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5 **Impact of Confinement on Proteins Concentrated**
6 **in Lithocholic Acid Based Organic Nanotubes**
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ABSTRACT

Organic nanotubes form in aqueous solution near physiological pH by self-assembly of lithocholic acid (LCA) with inner diameters of 20-40 nm. The encapsulation of enhanced green fluorescent protein (eGFP) and resultant confinement effect for eGFP within these nanotubes is studied via confocal microscopy. Timed release rate studies of eGFP encapsulated in LCA nanotubes and fluorescence recovery after photobleaching (FRAP) indicate that the diffusive transport of eGFP out of and/or within the nanotubes is very slow, in contrast to the rapid introduction of eGFP into the nanotubes. By encapsulating two fluorescent proteins in LCA nanotubes, eGFP and mCherry, as a fluorescence resonance energy transfer (FRET) pair, the FRET efficiencies are determined using FRET imaging microscopy at three different protein concentrations with a fixed donor-to-acceptor ratio of 1:1. Förster theory reveals that the proteins are spatially separated by 4.8 -7.2 nm in distance inside these nanotubes. The biomimetic nanochannels of LCA nanotubes not only afford a confining effect on eGFP that results in enhanced chemical and thermal stability under conditions of high denaturant concentration and temperature, but also function as protein concentrators for enriching protein in the nanochannels from a diluted protein solution by up to two orders of magnitude.

Keywords: lipid nanotubes; lithocholic acid; eGFP; confocal microscopy; stability

1. Introduction

Tubular nanoarchitectures [1] which are water soluble and comprised of nanochannels with dimensions comparable to the sizes of biomacromolecules, have great potential as nanocontainers in aqueous solution to encapsulate [2] and stabilize [3,4] biomacromolecules, and as nanocarriers for drug delivery [5,6]. The enhanced stabilities of encapsulated biomacromolecules, such as proteins, arise from the restriction of the unfolding of proteins under confinement [7,8]. A variety of amphiphilic molecules can self-assemble into tubular nanostructures, including lipids [9,10,11], glycolipids [12,13], peptides [14,15], and bile acids [16]. Lithocholic acid (LCA), one of the naturally occurring bile acids, has been shown to form nanotubes in aqueous solution [17] and exhibits interesting pH-switchable properties from spiral nanotubes assembled at alkaline pH to straight nanotubes near physiological pH [18]. Since LCA nanotubes are self-assembled in aqueous solution, they can be used to encapsulate biomacromolecules under conditions that are mild and biologically relevant, making them very attractive as biocompatible nanocontainers. In addition, because the self-assembling process involves weaker, reversible non-covalent interactions between molecules, the possibility exists for efficient release of the nanocontainer contents via manipulation of the pH or ionic strength.

Despite the potential associated with biomacromolecule encapsulation by organic nanotubes, to date there have been limited demonstrations of protein encapsulation using these types of nanocontainers (≤ 80 nm). Peptide nanotubes have been utilized for the encapsulation and stability enhancement of two different enzymes, lipase [19] and horseradish peroxidase [20]. The only other type of organic nanotube utilized for protein encapsulation has been glycolipids, as studied by Shimizu [3,21,22,23]. Self-assembled organic nanotubes derived from LCA have never been studied for protein encapsulation and stabilization, but are particularly attractive due

to the commercial availability and low cost of the bile acid building block, as well as the capability of forming nanotubes with relevant dimensions of tens of nanometers in diameter and tens of microns in length.

The encapsulation, confinement and enhanced stability of a fluorescent protein, enhanced green fluorescent protein (eGFP), in the nanochannels (20 – 40 nm) of LCA nanotubes prepared in aqueous solution near physiological pH are described, herein. The impact of organic nanotube diameter on protein encapsulation and stability was previously studied by Kameta et al. for GFP in glycolipid nanotubes [3]. Our results with LCA nanotubes corroborate many of their findings, that small diameter organic nanotubes 1) encapsulate proteins rapidly with subsequent release rates that are markedly lower, and 2) provide enhanced chemical and thermal stability for proteins while under confinement. In contrast to Kameta's study which reports a dramatic reduction in stability for proteins encapsulated within 20 nm inner diameter glycolipid nanotubes and an emphasis on electrostatic attraction as being the primary force retaining GFP inside their organic nanotubes, the LCA nanotubes reported here exhibit enhanced chemical and thermal stability for the entire size range from 20-40 nm inner diameter, and realize very slow release rates for encapsulated eGFP despite the fact that the inner surface of the LCA nanotubes carries the same negative charge as eGFP. FRET imaging spectroscopy is utilized to reveal for the first time the capability for organic nanotubes, and LCA nanotubes, in particular, to function as protein concentrators that enrich protein content within the nanochannels by up to two orders of magnitude. Explanation for this effect is supported by a theoretical study of the LCA nanotubes and interactions of proteins with the inner wall.

