Green Fluorescent Protein in Inertially Injected Aqueous Nanodroplets

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We inertially inject and study the contents of optically trappable aqueous nanodroplets (hydrosomes) emulsified in a perfluorinated matrix. A new piezoelectric actuated device for production of single hydrosomes on demand is introduced. Hydrosomes containing enhanced green fluorescent protein (EGFP) were injected, optically trapped, and held at the focus of an excitation laser in a confocal microscope, and single-molecule photobleaching events were observed. The rotational diffusion time of EGFP in trapped hydrosomes was measured using time-resolved fluorescence anisotropy. In free solution, the mean rotational diffusion time was determined to be 13.8 ± 0.1 ns at 3 μM and 14.0 ± 0.2 ns at 10 μM. In hydrosomes, the mean rotational diffusion time was similar and determined to be 12.6 ± 1.0 ns at 3 μM and 15.5 ± 1.6 ns at 10 μM. We conclude that the rotational motion inside the nanodroplets is consistent with rotation in free solution and that the protein therefore does not aggregate at the water−oil interface. Protein can be confined in hydrosomes with high efficiency using this technique, which provides an alternative to surface attachment or lipid encapsulation and opens up new avenues of research using single molecules contained in fluid nanovolumes.

Introduction

Water-in-oil (w/o) emulsions have been used to effectively sequester biomolecules into discrete micrometer-sized liquid droplets to monitor biochemical reactions. Single enzyme activity was first demonstrated using β-D-galactosidase in silicone oil emulsions.[1] Similar measurements have been made on chymotrypsin.[2] Amplification of single plasmid DNA templates using polymerase chain reaction (PCR) was demonstrated as was the detection of HIV RNA using reverse transcription PCR.[3] Similarly, expression of *Hae*III methyltransferase was shown to occur from a transcription/translation reaction mixture in aqueous droplets in mineral oil.[4] More recently, cell-free synthesis of enhanced green fluorescent protein (EGFP)[5] has been demonstrated in w/o emulsions with aqueous-phase droplets that are 1−100 μm in diameter.[6]

Here we extend the use of w/o emulsions by introducing a new method to make emulsified single aqueous nanodroplets on demand. We developed a piezoelectric actuated micropipet[7] to inertially inject individual (diameter ≥700 nm, Supporting Information), optically trappable, aqueous nanodroplets (hydrosomes) containing EGFP into an immiscible matrix composed of perfluorotriamylamine (available from 3M as FC-70). Fluorescent proteins couple fluorescence with structural integrity, making them an ideal system to evaluate protein confinement, immobilization, or isolation schemes. In particular, we would like to know how efficiently we can confine biomolecules and whether they remain functional in their confinement. Reiner et al.[8] previously demonstrated optical detection of single red fluorescent protein (RFP) molecules confined in optically trapped hydrosomes suspended in 2-(nonafluorobutyl)heptafluorofuran (available from 3M as FC-77 (index of refraction $n = 1.28$)). They used ultrasonication to generate w/o emulsions using 0.1% (v/v) Triton X-100 and found that half the RFP molecules survived agitation.[8] By contrast to ultrasonication, the method introduced here to create single emulsified aqueous nanodroplets is minimally destructive to the encapsulated protein.

To investigate the efficacy of this technique, we study the fluorescence of EGFP confined in hydrosomes that are optically trapped in the detection volume of a confocal microscope. Hydrosomes containing EGFP were held at the focus of a fluorescence excitation laser using a single-focus optical trap (Figure 1). The refractive index of FC-70 ($n = 1.30$) is lower than that of water ($n = 1.33$), which permits optical trapping. In a first set of experiments designed to demonstrate confinement of single EGFP molecules, continuous wave (cw) laser excitation was used to excite fluorescence from EGFP in injected hydrosomes, and single-step photobleaching events were observed. In a second set of experiments designed to elucidate the effects of confinement on EGFP dynamics, we measured the rotational diffusion time of EGFP using time-resolved fluorescence anisotropy. The rotational diffusion time of freely diffusing EGFP was measured and compared with that of ensembles of EGFP molecules confined in hydrosomes.

