil–water interfaces upon interfacial adsorption.

Figure 4. Dynamic interfacial tension of protein solutions vs Miglyol[®] 812 oil. Proteins were 0.1 mg/mL in 25 mM HEPES buffer at pH 7.5.

To understand macroscopic stability of a colloidal system, it is important to understand surface forces of the building blocks i.e., a single thin liquid film that separates the dispersed phase in a continuous phase.⁵⁷ In this study, the thin liquid film was studied by using a thin film pressure balance technique³⁹ on a free-standing horizontal liquid foam film, as a model system, that is compressed under controlled-capillary pressure (P_c). Typical images of D4S2 films (Figure 5a) were placed side by side to indicate visual changes in the thin films as the capillary pressure were increased progressively to reach instrument-limit pressure of 2500 Pa, as compared to DAMP4 films (Figure 5b). A weight concentration of 1.53 mg/mL protein solution in 25 mM buffer pH 7.5 was used similar to that used for emulsion preparation using D4S2. Both D4S2 and DAMP4 films exhibited heterogeneous film thickness as evident from the presence of dark and bright area. The bright phase regions are indicative of thicker surface materials, that can be due to the presence of protein aggregates at the film surfaces,⁵⁸ while the black areas can be considered as domains with a reduced thickness. D4S2 films were stable as the shape integrity remained intact at increasing pressure even when pressure reached 2500 Pa (Figure 5a). In

contrast, increasing capillary pressure on DAMP4 films triggered formation of a dark hole at the top edge of the surface films that grew larger occupying half of the film, an initiation of film rupture, upon increasing the pressure to 2500 Pa (Figure 5b). It is evident that surface films of D4S2 demonstrated higher stability than of DAMP4, which bears a positive charge and a near-zero charge, respectively, at pH 7.5, during film compression as a result of higher charge-charge repulsion of positively charged D4S2 at the investigated pH hence increased mechanical resistance of the film. DAMP4 protein bearing near-zero charge has been reported to have low film stability,⁴⁴ and that the film stability could be enhanced by increasing its positive charges through addition of Zn^{2+} ions which bound to histidine (H) residues within DAMP4.

Figure 5. Surface visualization of thin liquid films. (a) D4S2 films. (b) DAMP4 films. Proteins were 1.53 mg/mL in 25 mM HEPES buffer pH 7.5. P_c is the capillary pressure applied to compress the films with maximum pressure reached at 2500 Pa.

Emulsification activity. The surface activity of D4S2 was tested further for emulsifying Miglyol[®] 812 oil i.e., whether D4S2 is able to facilitate disruption of the oil phase into small

emulsion droplets upon sonication and stabilization of the droplets from aggregating with neighboring droplets. Emulsions of 10 v/v% Miglyol[®] 812 oil were prepared in various concentrations of D4S2 protein solutions (0.47–2.50 mg/mL) in HEPES buffer (25 mM, pH 7.5) via sonication that yielded emulsions having different physical properties (Figure 6). The size of emulsions produced at a D4S2 concentration of 0.47 mg/mL was 555 ± 25 nm with a dispersity ($\mathfrak{D}$) of 0.822, and the size decreased to 415 ± 8 nm ($\mathfrak{D} = 0.440$) when D4S2 concentration was increased to 0.93 mg/mL. The high $\mathfrak{D}$ s indicate aggregation probably because the protein was insufficiently available to adsorb at oil–water interfaces thus leaving gaps at the interfaces and, when two gaps on different droplets came into close proximity, resulting in droplet coalescence. Increasing D4S2 concentration to 1.53 mg/mL substantially decreased the droplet size to 203 ± 3 nm ($\mathfrak{D} = 0.210$). Further increase of D4S2 concentration resulted in only a slight decrease in size. Overall, D4S2 formed sufficiently stable emulsions at 1.53 mg/mL with highly positive charges (zeta potential = +38 mV) suggesting that electrostatic repulsion is a key mode of stabilization for D4S2.

Figure 6. Dependence of number-averaged diameter (d_n , ●) and the corresponding dispersity ($\mathfrak{D}$, ○) of emulsion droplets formed at various concentration of D4S2 in HEPES buffer (25 mM, pH 7.5) as determined by using dynamic light scattering (DLS).