2. Experimental

2.1 Chemicals

Lithocholic acid (LCA), sodium phosphate dibasic and monobasic salts, sodium hydroxide, sodium phosphotungstate, and urea were obtained from Sigma-Aldrich Co. All water utilized was dispensed from an EMD Millipore Milli-Q Advantage A10 system (18.2 M Ω ·cm). Enhanced green fluorescent protein (eGFP) and mCherry fluorescent protein were purchased from BioVision, Inc. and used as received. These two proteins are very similar in size and isoelectric point. They are both expressed and purified from transformed *E. coli* and belong to a family of structurally homologous fluorescent proteins, and so they are expected to behave very similarly in terms of their encapsulation by the LCA nanotubes.

2.2 Nanotube preparation and characterization

The preparation of LCA nanotubes followed the method reported in the literature [24] with some modifications. An aqueous LCA solution was prepared by dissolving 100 mg of LCA in 100 mL of 0.1 M NaOH at pH 13 using a magnetic stirrer. To this solution, HCl (1 M or 0.1 M) is added dropwise to lower the pH to ~7.4. The final, translucent colloidal suspension is left overnight under constant stirring, and the tube formation confirmed the next day by differential interference contrast (DIC) microscopy on a Nikon Eclipse Ti inverted confocal microscope with a 100X oil objective lens (CFI apochromat TIRF, numerical aperture of 1.49 and a working distance of 0.12mm). The pH of the colloidal suspension after the formation of the nanotubes eventually equilibrated to ~ 8.0, close to the pK_a of a pure monolayer of LCA [25]. This colloidal suspension is very stable; no settlement of the suspended nanotubes is observed over the course of three months.

LCA nanotubes were characterized using transmission electron microscopy (TEM) in both transmission (full-field) and scanning (STEM) mode using a JEOL 2200FS TEM operated at 200 keV. Prior to depositing the nanotubes on a holey carbon TEM grid (TedPella, Inc.), the

nanotubes are first collected and washed with copious amount of water using a Millipore Isopore polycarbonate membrane filter (0.2 μm GTTP) and then stained by dispersing the nanotubes from the membrane into a 2 wt% sodium phosphotungstate solution using a vortex (Labnet VX100).

AFM images were obtained using a TT-AFM (AFM Workshop). The cleaned LCA nanotubes are deposited on a freshly cleaved mica surface using a VTC-100 vacuum spin coater (MTI Corporation). All AFM scans are performed in vibrating mode at room temperature with silicon cantilevers bearing a normal spring constant of 58 N/m (AppNano). Fourier-transform infrared (FTIR) spectra were acquired using a Thermo Scientific Nicolet iS50 FT-IR Spectrometer with a built-in ATR module. A sample of dried LCA nanotubes are obtained by vacuum-drying the cleaned nanotubes in a vacuum desiccator for 24 hours. The sample is then scanned over the range of 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} .

2.3 Protein encapsulation and analysis

Stock solutions of eGFP and mCherry (600 μM) were prepared in phosphate buffer at pH 7.4. Appropriate aliquots of the stock solutions are mixed with nanotube suspensions and buffer solution to give three different concentrations of protein (6.0 μM , 30.0 μM , and 90.0 μM) in the final mixtures, while keeping the volume of the nanotube suspension constant. After vortexing for one minute, the mixtures are shaken gently overnight on a Labnet VX100, and then stored at 4 $^{\circ}\text{C}$ in a refrigerator until further use.

Absorbance measurements for the proteins were recorded on a Perkin Elmer Lamda 1050 WB InGaAs spectrophotometer. Fluorescence spectra of the proteins in bulk solution and encapsulated in nanotubes were recorded using a FluoroMax-3 spectrofluorimeter (Horiba Jobin

Yvon). Before measuring the fluorescence spectra of proteins encapsulated in the nanotubes, the proteins adsorbed on the outer surface of the nanotubes are removed. This is achieved via repeated washing during the filtration step, as described earlier in the sample preparation for TEM and confirmed by recording the fluorescence spectra of the filtrate collected in each wash until the fluorescence emission intensity (488 nm excitation for eGFP and 561 nm for mCherry) becomes negligible.