Materials and Methods

Materials. EGFP suspended in phosphate-buffered saline (PBS) at pH 7.2 was purchased from BioVision (Mountain View, CA, www.biovision.com, catalog no. 4999-100). Triton X-100 detergent was added at 0.1% (v/v) except where its absence is explicitly noted. Sulforhodamine B (SRB) was purchased from Sigma-Aldrich (St. Louis, MO, www.sigmaaldrich.com, catalog no. S-9012) and suspended in Tris−EDTA. Perfluorotriamylamine (FC-70) and 2-(nonafluorobutyl)heptafluorofuran (FC-77) were both acquired

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(1) Rotman, B. *Proc. Natl. Acad. Sci. U.S.A.* **1961**, *47*, 1981−1991.

(2) Lee, A. I.; Brody, J. P. *Biophys. J.* **2005**, *88*, 4303−4311.

(3) (a) Nakano, M.; Komatsu, J.; Matsuura, S.; Takashima, K.; Katsura, S.; Mizuno, A. *J. Biotechnol.* **2003**, *102*, 117−124. (b) Nakano, M.; Nakai, N.; Kurita, H.; Komatsu, J.; Takashima, K.; Katsura, S.; Mizuno, A. *J. Biosci. Bioeng.* **2005**, *99*, 293−295.

(4) Tawfik, D. S.; Griffiths, A. D. *Nat. Biotechnol.* **1998**, *16*, 652−656.

(5) Cormack, B. P.; Valdivia, R. H.; Falkow, S. *Gene* **1996**, *173*, 33−38.

(6) (a) Pietrini, A. V.; Luisi, P. L. *ChemBioChem* **2004**, *5*, 1055−1062. (b) Hase, M.; Yamada, A.; Hamada, T.; Baigl, D.; Yoshikawa, K. *Langmuir* **2007**, *23*, 348−352.

(7) Supporting Information and manuscript in preparation.

(8) Reiner, J. E.; Crawford, A. M.; Kishore, R. B.; Goldner, L. S.; Helmerson, K.; Gilson, M. K. *Appl. Phys. Lett.* **2006**, *89*, 013904.

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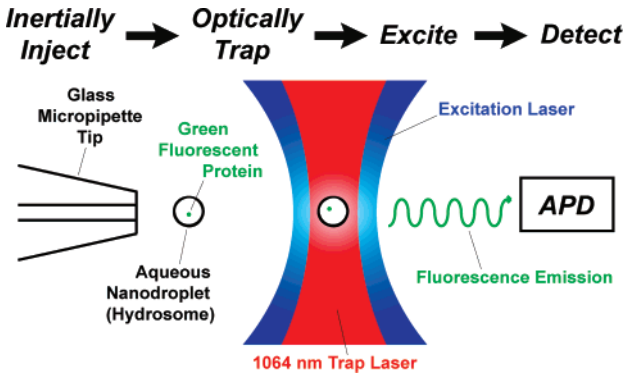

Figure 1. Schematic of hydrosomes inertially injected on demand into an immiscible perfluorotriamylamine (FC-70) liquid matrix using a piezoelectric actuated micropipet in a confocal microscope. EGFP molecules in the hydrosomes are optically excited, and fluorescence is detected with an avalanche photodiode (APD) connected to photon-counting electronics.

