

Contents lists available at [ScienceDirect](http://www.elsevier.com/locate/biophyschem)

Biophysical Chemistry

journal homepage: <http://www.elsevier.com/locate/biophyschem>

Aqueous solubilization of C₆₀ fullerene by natural protein surfactants, latherin and ranaspumin-2



Steven J. Vance^a, Vibhuti Desai^b, Brian O. Smith^b, Malcolm W. Kennedy^{b,c}, Alan Cooper^{a,*}

^a School of Chemistry, University of Glasgow, Glasgow, G12 8QQ, UK

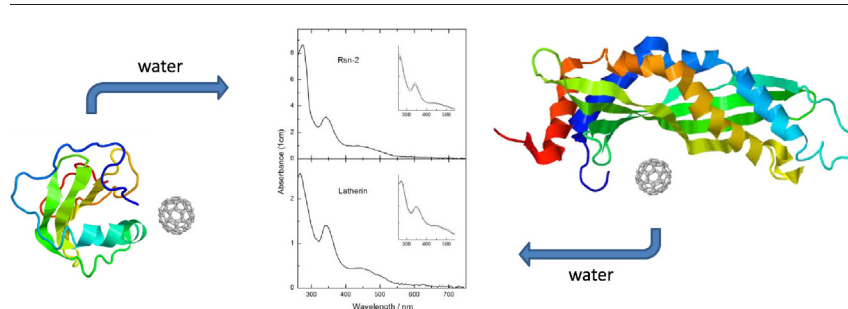
^b Institute of Molecular, Cell & Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, G12 8QQ, UK

^c Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, G12 8QQ, UK

HIGHLIGHTS

- C₆₀ solubilized in water by mixing with natural surfactant proteins.
- Aqueous fullerene: protein solutions have long term stability.
- C₆₀ cluster sizes are smaller than those obtained by more vigorous methods.
- Potential implications for nanoparticle toxicology and biotechnology

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 14 April 2016

Received in revised form 10 May 2016

Accepted 10 May 2016

Available online 13 May 2016

Abbreviations:

Rsn-2

Topic:

ranaspumin-2

PLUNC

Topic:

palate, lung, and nasal epithelium clone protein

SWNT

Topic:

single-wall nanotubes

BSA

Topic:

bovine serum albumin

ABSTRACT

C₆₀ fullerene is not soluble in water and dispersion usually requires organic solvents, sonication or vigorous mechanical mixing. However, we show here that mixing of pristine C₆₀ in water with natural surfactant proteins latherin and ranaspumin-2 (Rsn-2) at low concentrations yields stable aqueous dispersions with spectroscopic properties similar to those previously obtained by more vigorous methods. Particle sizes are significantly smaller than those achieved by mechanical dispersion alone, and concentrations are compatible with clusters approximating 1:1 protein:C₆₀ stoichiometry. These proteins can also be adsorbed onto more intractable carbon nanotubes. This promises to be a convenient way to interface a range of hydrophobic nanoparticles and related materials with biological macromolecules, with potential to exploit the versatility of recombinant protein engineering in the development of nano-bio interface devices. It also has potential consequences for toxicological aspects of these and similar nanoparticles.

© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Abbreviations: Rsn-2, ranaspumin-2; PLUNC, palate, lung, and nasal epithelium clone protein; SWNT, single-wall nanotubes; BSA, bovine serum albumin.

* Corresponding author.

E-mail address: alan.cooper@glasgow.ac.uk (A. Cooper).

<http://dx.doi.org/10.1016/j.bpc.2016.05.003>

0301-4622/© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Despite much initial promise, exploitation of fullerenes and other carbon-based nanomaterials in aqueous systems has been frustrated by the inevitable insolubility of such materials in water. In the course of our ongoing studies of natural surfactant proteins from frogs and horses [1–6] it occurred to us that solubilization or dispersion of fullerenes in water might be facilitated by interaction with such materials. Here we report on preliminary observations that indicate that this is indeed the case.

Ranaspumin-2 (Rsn-2) and latherin are potent surfactant proteins found in the foam nests of a species of tropical frog and horse sweat and saliva, respectively [1,7]. In its natural biological context, Rsn-2 is one of the proteins responsible for reducing aqueous surface tension to facilitate biocompatible foam nest formation, whereas latherin appears to act as a wetting agent to aid evaporative cooling in horses. These otherwise unrelated proteins are monomeric in solution, with structures that are typical of small water-soluble globular proteins (see Fig. 1), as determined by high-resolution protein NMR techniques [4,6]. Unusually however, for proteins in their native state, both have a strong propensity to adsorb onto hydrophobic surfaces or to partition into the air–water interface layer in ways consistent with their biological function [1]. Studies using small-angle neutron reflection measurements suggest that this involves relatively modest conformational changes which, at least in the case of Rsn-2, retain protein secondary structure, as shown by surface IR methods [4]. These proteins are highly soluble in their native state, with dimensions commensurate with possible interaction with fullerene molecules (Fig. 1). The van der Waals diameter of a C_{60} molecule is about 1.01 nm, with a nucleus to nucleus diameter of about 0.71 nm – small enough to fit inside the hydrophobic protein compartments that might be formed by modest hinge-bending conformational change, as envisaged for example in the case of Rsn-2 [4].