Samples for the confocal fluorescence microscope are prepared by depositing the cleaned nanotubes encapsulating fluorescent proteins on a glass coverslip (VWR, 24x50 mm, No. 1.5) or tissue culture dish with a cover glass bottom (World Precision FluoroDish, FD35-100). A Nikon confocal microscope with a 100X oil objective and two Coherent Sapphire lasers (488 nm and 561 nm, 100 mW each) are used in the acquisition of the fluorescent images.

For the fluorescence recovery after photobleaching (FRAP) studies, the photobleaching step is operated at 100 mW laser power for 2 s by focusing the laser beam on a small region of interest (ROI) along the length of a nanotube being interrogated. To minimize photobleaching when collecting images during the pre-bleach and recovery phases, the laser power is set to its lowest permissible value of 0.3 mW.

Fluorescence resonance energy transfer (FRET) measurements are realized on the same confocal microscope using Nikon's FRET module add-on. In order to properly correct for crosstalk and/or spectral bleedthrough, three sets of samples are prepared at three protein concentrations of 6.0 μM , 30.0 μM , and 90.0 μM : nanotubes encapsulated with eGFP alone (Donor), nanotubes encapsulated with mCherry alone (Acceptor), and a mixture of eGFP and mCherry in a 1:1 ratio (FRET pair). The images recorded for nanotubes encapsulating donor or acceptor alone are used to derive the coefficients for correcting spectral bleedthrough and

crosstalk. In all cases, three images are acquired: 1) 488 nm laser excitation with 515-555 nm emission filter set, 2) 488 nm laser excitation with 590-650 nm filter set, and 3) 561 nm laser excitation with 590-650 nm filter set. The method of Wouter et al. [26] is used in deriving the corrected FRET images. Förster's theory [27] is applied in calculating the mean distance between the donor and acceptor protein molecules r_{AD} , by using the following parameters: index of refraction, n , 1.33; overall quantum yield for eGFP fluorescence, Q_D , 0.6; factor reflecting the relative orientation of the transition dipole moments (rapid random orientation), κ^2 , 0.67; and an overlap integral for the overlap of the donor emission and acceptor absorbance spectra, J , of $1.4 \times 10^{-13} \text{ M}^{-1} \text{ cm}^{-1} \text{ nm}^4$ (see Fig. 1). In order to provide statistically relevant results, more than 40 nanotubes were analysed for each of the three concentrations.

Releasing studies of the encapsulated proteins from the inner space of the nanotubes at room temperature were carried out using the confocal microscope. The cleaned nanotubes encapsulating eGFP are deposited on the cover glass bottom of a tissue culture dish. Individual nanotubes are located and buffer solution is added to submerge the nanotubes, followed by capping the tissue culture dish to prevent buffer solution evaporation. Fluorescence images are recorded in 100-minute intervals at the minimum laser power for as long as 5 hours. The fluorescence intensities of the individual nanotubes are measured using area integration of the fluorescent images.

2.4 Protein denaturation studies

The stability of eGFP with respect to chemical denaturation, both in bulk solution and when encapsulated by nanotubes, is evaluated by adding free eGFP or nanotubes encapsulating eGFP into a buffer solution containing 6 M urea at pH 8.0 and measuring the change in fluorescence intensity over time on the spectrofluorimeter. For the thermal denaturation studies, a solution

(300 uL) containing free eGFP or nanotubes encapsulating eGFP is added to a cuvette with a secure cap and heated in a water bath at elevated temperatures. At various points in time, the cuvette is removed from the water bath and a fluorescence spectrum recorded using the spectrofluorimeter, whereupon the cuvette is immediately returned to the water bath for further thermal treatment.

3. Molecular dynamics (MD) simulations

A residue-level coarse grained model is adopted to simulate the dynamics of the green fluorescent protein (GFP) inside nanotubes. Each amino acid of a GFP is represented by a single bead centered about the corresponding C_α atom. Coordinates of C_α atoms for GFP are acquired from an experimental structure deposited in the Protein Data Bank (PDB ID: 1GFL). Nanotubes are constructed by beads of radius, 3 Å, with three different inner diameters of 20 nm, 30 nm and 40 nm. Interactions between GFPs are governed by pairwise potentials for all pairs of residues, given by Kim and Hummer [28]. These potential energies were developed to simulate protein-protein interactions and tested against various protein complexes and shown to yield binding constants and structures in agreement with experiments [29]. Interactions between GFPs and nanotubes are considered to have either the Lennard-Jones (LJ) potential between protein residues and beads of nanotubes for representing van der Waals (vdW) interactions or purely repulsive potentials. The LJ potential energy between a residue, i , of a GFP and a bead of a nanotube is given by,