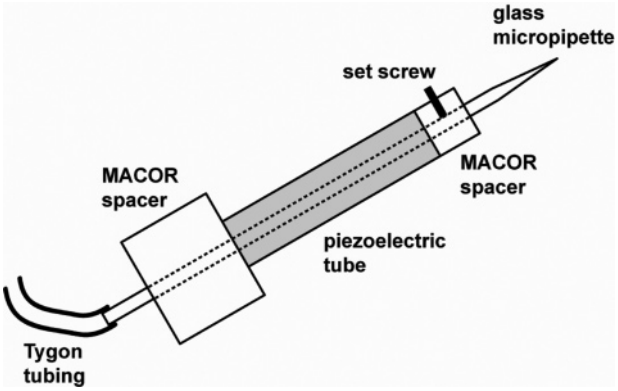

Figure 2. Schematic of the hydrosome injector assembly. The glass micropipet contains buffer solution with EGFP. The piezoelectric tube is glued to two MACOR spacers. The MACOR spacer near the Tygon tubing is mechanically coupled to an aluminum mount, and the MACOR spacer nearest the tip holds the glass pipet in place via a set screw. To inject a nanodroplet into the perfluorinated matrix, the piezoelectric tube is driven with individual cycles of a sawtooth waveform, which causes a quick retract motion of the glass micropipet, inertially injecting a droplet into the matrix.

from 3M (St. Paul, MN, www.3m.com). FC-70 was a generous gift from 3M. Both liquids were sparged with nitrogen to minimize the dissolved oxygen content.

Hydrosome Injector. The hydrosome injector is schematically shown with all of the key components (Figure 2). Devices similar to this have been developed by others, but they differ in either the details of construction or the mode of operation or both.[9] The micropipet is filled with buffer solution containing molecules, and the tip is immersed in perfluorinated solvent. The back end of the glass tube is connected to Tygon tubing. The Tygon tubing is connected to a FemtoJet pump (Eppendorf North America, Westbury, NY, www.eppendorfna.com) which generates a backing pressure to maintain a meniscus of aqueous solution at the pipet tip. The glass tube is mechanically coupled to a PZT piezoelectric tube with a single inner and a single outer Ni electrode (EBL Products Inc., East Hartford, CT, www.eblproducts.com, EBL no. 2 piezoceramic tube, 0.125 in. o.d. × 0.020 in. wall × 1.00 in. length). The piezoelectric tube is glued to two MACOR spacers. A steel set screw in the MACOR spacer nearest the tip holds the glass pipet in place. The back MACOR spacer is mechanically coupled to an aluminum mount. To inject a nanodroplet into the perfluorinated matrix, the piezoelectric tube is driven with a sawtooth waveform. Individual cycles of the sawtooth waveform cause a quick retract motion of the glass micropipet, inertially injecting a droplet into the matrix. The pipet tip is silanized (Sigmacote, Sigma-Aldrich Co., catalog no. SL2) to make it hydrophobic to prevent sticking of injected droplets to the glass.

Optical Trapping. For the EGFP data, optical trapping of injected hydrosomes is achieved using a linearly polarized 1064 nm cw laser (IPG Photonics, Oxford, MA, www.ipgphotonics.com, model YLD-5-1064-LP). The trap is formed in the detection volume of a confocal microscope using a high numerical aperture oil objective (Olympus America, Center Valley, PA, www.olympusamerica.com, model UPlanFl 100X/1.30 Oil). A 300 hPa backing pressure is used to generate a meniscus near the injector tip, and then one cycle of a 200 V sawtooth signal jettisons individual hydrosomes into the matrix. FC-70 is used instead of FC-77 because its higher viscosity imparts greater drag to slow the injected hydrosomes, making them easier to trap. Hydrosomes containing 3 nM EGFP in PBS at pH 7.2 are delivered from the micropipet tip and maneuvered into the optical trap. Triton X-100 at 0.1% (v/v) is added to the PBS. For SRB, data are collected on a similar apparatus using a 1064 nm cw laser for trapping (IPG Photonics, model YLD-5-1064) with an external polarizer.

Single-Molecule Detection. A 488 nm cw laser (Omnichrome model 532-AP-A01) was used to excite EGFP to observe single-molecule fluorescence. The laser was aligned through the same objective as the optical trap in a confocal arrangement. A dichroic mirror (Chroma Technology z488-1064 rpc) was used to separate the fluorescence from the excitation and trapping light. For rejection of background, excitation, and trap light in the fluorescence collection channel, two short-pass filters (Thorlabs, FGS900), one 500−550 nm filter (Omega Optical, 3RD millennium), one 500−600 nm filter (Omega Optical, 3RD millennium), and one 488 nm notch filter (Kaiser Optical Systems, Inc., Ann Arbor, MI, www.kosi.com, SuperNotch-Plus) were used in front of the avalanche photodiode (APD) detector (Perkin-Elmer, Waltham, MA, www.perkinelmer.com, SPCM-AQR-15).