For these initial exploratory studies we have examined the effects upon mixing of C_{60} fullerene in aqueous solutions of the surfactant proteins latherin and Rsn-2, together with control experiments using other proteins and cyclodextrins for comparison. Samples were analysed by UV–vis and NMR spectroscopy, together with protein concentration analysis where appropriate. We find that these two surfactant proteins do indeed interact with and disperse C_{60} fullerene into the aqueous phase, and that they may also interact with carbon nanotubes.

2. Experimental

Materials: recombinant proteins Rsn-2 and latherin, both natural abundance and ^{15}N -enriched for NMR, were prepared, purified and characterised as previously described [3–6]. Control proteins and other reagents were obtained from commercial sources as follows, and used as received: fullerene- C_{60} (Sigma-Aldrich 483036), single-wall nanotubes (SWNT) (Sigma-Aldrich 704121; carbon nanotube,

single-walled, 0.7–1.1 nm diameter), hen egg white lysozyme (Sigma L-6876), bovine serum albumin (BSA; Sigma A-6003), β -cyclodextrin (Sigma C-4767), hydroxypropyl- β -cyclodextrin (Aldrich 33,260-7). Natural frog foam fluid containing a natural cocktail of proteins and carbohydrate components was isolated by centrifugation from nest foams of the tropical túngara frog *Engystomops* (= *Physalaemus*) *pustulosus*, as described in [2,3]. Aqueous samples were dissolved either in deionized water (cyclodextrins) or in 20 mM sodium phosphate/50 mM NaCl buffer, pH 7.5 (proteins).

Sample preparation: typically, about 10 mg of solid C_{60} was suspended in 1–2 ml of buffer containing the test protein (1–7 mg/ml), or control, in a 7 ml polystyrene bijoux tube, and stirred for several days (1–15 days) at 4 °C using a magnetic stirrer with ca. 5 mm stir bars. The stirring speed was adjusted to be fast enough to keep solid particles in suspension, but to avoid foaming. Aliquots were taken periodically at intervals for UV–vis spectroscopic analysis using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA) 0.1 or 1 mm path length, following brief centrifugation (Heraeus Biofuge Fresco bench microfuge, 16,000 $\times g$) to remove undissolved material.

Protein concentrations in solution were determined from the 280 nm UV absorbance using molar extinction coefficients, ϵ_{280} (1 cm), 6085 (Rsn-2, mw 13,415) and 10,095 (latherin, mw 22,899). For samples in which the 280 nm protein absorbance was masked by other components (C_{60} , etc.), the protein concentration was measured using a standard colorimetric method (Bio-Rad Inc. “Quick Start™” Bradford Protein Assay), calibrated using standard Rsn-2 or latherin solutions, as appropriate.

NMR: Protein- C_{60} solutions were monitored by solution NMR spectroscopy to examine the state of the protein. The ^{15}N -labelled Rsn-2 mixed with C_{60} as described above was analysed by 1D ^1H double pulsed field gradient spin echo [8] and 2D ^{15}N , ^1H heteronuclear single quantum correlation (HSQC) spectroscopy [9] at 14.1 Tesla using a Bruker AVANCE spectrometer equipped with a TCI cryoprobe, with ^{15}N -Rsn-2 concentrations of up to 530 μM .

3. Results & discussion

Stirring of solid pristine C_{60} in dilute aqueous solutions of either latherin or Rsn-2 (1–7 mg/ml) gave pale brown suspensions typical of the fullerene dispersions produced by more aggressive techniques such as grinding or sonication [10,11]. After centrifugation to remove excess C_{60} and other particulate matter, the supernatant was water-clear and straw-coloured with prominent UV–vis absorbance bands around 342 nm together with a much broader peak in the 440–460 nm range (Fig. 2; Table 1), as is typically observed for aqueous dispersions of C_{60} produced by other methods [11–18]. For comparison, control experiments using β -cyclodextrin or hydroxypropyl- β -cyclodextrin solutions (2% w/v) under the same conditions gave clear supernatants with C_{60} absorbance bands at 263(± 3), 343(± 3) and 445(± 5) nm, as previously reported [11,15,16]. Note that the shorter

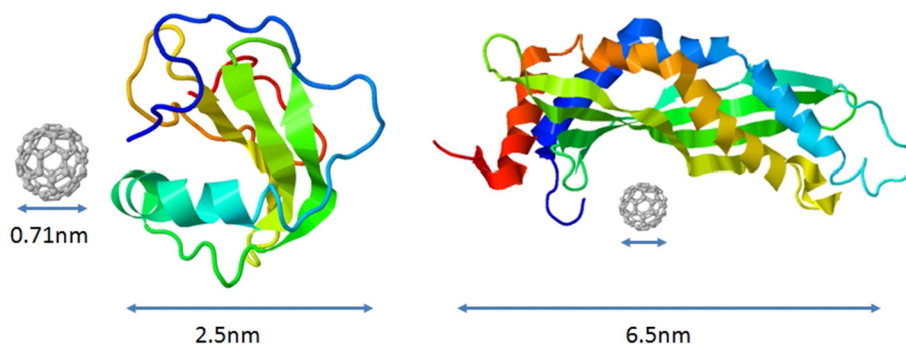


Fig. 1. Cartoon illustrating the size of C_{60} in comparison to the surfactant proteins Rsn-2 (mw 13,415 Da; left) and latherin (mw 22,899 Da; right), approximately to scale. The structures for Rsn-2 (pdb: 2WGO) and latherin (pdb: 3ZPM) are taken from [4] and [6], respectively.