$$u_i(r) = 4\varepsilon \left[\left(\frac{\sigma_i}{r} \right)^{12} - \left(\frac{\sigma_i}{r} \right)^6 \right] \quad (1)$$

where ε is the interaction strength, σ_i is the interaction range given by the sum of the vdW radius of the residue, i , and the bead radius, 3 Å, and r is the distance. For repulsive interactions, only

the first term in the above equation is retained. For attractive LJ potential, ϵ of 0.1, 0.2, and 0.5 kcal/mole is chosen, and for the repulsive potential, $\epsilon = 1.0$ kcal/mole is used.

To estimate the diffusion constants of the GFP inside LCA nanotubes, molecular dynamics (MD) simulations are performed using the LAMMPS software [30]. A periodic boundary condition along the axis of a nanotube is considered to simulate the infinitely long tube. During the course of the simulations, the nanotubes are immobilized, since our goal is to estimate the diffusion constants for GFPs inside nanotubes. Furthermore, GFPs are treated as rigid bodies, since they remain structurally intact. To compare the data with those in bulk, MD simulations are performed on GFPs in a cubic box with periodic boundary conditions at experimentally derived protein concentrations (6 μM , 30 μM , and 90 μM). Each simulation run lasts for a total of 100 fs with a time-step of 100 fs; the configurations are stored for analysis every 100 ps. The trajectories of the first 500 ns are discarded, as it is shown that the system is well equilibrated after that.

The translational diffusion coefficient of GFP is estimated by calculating the mean square displacement (MSD) of the center of mass of a GFP, given by,

$$\mathbf{MSD}(t) = \langle (\mathbf{r}(t + t_0) - \mathbf{r}(t_0))^2 \rangle \quad (2)$$

where $\mathbf{r}(t)$ is the three-dimensional position of the center of mass of a GFP at time t , while $\langle \rangle$ denotes the time average over an entire trajectory. The translational diffusion coefficient, D , for a protein in three-dimensions is then given by,

$$\mathbf{MSD}(t) = 6Dt \quad (3)$$

while inside nanotubes (which are effectively one-dimensional systems for small inner diameters), one has,

$$MSD(t) = 2Dt \quad (4)$$

4. Results and Discussion

4.1 LCA nanotubes

TEM (Fig. 2a) and AFM images (Fig. 2b) confirm the formation of LCA nanotubes whose inner diameters are comparable with the size of protein molecules. Analysis of TEM and AFM images reveal that the outer diameters of LCA nanotubes vary from 30 – 50 nm with inner diameters ranging from 20-40 nm. The lengths of the nanotubes are in the range of 15 - 30 μ m, and the measured wall thickness of the nanotubes, ~5 nm, is close to the molecular length of LCA trimers linked via hydrogen bonds in head-to-tail order and arranged in a fashion of alternating concave and convex orientation (Fig. 3). The mechanism of tube formation from LCA likely involves twisted and helical ribbons, and theories based on molecular chirality (LCA is chiral) describe molecular packing as taking place at a non-zero angle with respect to the nearest neighbors, further inducing a twist and the formation of cylindrical morphologies [17]. LCA nanotubes are very robust to the electron beam and do not appear to change in either TEM or STEM mode under high-dose irradiation.

The FTIR spectrum from a dried sample of LCA nanotubes is presented in Fig. 4a. This spectrum bears a sharp, strong band due to the C=O stretching of the carboxyl group –COOH at ~1698 cm^{-1} , in addition to a band of medium intensity at ~1536 cm^{-1} , due to the asymmetric COO^- stretching of the carboxylate anion. The FTIR results reveal the co-existence of –COOH and – COO^- groups in the LCA nanotubes and that the majority of –OH groups are involved in

hydrogen bonding, since the IR bands located above 3500 cm^{-1} (3572 and 3647 cm^{-1}) are very weak. It can be postulated that the initial aggregation of LCA molecules involves the combination of the intermolecular H-bonding between the OH group on the concave α -face of one LCA molecule and the $-\text{COOH}/-\text{COO}^-$ group of another, and the hydrophobic interaction between the convex β -faces, in an alternating manner (Fig. 3). It is likely that the inner and outer surfaces of the nanotube are identical, carrying some negative charges due to the partial deprotonation of the $-\text{COOH}$ moieties.