For SRB, data were collected on a similar apparatus with a 532 nm cw solid-state laser (CrystaLaser, Reno, NV, www.crystalaser.com, model GCL-532-L) for excitation and a dichroic mirror to separate fluorescence from excitation and trapping light (Omega Optical 540DCLP). One 550−650 nm band-pass filter (Omega Optical, 3RD millennium), one 532/1064 nm dual-band notch filter (Omega Optical XB11), and one dichroic mirror (Omega Optical 630DCLP) were used in front of the APD (Perkin-Elmer, Waltham, MA, www.perkinelmer.com, SPCM-AQR-14). In the case of SRB, a pair of acousto-optic modulators were used to temporally separate the trap and excitation light at a frequency of 50 kHz.

Time-Resolved Fluorescence Anisotropy. Experiments were performed on a confocal microscope using a high numerical aperture oil objective (Olympus America, model UPlanFl 100X/1.30 Oil). Pulses from a linearly polarized, mode-locked Ti:sapphire laser (Coherent, model Mira 900), frequency doubled to 461 nm with a 76 MHz repetition rate and nominal pulse width of 200 fs, were used to excite EGFP. Fluorescence light was separated from excitation and trapping light using a dichroic mirror and filters similar to those used in the single-molecule experiment except without the 488 nm notch filter. A polarizing beamsplitter separated the fluorescence into components parallel to and perpendicular to the excitation polarization. We refer to the two detected intensities as $I_{\parallel}(t)$ and $I_{\perp}(t)$. The fluorescence was focused onto and detected by two separate photon-counting APDs (Micro Photon Devices, Bolzano, Italy, www.micro-photon-devices.com, model PDM 50ct) with a full width at half-maximum response time of less than 40 ps (Supporting Information Figure S3). Arrival time histograms were determined using time-correlated single-photon-counting electronics with 3 ps A/D resolution (Becker & Hickl, Berlin, Germany, www.becker-hickl.de, model SPC-830).

Hydrosome Experiments. As in the single-molecule experiments, optical trapping simultaneous with laser excitation was accomplished with a linearly polarized 1064 nm cw laser (IPG Photonics, model YLR-5-1064-LP). The IR laser trapping power was approximately

(9) (a) Zhang, W.; Hou, L.; Mu, L.; Zhu, L. *Proc. SPIE* **2003**, *5345*, 220−229. (b) Lee, C.-H.; Lal, A. *IEEE Trans. Ultrason., Ferroelectr., Freq. Control* **2004**, *51*, 1514−1522.

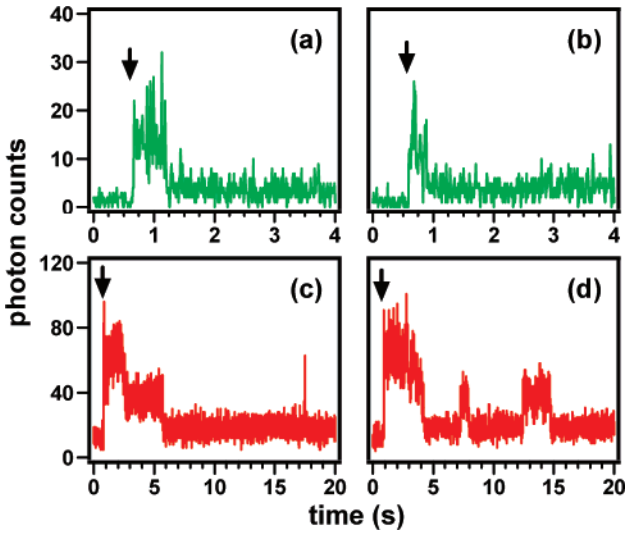

Figure 3. Single-molecule fluorescence from (a, b) EGFP and (c, d) SRB isolated in hydrosomes. The arrows indicate when the excitation is switched on. Two SRB molecules are likely present in (c) and (d) as indicated by steps in the photobleaching. Counts are in 10 ms bins.