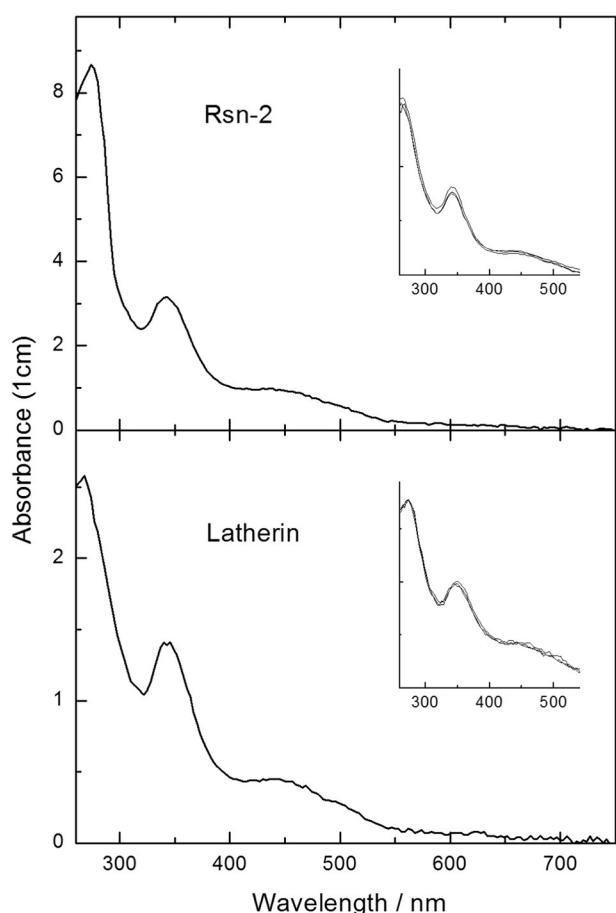


Fig. 2. Examples of UV-vis spectra of C_{60} dispersed in aqueous solutions of Rsn-2 (upper panel) or latherin (lower panel). The inserts show superimposed spectra of samples after storage at 4 °C for approximately 1, 2 and 6 months. (Absorbances measured on a Nanodrop using 0.1 or 1 mm path length, but scaled here to 1 cm path length for convenience.)

wavelength band is obscured in the latherin/Rsn-2 experiments by the intrinsic UV absorbance of the proteins in this region. No aqueous dispersion of C_{60} was seen in the absence of additives under these relatively gentle stirring conditions.

As commonly observed with heterogeneous particulate materials such as these, the rates and extents of dispersion can depend unpredictably on numerous physical factors such as stirrer shape, speed of stirring, etc. However, in our experiments, solution/dispersion with

latherin or Rsn-2 was usually apparent within 1–5 days under most circumstances.

Control experiments involving mixing of C_{60} with marginally surfactant or non-surfactant proteins (BSA and lysozyme, respectively) gave erratic and unpredictable results. As described previously by others [19,20], BSA did give some dispersion but, in our hands under these conditions, this was generally transient and the mixtures rapidly coagulated and precipitated, leaving supernatants devoid of both C_{60} and BSA, as determined by UV absorbance and colorimetric protein analysis (Bradford assay). This is consistent with the surface denaturation or partial unfolding of BSA thought to be the basis for the non-specific surface coating properties of such marginally surfactant proteins [20–22]. Similar variability involving transient dispersion and precipitation was observed with lysozyme, with no stable C_{60} dispersions obtained.

These observations with BSA and lysozyme were in marked contrast to our results with natural surfactant proteins, where the C_{60} solutions in Rsn-2 or latherin mixtures remained water-clear, stable and spectroscopically unchanged for long periods of time – up to six months in trials so far – when stored at 4 °C (see Fig. 1). Gel electrophoresis (SDS-PAGE, data not shown) showed no protein degradation in the presence of C_{60} under similar conditions. Analysis of the protein content of the C_{60} supernatants (using the Bradford assay) showed that most of the Rsn-2 or latherin (>90%) remained in solution and was not co-precipitated in the excess C_{60} pellet. Since denatured/unfolded proteins are generally very sticky and insoluble, this suggests that the natural surfactant proteins could retain significant native structure under these conditions, as we have observed elsewhere as part of their natural surfactant activities [1,3,5]. We have shown, for example, that latherin in dilute aqueous solution adheres strongly to a non-polar surface, whereas lysozyme does not under the same mild conditions [5]. It appears to be characteristic of natural surfactant proteins that interaction with non-polar interfaces can be accomplished by relatively subtle conformational change, rather than by more disruptive denaturation.