4.2 Stabilities of LCA nanotubes

Jean et al.³¹ report that LCA nanotubes obtained at pH 12.5 maintain their structural properties with increasing temperature up to $57.5\text{ }^{\circ}\text{C}$, but then disintegrate over a short temperature window of ca. 3°C , as studied using small-angle X-ray scattering (SAXS). Their cryo-TEM results reveal that the heating process induces a transition to much larger structures prior to their final dissolution. In this study, the thermal stability of LCA nanotubes prepared at pH 7.4 is evaluated by qualitatively monitoring the visual appearance of the nanotube suspension during heating, measuring the pH change of the suspension after heating, and examining the nanotubes collected after heating by DIC microscopy and FTIR. When heating the nanotube suspension at 80°C , no visible change is observed from the appearance of the nanotube suspension, suggesting no significant dissolution at this temperature for up to 90 min. However, the pH of the nanotube suspension increases by 0.8 units, and DIC microscopy shows the appearance of larger diameter nanotubes when comparing to the sample before heating. The relative intensities of infrared C=O stretching bands (Fig. 4b) show an increase in the ratio of $-\text{COOH}/-\text{COO}^-$, implying that the increase in pH is caused by protonation of $-\text{COO}^-$ groups initially present in the nanotubes during the heating treatment, and the creation of larger

structures, likely related to the dominance of the now protonated LCA molecules. Nanotube suspensions and the isolated nanotubes were also examined after heating at 70°C for 90 min. The pH of the suspension in this case only experiences 0.3 units of increase, and there are no noticeable differences in IR spectra for nanotubes before and after the heat treatment.

Chemical stability in the presence of the denaturant, urea, is investigated by monitoring individual nanotubes immersed in a buffer solution containing 6 M urea using DIC microscopy. No change or dissolution of the nanotubes is observed for up to 5 hrs.

4.3 Protein encapsulation and confinement

Kameta et al. [Error! Bookmark not defined.] used a fluorescent acceptor dye immobilized on the inner surface of their 10, 20 and 80 nm i.d. bolaamphiphile nanotubes to visualize the transportation of GFP in the nanochannels and to detect release of the encapsulated GFP to the bulk solution in real time, based on FRET. They found that the diffusion constants and release rates of GFP markedly decrease as the inner diameter of the nanotubes are decreased. Despite this decrease, the GFP migrated into a 2 μ m length of 20 nm i.d. nanotube, for example, in less than a second. Visualization of the transportation of eGFP in and out of the nanochannels of LCA nanotubes in real time could not be performed here due to the absence of immobilized fluorescence acceptors on the inner surface of the nanotubes. In order to gain some insight into the transport of eGFP into the LCA nanotubes, eGFP was mixed with the LCA nanotubes for a period of five minutes, cleaned according to the experimental protocol, and examined using confocal fluorescence microscopy. The results indicate that eGFP is encapsulated inside the nanochannel within this time period of five minutes.

In contrast to the relatively rapid introduction of eGFP into the nanotubes, the release rate for the encapsulated eGFP from the nanotubes is very slow at room temperature. The release rate

studies are performed using the confocal fluorescence microscope to monitor the change in average fluorescence intensity of individual nanotubes immersed in a buffer solution. Regardless of whether the nanotubes are equilibrated in 6.0 μM , 30.0 μM , or 90.0 μM concentrations of eGFP prior to the harvesting and cleaning steps, the loss of fluorescence intensity is less than 20% after 5 hrs (Fig. 5). In the 6.0 μM case, the selected nanotubes remained inside the microscope's field of view in a pool of buffer solution for a total of 18 hrs, reporting just a 26 % loss in fluorescence intensity.

In addition to characterizing the release behaviour for encapsulated eGFP from the inner volume of the nanotubes to the surrounding aqueous environment, the transportation behaviour of eGFP within the nanochannel of the nanotubes is also of interest. FRAP is an effective method for assessing this transport. The FRAP measurements are performed following the procedure described in the experimental section with a high laser power to photobleach a localized ROI on an individual nanotube, and the lowest permissible laser power to acquire the images both before and after the photobleaching step. The FRAP results presented in Fig. 6 are in agreement with the results obtained in the release rate studies: the transportation of eGFP within the nanotube is very slow and there is little if any recovery in fluorescence intensity 60 min after photobleaching.