70 mW. PBS buffer with 0.1% (v/v) Triton X-100 and 3 or 10 μM EGFP was loaded into the glass micropipets and injected into FC-70. $I_{||}(t)$ and $I_{\perp}(t)$ were collected until most of the protein had photobleached, typically 10−20 s. The total counts for these histograms were typically between 0.5×10^6 and 1×10^6, and the peak count in the parallel channel was on the order of 1000 counts. Variation in the count rate and total counts was due to differences in the hydrosome size. The excitation power was approximately 50 nW for the 3 μM samples and 15 nW for the 10 μM samples. At both excitation power levels, the initial fluorescence count rate from the EGFP in hydrosomes (typically between 50 and 100 kHz) was similar to the count rate from the EGFP in free solution (approximately 70 kHz).

Free Solution Experiments. Experiments were performed in the same apparatus described immediately above. Closed fluid cells were fabricated using a microscope slide, a coverslip, and Parafilm. Each slide had two clearance holes approximately 1 mm in diameter separated by 38 mm. A 43 mm × 5 mm rectangle was cut in the middle of a piece of Parafilm 24 mm × 60 mm in size. The Parafilm was sandwiched between a 24 mm × 60 mm coverslip and the slide and heated such that the two holes were connected via a closed channel. A 10 μL volume of PBS buffer with 0.1% (v/v) Triton X-100 and 3 or 10 μM EGFP was pipetted into the channel. The confocal detection volume was positioned 25 μm above the coverslip surface. To investigate any effect of the surfactant and/or the IR laser, data were collected with and without 0.1% (v/v) Triton X-100 added to the PBS buffer and with and without the trapping laser on. $I_{||}$ and $I_{\perp}$ histograms were collected for 180 s at count rates of approximately 70 kHz. The parallel channel typically contained a total of approximately 1×10^7 counts and a peak count of about 1×10^4. The perpendicular channel typically contained a total of approximately 4×10^6 counts and a peak count of about 4×10^3.

Results and Discussion

By injecting hydrosomes into FC-70 as described above, we were able to confine EGPF in the emulsified aqueous phase with no apparent damage to the protein. Fluorescence followed by a discrete photobleaching event indicated the presence of single EGFP molecules in optically trapped hydrosomes illuminated with a 488 nm cw laser containing 3 nM protein in PBS (Figure 3a,b). For comparison, single-molecule bleaching events were also detected from hydrosomes containing 1 nM SRB in Tris−EDTA buffer when illuminated with a 532 nm cw laser (Figure 3c,d).

We can introduce hydrosomes containing EGFP into FC-70 without loss of contents using the injector. A small amount of surfactant in the buffer solution is necessary to prevent partitioning into the continuous phase (Supporting Information). In contrast, the use of ultrasonication to form an emulsion can cause loss or destruction of contents.[8] Using the same ultrasonication protocol of Reiner et al.,[8] we confirmed that EGFP can be detected in hydrosomes in FC-77 as long as surfactant is present. However, no fluorescence could be observed when buffer containing EGFP was emulsified by ultrasonication in FC-70 even in the presence of surfactant. FC-70 is nearly 20 times more viscous than FC-77 and requires more vigorous agitation to form an emulsion.

To obtain useful information about the dynamics, kinetics, or conformational changes of confined individual protein molecules, it is critical to ensure that molecules are freely diffusing and not sticking at the water−surfactant−oil interface. Fluorescence images of a large droplet containing 3 μM EGFP showed no obvious sticking of EGFP at the boundary, consistent with free diffusion of the encapsulated protein (Supporting Information Figure S1). To quantify the rotational dynamics, we determined the rotational diffusion time using time-resolved fluorescence anisotropy.[10−14] We computed the anisotropy, $R(t) = D(t)/S(t)$, from the sum, $S(t) = I_{||}(t) + 2gI_{\perp}(t)$, and difference, $D(t) = I_{||}(t) - gI_{\perp}(t)$, of the fluorescence components.[14] The factor g accounts for detector imbalance and is determined with Alexa 488. Since the rotational diffusion time is much greater than the APD response time, the anisotropy was calculated without deconvolving the instrument response and modeled by a single-exponential decay, $R(t) \approx \alpha e^{-t/\theta}$, where θ is the rotational diffusion time and α is the fundamental anisotropy of the molecule (Supporting Information).