Mechanical dispersion of fullerene in water usually results in heterogeneous mixtures of C_{60} aggregates with a wide range of particle sizes, and with spectral properties that depend upon the particle size distribution [12,13], and this allows us to estimate possible limits on the C_{60} cluster sizes that we observe here. Careful studies elsewhere have shown that there is an empirical relationship between C_{60} cluster size and near-UV absorbance wavelength [10,12,13]. In particular, the position of the near-UV peak ranges from 350 to 360 nm in aqueous C_{60} clusters produced by mechanical dispersion, and is strongly correlated with cluster size, with an extrapolated λ_{\max} of around 340 nm for the hydrated monomer. For the range of Rsn-2- C_{60} samples that we have examined so far, we observed absorbance λ_{\max} values of 342 nm (± 2 nm; $n = 16$), approaching what might be expected for a hydrated C_{60} monomer [10,13]. We saw slightly longer λ_{\max} values for latherin-

Table 1
Spectroscopic properties of aqueous C_{60} dispersions.^a

	UV-vis peaks λ_{\max}/nm	Absorbance ratio A_{450}/A_{360}	Notes <i>Except where indicated, all samples were in aqueous buffer, pH 7.5</i>
Rsn-2 ($n = 16$)	342 (± 2) 440–450	0.30 (± 0.04)	Stable for at least 6 months at 4 °C Protein concentration range 1–7 mg/ml
Latherin ($n = 4$)	346 (± 3) 440–460	0.38 (± 0.06)	Stable for weeks, with no precipitation 1–2 mg/ml
β -cyclodextrin ($n = 1$)	346 450	0.32	In water (2% w/v)
Hydroxypropyl- β -CD ($n = 2$)	340 440–450	0.27	In water (2% w/v)
BSA ($n = 1$) (<i>before precipitation</i>)	352 450–460	0.43	Transient – precipitates erratically 1–10 mg/ml
Lysozyme ($n = 1$) (<i>before precipitation</i>)	344 450	0.32	Transient – precipitates erratically 1 mg/ml
Frog foam fluid ($n = 1$)	340–350	0.42	Natural mixture of Rsn-2 with a range of other proteins and carbohydrates. Total protein ca. 1 mg/ml, of which roughly 10% is Rsn-2

^a n is the number of separate observations. Solid C_{60} present in excess.

C₆₀ samples at 346 nm (± 3 nm; $n = 4$), suggesting slightly larger C₆₀ clusters (or less solvated environment) with this protein. For comparison, single experiments with cyclodextrins yield λ_{\max} values of 346 and 340 nm for β -cyclodextrin and hydroxypropyl- β -cyclodextrin solutions, respectively (Table 1).

Interestingly, for transient BSA-C₆₀ solutions prior to spontaneous precipitation, absorbance λ_{\max} was about 352 nm, consistent with the larger clusters that might be anticipated here for dispersion involving non-specific protein denaturation and aggregation. This, however, apparently conflicts with previous studies reporting stable BSA-C₆₀ dispersions with λ_{\max} of about 343 nm [20]. We are currently unable to account for this discrepancy, but it seems to reflect inherent difficulties commonly experienced with replication in these systems.

Additional particle size information can be gleaned from other aspects of the UV-vis absorbance spectra (Figs. 2, 3). In particular, it is clear that the subsidiary absorbance band around 450 nm is significantly less pronounced here than is observed for larger C₆₀ clusters produced by mechanical dispersion in the absence of additives [13]. Empirical data show a strong correlation between C₆₀ cluster size and the A_{450}/A_{360} absorbance band amplitude ratios, with A_{450}/A_{360} values increasing from 0.4 to 0.9 over the 120–400 nm hydrodynamic diameter range accessible from simple mechanical dispersion [12,13]. As shown in Table 1, A_{450}/A_{360} absorbance ratios for the natural surfactant protein dispersions presented here are significantly lower (consistent with cluster diameters probably much smaller than 100 nm) and smaller than the clusters formed by most other techniques [11,15]. However, some caution is warranted here because the correlations in absorbance properties reported for mechanical dispersions [10,12,13] relate (presumably) to fully hydrated C₆₀ clusters, whereas our observations are for clusters stabilised by interaction with protein. This may affect spectral properties of the clusters that are hard to predict at this stage.

We have no direct evidence yet for the state of the C₆₀ molecules in the latherin/Rsn-2 preparations, but one can propose various ways in which surfactant proteins might assist solubilization or dispersion in water. This might include the formation of specific stoichiometric (1:1) protein-C₆₀ monomeric complexes or, alternatively, more heterogeneous C₆₀ clusters stabilised by multiple protein interactions, either acting as a glue or coat for larger clusters in the aqueous environment. However, the existence of very large protein-C₆₀ aggregates seems to be ruled out by persistence of the protein-C₆₀ in solution, and the optical

clarity of the solutions, as well as by the UV-vis spectral properties described above.