Biomimetic-silicification activity. To determine the effectiveness of D4S2 for making silica nanocapsules, the nanoemulsions formed using 1.53 mg/mL D4S2 in HEPES buffer (25 mM, pH 7.5) were reacted with silica precursor tetraethoxysilane (TEOS) molecules. Following addition of TEOS to the nanoemulsions, silica nanocapsule was formed after 20-h reaction at pH 7.5 and room temperature (Figure 7). The outer diameter and shell thickness of the nanocapsules as measured by TEM were 249 ± 29 nm and 26 ± 2 nm. The biosilicification process at the oil–water interface was due to the catalytic role of Si module, which comprises densely packed cationic arginine (R) and lysine (K) residues, projecting toward bulk aqueous phase as well as polar residues of DAMP4 variant module that induced hydrolysis of TEOS and polycondensation at the oil–water interfaces forming silica shell encapsulating the oil core.

Figure 7. Silica nanocapsules formed after reaction of nanoemulsions stabilized by D4S2 (1.53 mg/mL) with TEOS (32 μ mol) in HEPES buffer (25 mM) at pH 7.5 and room temperature for 20 h. (a) Transmission electron microscopy (TEM) image of the silica nanocapsules (scale bar is 200 nm). (b) Dynamic light scattering (DLS) showing size distribution profiles of the silica nanocapsules.

CONCLUSIONS

This study demonstrates the parallel biomolecular, scale-up and interfacial catalytic design strategies for development of a 116-residue protein, D4S2, to catalyze interfacial synthesis of

silica nanocapsules. D4S2 was designed, at the molecular level, by modularizing a DAMP4 variant protein, expected to have four-helix bundle structure and surface activity as imparted by DAMP4 protein, with a Si peptide rich in cationic amino acids known for biosilicification activity derived from SurSi peptide. This modular protein could be synthesized in cell factory *Escherichia coli* and extracted from the cells by heating and mixing with a suitably high concentration of Na_2SO_4 to disrupt the cells and precipitate most protein contaminants while maintaining D4S2 solubility, respectively. The stability of D4S2 to high temperature and chemical denaturant was related to its four-helix bundle structure in bulk, as designed. D4S2 protein was further purified by means of immobilized-metal affinity chromatography and then desalting chromatography to remove DNAs and salts, respectively. Properties of the purified D4S2 including molecular mass (13.3 kDa) and secondary structure (α -helix) were confirmed by using mass spectrometry and circular dichroism spectroscopy, respectively. Surface activity of D4S2 was evidenced as it substantially lowered the dynamic interfacial tension of freshly formed Miglyol[®] 812 oil droplet upon interfacial adsorption. Thin film of D4S2 demonstrated stability against rupture when compressed at increasing pressure likely due to high electrostatic repulsion of D4S2 at the pH that imparted mechanical resistance to the film. Finally, D4S2 was shown able to facilitate formation and stabilization of nanoemulsions through its surface-active module and, upon addition of silica precursor tetraethoxysilane (TEOS), direct nucleation and growth of a silica layer encasing the nanoemulsion core through its biosilicification-active module under near-neutral pH and ambient temperature without using any toxic organic solvents. The present work provides an environmentally-friendly approach to synthesize biocompatible oil-core silica-shell nanocapsules using a recombinant catalytic modular protein (ReCaMoP) offering the

advantages of sustainable and scalable bioproduction, suitable for use as controlled-release nanocarrier of various actives in biomedical and agricultural fields.

ASSOCIATED CONTENT

Supporting Information

Qualitative analysis of protein, quantitation of DNA, transformation of recombinant DNA encoded for D4S2 protein into *E. coli*, expression of recombinant D4S2 protein in *E. coli*, and the subsequent purification processes, as well as Figures S1–S4. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

A, alanine; D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; K, lysine; L, leucine; M, methionine; P, proline; Q, glutamine; R, arginine; S, serine; V, valine; Y, tyrosine; CD, circular dichroism; Da, Dalton; DLS, dynamic light scattering; DNA, deoxyribonucleic acid; HEPES, sodium 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate; IMAC, immobilized-metal affinity chromatography; LC, liquid chromatography; MS, mass spectrometry; *M*, molecular mass; *Đ*, dispersity; *pI*, isoelectric point; ReCaMoP, recombinant catalytic modular protein; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TEOS, tetraethoxysilane; TFA, trifluoroacetic acid; UV, ultraviolet

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