The fluorescence histograms and corresponding anisotropy profiles from two typical samples at 3 μM in free solution (Figure 4a,c) and in a hydrosome (Figure 4b,d) indicate very similar behavior of EGFP in both environments. Fitting the anisotropy using a single-exponential decay (black line) yielded rotational diffusion times of 13.8 ± 0.2 ns in a typical free solution sample (Figure 4c) and 12.6 ± 1.0 ns in a typical hydrosome sample (Figure 4d). Table 1 summarizes the results of the time-resolved fluorescence anisotropy experiments on EGFP in hydrosomes and free solution. The column labeled "*N*" represents the number of data sets that were collected at each set of experimental conditions. Here we tabulate the mean rotational diffusion time $\bar{\theta}$ and standard deviation σ_θ for the N values of θ. Note that the free solution experiments with the IR laser on and surfactant added were done twice. In free solution, the mean rotational diffusion time was determined to be 13.8 ± 0.1 ns from 11 data sets at 3 μM and 14.0 ± 0.2 ns from 11 data sets at 10 μM. This is in reasonable agreement with previous reports of EGFP and wild-type GFP rotational times of 13.5−20 ns.[11,12,15] In hydrosomes, the mean rotational diffusion time was determined to be 12.6 ± 1.0 ns from 41 data sets at 3 μM and 15.5 ± 1.6 ns from 18 data sets at 10 μM. We therefore conclude that EGFP does not congregate at the water−oil interface in the presence

(10) (a) Schaffer, J.; Volkmer, A.; Eggeling, C.; Subramaniam, V.; Striker, G.; Seidel, C. A. M. *J. Phys. Chem. A* **1999**, *103*, 331−336. (b) Hu, D.; Lu, H. P. *J. Phys. Chem. B* **2003**, *107*, 618−626.
(11) (a) Striker, G.; Subramaniam, V.; Seidel, C. A. M.; Volkmer, A. *J. Phys. Chem B* **1999**, *103*, 8612−8617. (b) Volkmer, A.; Subramaniam, V.; Birch, D. J. S.; Jovin, T. M. *Biophys. J.* **2000**, *78*, 1589−1598.
(12) Uskova, M. A.; Borst, J.-W.; Hink, M. A.; van Hoek, A.; Schots, A.; Klyachko, N. L.; Visser, A. J. W. G. *Biophys. Chem.* **2000**, *87*, 73−84.
(13) Heikal, A. A.; Hess, S. T.; Baird, G. S.; Tsien, R. Y.; Webb, W. W. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 11996−12001.
(14) Cross, A. J.; Fleming, G. R. *Biophys. J.* **1984**, *46*, 45−56.
(15) Swaminathan, R.; Hoang, C. P.; Verkman, A. S. *Biophys. J.* **1997**, *72*, 1900−1907.