The stoichiometries of the soluble protein-C₆₀ complexes are difficult to estimate precisely from spectral data because of major uncertainties in the UV-vis molar extinction coefficients of C₆₀ in aqueous dispersions. Literature values for the characteristic 360 nm band vary widely over the range 30,000–60,000 M⁻¹ cm⁻¹ for ϵ_{360} in water [11–14,18], with much of this variability arising from the different C₆₀ cluster sizes obtained by different methods of preparation [12]. Consequently, it is possible only to make order-of-magnitude estimates of relative concentrations here. For purposes of estimation, we have assumed a representative ϵ_{360} value of 60,000 M⁻¹ cm⁻¹ appropriate for small C₆₀ clusters [12] in order to estimate solubilized C₆₀ concentrations. In a series of experiments involving Rsn-2 concentrations in the range 1–7 mg/ml (75–530 μ M), with incubation/mixing times of 3–15 days, the molar C₆₀ concentrations in solution were always less than or equal to that of the Rsn-2. This is consistent with a stoichiometric C₆₀:Rsn-2 ratio not exceeding 1:1 in the clusters.

However, this does not necessarily imply that clusters are monomeric, and does not rule out the possibility of larger protein/C₆₀ clusters containing both components in similar proportions. In order to address this question further we have performed solution NMR experiments using ¹⁵N-enriched Rsn-2 to follow the state of the protein as C₆₀ complexes developed. In 1D-NMR and ¹⁵N-HSQC experiments with Rsn-2/C₆₀ samples (Fig. 4) we observed that the positions and linewidths of protein NMR peaks were unaltered, but their intensities were uniformly decreased (by ~15% after ~3 days and ~45% after ~10 days) showing that a proportion of the protein remained structured and monomeric in solution, while the remainder became invisible to solution NMR in the presence of C₆₀ under these conditions. This also confirms the lack of chemical degradation of Rsn-2. UV-vis analysis of the same samples indicated that, with a ¹⁵N-Rsn-2 starting concentration of 530 μ M, the C₆₀ concentration in solution increased to 45 μ M (3 days) and 280 μ M (10 days), respectively. Assuming 1:1 C₆₀:Rsn-2 stoichiometry, this corresponds to roughly 10% and 50% of the protein involved in C₆₀ clusters at 3 and 10 days, respectively. These values are of similar magnitude to those relating to loss of NMR peak intensities in the same samples, indicating that the C₆₀/Rsn-2 clusters are probably larger than monomeric, with aggregate molecular weights of at least 100 kDa, resulting in long tumbling times and linewidth broadening that make them invisible to NMR.

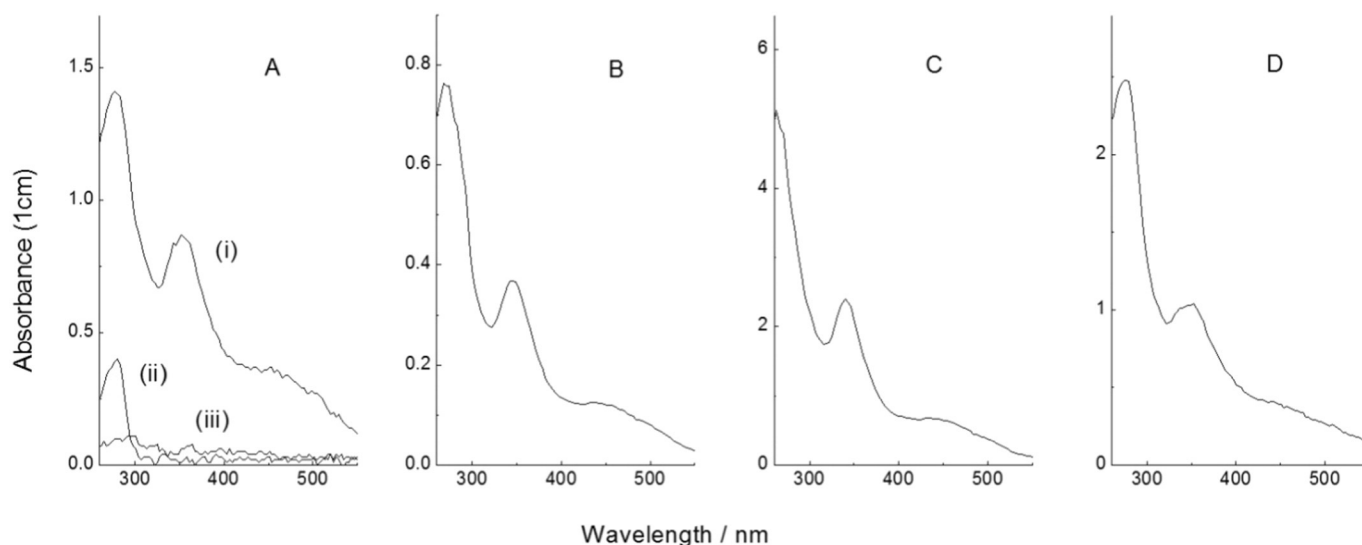


Fig. 3. Examples of UV-vis spectra of C₆₀ dispersed in aqueous solutions of: (A) BSA; (B) lysozyme; (C) hydroxypropyl- β -cyclodextrin; (D) natural frog foam nest fluid. Note that the BSA and lysozyme samples are unstable and precipitate irreversibly shortly after formation. This is illustrated in panel (A), where line (i) is the spectrum of the BSA-C₆₀ dispersion after 1 day mixing, together with the spectrum of the BSA solution alone (ii) showing the typical 280 nm protein absorbance band. Both these bands disappear after spontaneous precipitation (iii) a few days later.