The rapid introduction of eGFP into the interior of the LCA nanotube and in contrast, its much slower release rate, were observed similarly by Kameta et al.^{Error! Bookmark not defined.} for their bolaamphiphile organic nanotubes. This behaviour is ascribed to an electrostatic attraction between the cationic charge on the inner wall and the anionic GFP, in addition to a strong reduction in diffusion within restricted nanospaces [32]. However, when they raised the pH to the point that the amino groups on the inner wall of the nanotubes became deprotonated, they noticed a significant increase in the release rates from all three different sized nanotubes (10, 20,

80 nm), resulting in a 26 -58 % release ratio after 20 hrs, which they attributed to a decrease in the electrostatic attraction. In the case of the LCA nanotubes examined here, the wall of the nanotube is negatively charged due to the partial deprotonation of –COOH groups, as supported by the IR spectrum, suggesting the existence of electrostatic repulsion. Despite this electrostatic repulsion, the release rates for eGFP from LCA nanotubes equilibrated with three different initial concentrations are comparable to the release rates determined by Kameta et al.^{Error! Bookmark not defined.} for their 10 and 20 nm id nanotubes at pH 8.9. This implies that there are additional interactions between the eGFP and the inner surface of the LCA nanotubes that overwhelm the electrostatic repulsion. More than likely, these interactions include a combination of hydrogen bonds and vdW interactions that play a major role in retaining eGFP inside the LCA nanotubes.

FRET imaging microscopy is a powerful technique for studying molecular interactions inside living cells and enabling a visualization of the spatial distribution of FRET efficiency throughout the image, rather than registering only an average over the entire cell or population, such as evident in macroscopic solution measurements of FRET [33]. To understand the extent of confinement or crowding for eGFP in LCA nanotubes, as exemplified by protein to protein distances, two fluorescent proteins of similar size, eGFP and mCherry, were chosen as a FRET pair. As shown in Fig. 1, the emission of eGFP overlaps with the absorbance spectrum of mCherry, such that when these two fluorescent proteins are within very close proximity (1-10 nm), the excitation of eGFP results in fluorescent emission from mCherry through non-radiative dipole-dipole coupling. FRET imaging microscopy is utilized here to study the interactions of this FRET pair when co-encapsulated in LCA nanotubes. The TEM results indicate that the LCA nanotubes' outer diameters range from 30-50 nm and DIC microscopy is sufficient to resolve two major populations of nanotubes, referred to as smaller and larger nanotubes in Fig.

7a. The FRET efficiencies derived from these two major populations of LCA nanotubes are plotted against three initial equilibrating protein concentrations, together with the calculated mean distance between the donor and the acceptor protein molecules, r_{DA} . The FRET efficiencies range from 13% to 51% for the smaller i.d. tubes and from 8% to 38 % for the larger i.d. tubes, as the initial concentration of each protein increases from 6 μ M to 90 μ M. Accordingly, the calculated mean distance between donor and acceptor protein molecules, r_{DA} , decreases from 6.6 nm to 4.8 nm in the smaller nanotubes, and from 7.2 nm to 5.2 nm in the larger nanotubes. Since the ratio of donor concentration/acceptor concentration is 1, the mean distance between the donor and acceptor can be assumed to be the same for each pair. Fig. 7b is a corrected FRET image for two LCA nanotubes encapsulating both eGFP and mCherry after equilibrating with a solution containing 90 μ M of each protein.

In order to appreciate the significance of these small r_{DA} values, a comparison to the mean nearest-neighbour distance in a random mixture of identical protein concentrations in free solution provides some critical insight. Assuming the initial concentration of each protein is 30 μ M and the total protein concentration is 60 μ M, the mean nearest neighbor distance, d , in bulk solution will be ~30 nm ($d = (1/6.02 \times 10^{23} \times C)^{1/3}$ where C is the molar concentration). By comparing the mean nearest neighbor distance, d , in bulk solution to the mean distance between donor and acceptor, r_{DA} , for proteins encapsulated in the LCA nanotubes, the estimated concentration factor resulting from the encapsulation ranges from 83 to 480 times, a factor which increases as the bulk concentration decreases. It is clear that the high inner surface to volume ratio of the LCA nanotubes facilitates substantial enrichment as well as the confinement of the proteins inside the LCA nanotubes.