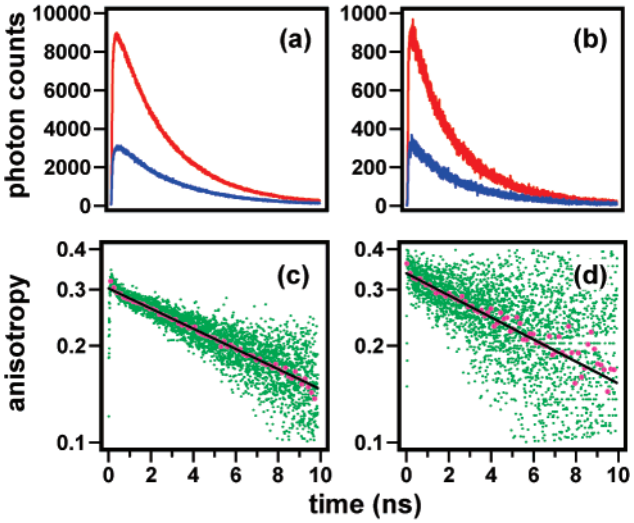

Figure 4. Examples of time-resolved fluorescence anisotropy of EGFP at 3 μM (a, c) in free solution and (b, d) in a hydrosome, corresponding to about 1000 molecules in a 1 μm diameter hydrosome. The anisotropy (green) was computed from the parallel (red) and perpendicular (blue) intensities every 3 ps. Fits using a single-exponential decay (black line) yield rotational diffusion times of (c) 13.8 $\pm$ 0.2 ns in free solution and (d) 12.6 $\pm$ 1.0 ns in a hydrosome. For clarity, the anisotropy averaged every 150 ps (magenta) shows agreement of the fit with the data.

Table 1. Results of Time-Resolved Fluorescence Anisotropy Measurements on EGFP

concn (μM)	sample	N	IR laser	Triton X-100, 0.1% (v/v)	mean $\bar{\theta}$ (ns)	σ_θ (ns)
3	hydrosome	41	on	yes	12.63	0.97
3	solution 1	21	on	yes	13.84	0.15
3	solution 2	12	off	no	11.97	0.28
3	solution 3	11	on	no	10.99	0.09
3	solution 4	13	off	yes	14.67	0.16
3	solution 5	11	on	yes	13.82	0.13
10	hydrosome	18	on	yes	15.50	1.60
10	solution 1	20	on	yes	14.10	0.18
10	solution 2	13	off	no	12.55	0.40
10	solution 3	10	on	no	12.61	0.17
10	solution 4	11	off	yes	14.19	0.39
10	solution 5	11	on	yes	14.03	0.19

of surfactant and that the rotational motion inside the nanodroplets is consistent with Brownian rotation of EGFP in free solution.

From the data in Table 1, two further conclusions can be drawn. First, the rotational diffusion time is unaffected by the presence of the IR laser. Second, at both concentrations, addition of Triton X-100 increases the rotational time by about 2 ns. This latter conclusion is not surprising since micelle formation around proteins is a well-documented effect. In the case of EGFP, the rotational times in reversed bis(2-ethylhexyl)sulfosuccinate micelles have been previously measured by Uskova et al. to be in the range of 24−33 ns in isooctane and 53−63 ns in dodecane.[12] These studies also contained much more surfactant than used here. They determined the rotational diffusion time to be 13.5 ns in Tris−HCl buffer at pH 8.2 in the absence of surfactant, which agrees reasonably well with our free solution results.

Conclusion

Here we have introduced a new method to make emulsified single hydrosomes containing EGFP molecules on demand. This method is minimally destructive or nondestructive to the protein and can therefore be used to confine proteins, and by extension any hydrophilic molecule or functionalized nanoparticles, with high efficiency. EGFP is shown to exhibit free rotational diffusion inside the hydrosomes, indicating that sticking at the boundaries is not significant. These results suggest that this technique might be successfully used to confine and study the dynamics and/or folding of single protein molecules or molecular complexes whose activity when confined on a surface might be suspect. Since hydrosomes can be maneuvered and fused without loss of contents,[8] it will be possible to study single molecular complex formation and nanochemistry using injection and mixing of hydrosomes containing different moieties.[8,16] Finally, we note that the method described here to produce single hydrosomes eliminates the risk of contamination from unintentional fusion that exists with ensembles of droplets.[17]

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Supporting Information Available: Details of the materials, experimental apparatus, and data analysis. This information is available free of charge via the Internet at http://pubs.acs.org.

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(16) Lorenz, R. M.; Edgar, J. S.; Jeffries, G. D. M.; Chui, D. T. *Anal. Chem.* **2006**, *78*, 6433−6439.
(17) Lorenz, R. M.; Edgar, J. S.; Jeffries, G. D. M.; Zhao, Y.; McGloin, D.; Chui, D. T. *Anal. Chem.* **2007**, *79*, 224−228.