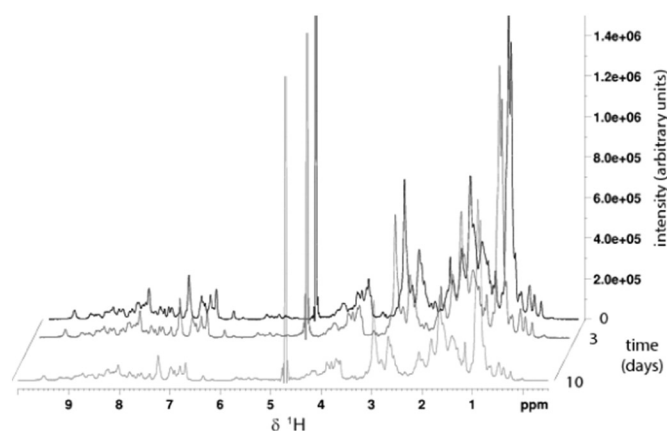


Fig. 4. 1D ^1H NMR spectra of Rsn-2 before addition of C_{60} and at 3 and 10 days after incubation with fullerene. Spectra were recorded on ^{15}N labelled Rsn-2 using a double pulsed field gradient spin echo pulse sequence incorporating ^{15}N decoupling during acquisition and processed with a 90° shifted sinebell squared window function. The signal intensities are reduced by approximately 15 and 45% after 3 and 10 days respectively. (Note: the peak at about 4.7 ppm comes from residual water.)

A cluster size of (say) 10 C_{60} molecules, each together with an accompanying Rsn-2 molecule (mw 13.4 kDa) would tumble too slowly in solution to be visible by NMR, yet would still be soluble and small enough to give the observed UV-vis spectral properties consistent with extrapolated values for small clusters [10,13].

Having demonstrated significant protein-mediated solubilization of fullerene with Rsn-2 and latherin, it is tempting to examine what might happen with other carbon allotropes of interest. Although we have not (yet) observed true solubilization, our preliminary trials with single-wall carbon nanotubes (SWNT) in water look promising. Full solubilization of SWNT with surfactant proteins might be unlikely given the large size, rod-like aspect ratio and size heterogeneity of the nanotubes, but we found that mixing SWNT with either of our proteins brings about significant changes in the suspension's visual appearance (black to pale-grey turbid suspension) that are characteristic of nanotube dispersion in water [23]. This is accompanied by total loss of protein from the supernatant (Fig. 5) upon centrifugation, consistent with adsorption of the protein onto nanotubes.

In view of potential environmental concerns, and in light of projected large-scale industrial production and application of fullerenes

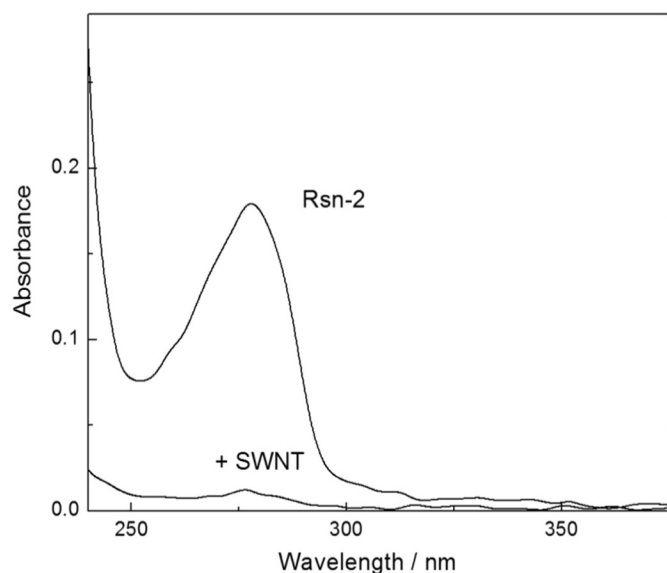


Fig. 5. UV-vis spectra illustrating the loss of the characteristic protein absorbance of Rsn-2 upon incubation with carbon nanotubes (SWNT).