4.4 Chemical and thermal denaturation

The confinement effect for proteins encapsulated in nanostructures with dimensions that are comparable to the diameters of proteins has been demonstrated to result in stabilization of the proteins, giving rise to an enhanced resistance to chemical and thermal denaturation. It is evident from Fig. 8 that the confinement effect for eGFP encapsulated in LCA nanotubes, likewise, results in an enhanced resistance towards chemical denaturation by 6 M urea, when compared to free eGFP in solution. Note that within the first 25 minutes of exposure to 6M urea, the free eGFP solution loses 45% of its fluorescence intensity, while the solution containing LCA nanotubes encapsulating eGFP only reports a 2% decrease. After mixing with 6 M urea for 5 hrs, the relative fluorescence intensity of the free eGFP drops to 34%, while the eGFP confined inside LCA nanotubes maintains a relative fluorescence intensity as high as 84% for all three initial eGFP concentrations.

Thermal stability studies of LCA nanotubes prepared at pH 12.5 by Jean et al. [31] reveal a threshold at 57.5°C upon which structural changes occur. Bearing this in mind, thermal denaturation studies of eGFP in bulk solution and encapsulated in LCA nanotubes were carried out at 57°C. The results presented in Fig. 9 demonstrate the enhanced thermal stability for eGFPs when confined inside LCA nanotubes. After 3 hrs of heating at 57°C, the decrease in relative fluorescence intensity for free eGFP is more than double that of the eGFP encapsulated in LCA nanotubes. It is noteworthy that when heating the nanotubes containing eGFP to 62°C, a temperature above the structural threshold for the disintegration of the LCA nanotubes determined by Jean et al. [25], the results appear identical to the 57°C temperature study for the first 30 min. After the first 30 min, a steeper decline in relative fluorescence intensity is observed, followed by a steady decrease in fluorescence intensity with nearly the same slope as the 57°C study. This suggests that even though some structural changes likely take place at the

elevated temperature of 62°C, there is no major disintegration of the nanotubes. Regardless of the structures formed during the heat treatment, they still afford a good deal of protection for encapsulated eGFP's against thermal denaturation.

The enhanced chemical and thermal stabilities observed here for eGFP encapsulated by LCA nanotubes with diameters in the 20-40 nm range are in sharp contrast to the results reported by Kameta et al. [3]. In the previous study Kameta et al. observed that the confinement of GFP by glycolipid nanotubes with 20 nm inner diameter actually destabilized the protein, resulting in lower chemical and thermal stabilities when compared to free GFP in solution. No explanation was provided for this result which was in contrast to the improved stability of GFP encapsulated in glycolipid nanotubes whose diameters were 10 and 80 nm.

4.5 MD simulations

An example of a simulation set-up for GFPs encapsulated by a nanotube is shown in Fig. 10a. Multiple copies of GFP are present in order to realize estimated GFP concentrations in LCA nanotubes that mirror the factor of 100 increase in concentration described earlier from the FRET results (600 μ M, 3 mM, and 9 mM). The MD simulations show that GFPs in bulk follow a random diffusive motion. Fig.10b shows the MSD calculated for 600 μ M concentration (taking into account the two orders of magnitude increase in concentration) and the excellent fit using equation (3). The estimated diffusion coefficient is approximately 10^{-6} cm²/s, which is close to the experimental value, 8.7×10^{-7} cm²/s [34]. This confirms that the coarse-grained model with implicit solvent is adequate to study the dynamics of GFPs. GFPs generally do not form dimers or oligomers in the simulation runs, as the interactions between GFPs are shown to be weak. This, again, agrees with experimental observation.

Inside nanotubes, GFPs still undergo a diffusive motion for both repulsive and attractive

walls, as shown in Fig. 10b. The diffusion coefficients for GFP inside nanotubes relative to that in bulk are shown in Fig. 10c for the repulsive wall and the vdW wall with $\varepsilon = 0.1, 0.2$ and 0.5 kcal/mole. For the repulsive wall, the diffusion constant decreases by a factor of four for the nanotubes, while in the presence of attractive vdW interactions, the diffusion of GFP is slowed down by an order of three at the highest attraction (0.5 kcal/mole). Furthermore, some GFPs ($\sim 20\text{-}30\%$) are observed to be immobilized inside nanotubes due to the attractive wall at $\varepsilon \geq 0.5$ kcal/mole. Even for small ε ($= 0.1$ kcal/mole), some GFPs are adsorbed on the wall and diffuse along the nanotube. The dependence of the diffusion coefficient on the GFP concentration has been shown to be minimal for the values chosen in the simulations ($600\text{ }\mu\text{M}$, 3mM and 9 mM).