and related materials together with the inevitable contamination issues, it is important to explore the ways in which such materials may interact and disperse in the wider natural environment. Although the particular proteins we deal with here are unlikely to represent significant vehicles for the environmental dispersal of C_{60} -like nanoparticles, their behaviour may exemplify such effects with natural surfactant proteins from more widespread sources. Such proteins may occur more widely in nature than currently realised [1,24], and will likely occur in complex mixtures. One of the proteins that we have been using here (Rsn-2) is merely one component in a cocktail of ranaspumin proteins that we have identified in the foam nest fluids of a tropical frog [3]. This fluid, collected in the field as part of other projects [2–4], gives very similar C_{60} dispersion to that obtained using recombinant Rsn-2 on its own. Although Rsn-2 is just one component in this cocktail, mixing of the natural foam fluid with C_{60} leads to dispersion/solubilization with UV-vis spectra similar to those obtained with pure recombinant Rsn-2 (Fig. 3, Table 1). Interestingly, judging from the λ_{max} and absorbance ratios, the apparent cluster size might be somewhat larger in this case, possibly because of the much lower protein concentration and the presence of other components in the natural fluid. Bearing in mind the way in which these frogs construct the nests in water, using a rapid “egg-beater” mixing process, it is not hard to envisage how carbon and other nanoparticles might enter the biological milieu, with subsequent uptake into biological cells and systems. These observations also have potential consequences for toxicological aspects of nanoparticles, with particular relevance to considerations of toxicity of fullerenes and carbon nanotubes, where, for example, lung surfactants and the latherin-related PLUNC proteins of the human oral and upper respiratory tract [25–27], might be significant in the uptake, tissue penetration, and/or clearance of airborne particles.

As indicated by references cited herein, non-specific aqueous dispersion of fullerenes by proteins and protein interactions with nanotubes have been previously reported, and more recent reports show that this can also be achieved by appropriately-designed peptides [28]. In the work we have presented here, we have shown how it can be possible to take advantage of the specific structural and functional properties of natural surfactant proteins (such as latherin and Rsn-2) to effect solubilization and interaction with fullerenes and related materials, avoiding more aggressive sonication or chemical modification techniques that have been employed elsewhere (see [29,30] for recent examples). This offers a convenient way in which to design biological molecules specifically for interactions with hydrophobic nanoparticles and surfaces. Recombinant protein engineering may further release the potential versatility and flexibility of natural surfactant proteins in the development of nanomolecular/biomolecular interfaces using, for example, fusions of proteins such as latherin and Rsn-2 with enzymes or other proteins with useful properties.

Author contributions

Original concept: AC.
Sample preparation and experiments: AC, SV, VD.
Data analysis and interpretation: AC, BOS, MWK.
Wrote paper: AC, with input from all co-authors.

Acknowledgements

This study stems from work originally supported by grants to M.W.K., A.C. and B.O.S. from the Wellcome Trust (GR070994MA), with studentship support from the UK Biotechnology and Biological Sciences Research Council (S.J.V.) and the University of Glasgow (V.D.).

References

- [1] A. Cooper, M.W. Kennedy, Biofoams and natural protein surfactants, *Biophys. Chem.* 151 (3) (2010) 96–104.

- [2] A. Cooper, M.W. Kennedy, R.I. Fleming, E.H. Wilson, H. Videler, D.L. Wokosin, et al., Adsorption of frog foam nest proteins at the air-water interface, *Biophys. J.* 88 (3) (2005) 2114–2125.
- [3] R.I. Fleming, C.D. Mackenzie, A. Cooper, M.W. Kennedy, Foam nest components of the tungara frog: a cocktail of proteins conferring physical and biological resilience, *Proc. R. Soc. B Biol. Sci.* 276 (1663) (2009) 1787–1795.
- [4] C.D. Mackenzie, B.O. Smith, A. Meister, A. Blume, X. Zhao, J.R. Lu, et al., Ranaspumin-2: structure and function of a surfactant protein from the foam nests of a tropical frog, *Biophys. J.* 96 (12) (2009) 4984–4992.
- [5] R.E. McDonald, R.I. Fleming, J.G. Beeley, D.L. Bovell, J.R. Lu, X. Zhao, et al., Latherin: a surfactant protein of horse sweat and saliva, *PLoS One* 4 (5) (2009).
- [6] S.J. Vance, R.E. McDonald, A. Cooper, B.O. Smith, M.W. Kennedy, The structure of latherin, a surfactant allergen protein from horse sweat and saliva, *J. R. Soc. Interface* 10 (85) (2013).
- [7] J.G. Beeley, R. Eason, D.H. Snow, Isolation and characterization of latherin, a surface-active protein from horse sweat, *Biochem. J.* 235 (3) (1986) 645–650.
- [8] T.L. Hwang, A.J. Shaka, Water suppression that works — excitation sculpting using arbitrary wave-forms and pulsed-field gradients, *J. Magn. Reson. Ser. A* 112 (2) (1995) 275–279.
- [9] S. Mori, C. Abeygunawardana, M.O. Johnson, P.C.M. Vanzijl, Improved sensitivity of hsqc spectra of exchanging protons at short interscan delays using a new fast hsqc (Fhsqc) detection scheme that avoids water saturation, *J. Magn. Reson. Ser. B* 108 (1) (1995) 94–98.
- [10] S. Deguchi, S. Mukai, T. Yamazaki, M. Tsudome, K. Horikoshi, Nanoparticles of fullerene C-60 from engineering of antiquity, *J. Phys. Chem. C* 114 (2) (2010) 849–856.
- [11] N.O. Mchedlov-Petrosyan, Fullerenes in liquid media: an unsettling intrusion into the solution chemistry, *Chem. Rev.* 113 (7) (2013) 5149–5193.
- [12] X.J. Chang, P.J. Vikesland, Uncontrolled variability in the extinction spectra of C-60 nanoparticle suspensions, *Langmuir* 29 (31) (2013) 9685–9693.
- [13] X.J. Chang, P.J. Vikesland, UV-vis spectroscopic properties of nC(60) produced via extended mixing, *Environ. Sci. Technol.* 45 (23) (2011) 9967–9974.
- [14] G.V. Andrievsky, V.K. Klochkov, A.B. Bordyuh, G.I. Dovbeshko, Comparative analysis of two aqueous-colloidal solutions of C-60 fullerene with help of FTIR reflectance and UV-Vis spectroscopy, *Chem. Phys. Lett.* 364 (1–2) (2002) 8–17.
- [15] A. Buvári-Barcza, J. Rohonczy, N. Rozlosnik, T. Gilanyi, B. Szabo, G. Lovas, et al., Aqueous solubilization of 60 fullerene via inclusion complex formation and the hydration of C-60, *J. Chem. Soc. Perkin Trans. 2* (2) (2001) 191–196.
- [16] C.N. Murthy, K.E. Geckeler, The water-soluble beta-cyclodextrin-60 fullerene complex, *Chem. Commun.* 13 (2001) 1194–1195.
- [17] P. Scharff, K. Risch, L. Carta-Abelmann, I.M. Dmytruk, M.M. Bilyi, O.A. Golub, et al., Structure of C-60 fullerene in water: spectroscopic data, *Carbon* 42 (5–6) (2004) 1203–1206.
- [18] V.M. Torres, M. Posa, B. Srdjenovic, A.L. Simplicio, Solubilization of fullerene C-60 in micellar solutions of different solubilizers, *Colloids Surf. B: Biointerfaces* 82 (1) (2011) 46–53.
- [19] S. Deguchi, T. Yamazaki, M. S-a, R. Usami, K. Horikoshi, Stabilization of C-60 nanoparticles by protein adsorption and its implications for toxicity studies, *Chem. Res. Toxicol.* 20 (6) (2007) 854–858.
- [20] H. Wu, L. Lin, P. Wang, S. Jiang, Z. Dai, X. Zou, Solubilization of pristine fullerene by the unfolding mechanism of bovine serum albumin for cytotoxic application, *Chem. Commun.* 47 (38) (2011) 10659–10661.
- [21] K. Nakanishi, T. Sakiyama, K. Imamura, On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon, *J. Biosci. Bioeng.* 91 (3) (2011) 233–244.
- [22] M. Rabe, D. Verdes, S. Seeger, Understanding protein adsorption phenomena at solid surfaces, *Adv. Colloid Interf. Sci.* 162 (1–2) (2011) 87–106.
- [23] D. Nepal, K.E. Geckeler, Proteins and carbon nanotubes: close encounter in water, *Small* 3 (7) (2007) 1259–1265.
- [24] N.R. Stanley-Wall, C.E. MacPhee, Connecting the dots between bacterial biofilms and ice cream, *Phys. Biol.* 12 (6) (2015).
- [25] J. Barnoud, L. Urbini, L. Monticelli, C-60 fullerene promotes lung monolayer collapse, *J. R. Soc. Interface* 12 (104) (2015).
- [26] K. Donaldson, R. Aitken, L. Tran, V. Stone, R. Duffin, G. Forrester, et al., Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety, *Toxicol. Sci.* 92 (1) (2006) 5–22.
- [27] L. Gakhar, J.A. Bartlett, J. Penterman, D. Mizrahi, P.K. Singh, R.K. Mallampalli, et al., PLUNC is a novel airway surfactant protein with anti-Biofilm activity, *PLoS One* 5 (2) (2010).
- [28] S. Bartocci, D. Mazzier, A. Moretto, M. Mba, A peptide topological template for the dispersion of 60 fullerene in water, *Org. Biomol. Chem.* 13 (2) (2015) 348–352.
- [29] M. Calvaresi, F. Arnesano, S. Bonacchi, A. Bottoni, V. Calò, S. Conte, et al., C60@Lysozyme: direct observation by nuclear magnetic resonance of a 1:1 fullerene protein adduct, *ACS Nano* 8 (2) (2014) 1871–1877.
- [30] S. Zanzoni, A. Ceccon, M. Assfalg, R.K. Singh, D. Fushman, M. D'Onofrio, Polyhydroxylated [60]fullerene binds specifically to functional recognition sites on a monomeric and a dimeric ubiquitin, *Nanoscale* 7 (16) (2015) 7197–7205.