Conclusions

LCA nanotubes are prepared via self-assembly in aqueous solution at pH 7.4 using naturally occurring lithocholic acid. The inner diameters of these nanotubes range from $20 - 40\text{ nm}$, making them comparable to the size of protein molecules. The nanotubes, themselves, exhibit excellent stability to elevated temperature and high concentrations of protein denaturant exposure. They encapsulate eGFP from bulk solution and provide a confined environment to enhance its thermal and chemical stabilities. These attributes can be very attractive for applications requiring protein stabilization under harsh environmental conditions. Despite the fact that the inner surface of the LCA nanotubes carries the same negative charge as eGFP, the release rate of encapsulated eGFPs from the nanotubes is very slow. Once inside, the transport rate of eGFP is also very low, a result which agrees with the slow release rate and can be attributed to the attractive forces evident between the inner surface of the LCA nanotubes and the protein via hydrogen bond formation and vdW interactions. Comparing the mean distances of protein molecules in the nanotubes, as derived from FRET, with that in bulk solution, reveals

that LCA nanotubes not only provide a biologically friendly environment to protect proteins against chemical and thermal denaturation, they also act as a protein concentrator to enrich protein molecules within their inner volume. MD simulations support the experimental findings: under the attractive wall condition, the diffusion of a GFP can be slowed down by as much as three orders of magnitude when compared to a GFP in bulk solution. Additionally, if the interaction strength, ε , is greater than 0.5 kcal/mole, some portion of GFP molecules are immobilized on the inner surface of the nanotubes.

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Figure Captions

Fig. 1. Normalized fluorescence emission intensity for eGFP (green dashed line), absorbance intensity for mCherry (red solid line) and mCherry fluorescence emission intensity (magenta dashed dot line).

Fig. 2. Bright-field TEM micrograph (a) and AFM image (b) of the LCA nanotubes.

Fig. 3. Schematic showing hydrogen bonding and hydrophobic interactions among LCA molecules and their self-assembly into nanotubes.

Fig. 4. FTIR spectra of LCA nanotubes isolated from a) the original suspension at pH 8.2, and b) the heat-treated solution after 90 min at 80°C and a final pH of 9.0.

Fig. 5. Release rate of eGFP from the LCA nanotubes with initial equilibrating concentrations of 6 μM (black square), 30 μM (blue triangle) and 90 μM (red sphere). Due to the experimental challenge of maintaining the same LCA nanotube submerged in a pool of buffer solution in the field of view of the confocal microscope for an extended period of time, 1080 minutes for the 6 μM initial eGFP equilibrating concentration, only a single data point was collected for this experiment and so no error bar is available.

Fig. 6. FRAP results showing the fluorescence intensity during the pre-bleach stage (0-60 s), and after the photobleaching step (60-3660 s) for an eGFP encapsulated lipid nanotube. The inset images show the nanotube prior to (a), immediately (b) and 3600 s (c) after the photobleaching step.

Fig. 7. a) Corrected FRET image, and b) FRET efficiencies (red sphere – smaller nanotubes, black square – larger nanotubes) and mean donor to acceptor distances, r_{DA} , (magenta diamond – smaller nanotubes, blue triangle – larger nanotubes) vs initial protein concentrations.

Fig. 8. Chemical denaturation in 6M urea at pH 7.4 of eGFP in bulk solution (magenta diamond) and eGFP encapsulated in LCA nanotubes with initial equilibrating concentrations of 6 μM (black square), 30 μM (blue triangle) and 90 μM (red sphere).

Fig. 9. Change in relative fluorescence intensity after heating solutions containing free eGFP at 57 $^{\circ}\text{C}$ (red sphere) and eGFP encapsulated in LCA nanotubes at 57 $^{\circ}\text{C}$ (black square) and 62 $^{\circ}\text{C}$ (blue triangle).

Fig. 10. Diffusion of GFP inside nanotubes. a) Schematics of a simulation setup for GFPs inside a 20 nm nanotube. Beads of the nanotube are depicted in green, while GFP molecules are in a different color scheme. b) Mean square distance (MSD) of a GFP as a function of time for bulk (red), 20 nm nanotube with repulsive interactions (blue), and 20 nm nanotube with attractive interactions (green; $\varepsilon = 0.1$ kcal/mole). The dashed lines are the least-square fits to equations (3) and (4). c) Measured diffusion coefficients inside nanotubes relative to that in bulk for repulsive walls (red) and attractive walls with $\varepsilon = 0.1$ (blue), $\varepsilon = 0.2$ (purple), and $\varepsilon = 0.5$ (green) at 600 μM concentration.

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Fig. 6.

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524 **Fig. 9.**

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528 a)

b)

c)