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Quantitative Measure of the Size Dispersity in Ultrasmall Fluorescent Organic-Inorganic Hybrid Core-Shell Silica Nanoparticles by Small-angle X-ray Scattering

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Abstract

Small-angle X-ray scattering (SAXS) was performed on dispersions of ultrasmall ($d < 10$ nm) fluorescent organic-inorganic hybrid core-shell silica nanoparticles synthesized in aqueous solutions (C' dots) by using an oscillating flow cell to overcome beam induced particle degradation. Form factor analysis and fitting was used to determine the size and size dispersity of the internal silica core containing covalently encapsulated fluorophores. The structure of the organic poly(ethylene glycol) (PEG) shell was modelled as a monodisperse corona containing concentrated and semi-dilute regimes of decaying density and as a simple polydisperse shell to determine the bounds of dispersity in the overall hybrid particle. C' dots containing single growth step silica cores have dispersities of 0.19–0.21; growth of additional silica shells onto the core produces a thin, dense silica layer, and increases the dispersity to 0.22–0.23. Comparison to FCS and DLS measures of size shows good agreement with SAXS measured and modelled sizes and size dispersities. Finally, comparison of a set of same sized and purified particles demonstrates that SAXS is sensitive to the skewness of the gel permeation chromatography elugrams of the original as-made materials. These and other insights provided by quantitative SAXS assessments may become useful for generation of robust nanoparticle design criteria necessary for their successful and safe use, for example in nanomedicine and oncology applications.

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Author Contributions

KPB and UW designed the work. KPB performed the SAXS and BIOSAXS measurements and analyzed the data. KM and FFEK synthesized the particles and conducted FCS and DLS measurements. KM also conducted TEM measurements. TG and FFEK assisted in sample preparation for SAXS measurements. PAB assisted in design and collection of static SAXS measurements. REG provided experimental support and valuable discussion for BIOSAXS measurements. KPB wrote the manuscript with feedback from UW. All authors read and commented on the final manuscript.

Supporting Information. Supporting figures and tables including particle characterization and Guinier fits can be found in the supporting material. This material is available free of charge via the Internet at <http://pubs.acs.org>

Notes

KM and UW have a financial interest in Elucida Oncology, Inc. The remaining authors declare no competing financial interest.

Graphical Abstract

INTRODUCTION

There has been growing interest in ultrasmall (diameters < 10 nm) nanoparticles in recent years due to their size dependent catalytic or optical properties.^{1–3} In order to improve colloidal stability, such nanoparticles are typically surface coated with organic molecules or ligands, leading to organic-inorganic hybrid core-shell nanoparticles with unique properties offered by the combination of, and interfaces created between, the two components. In terms of ligands, particular focus has been on poly(ethylene glycol) (PEG) surface functionalized (PEGylated) nanoparticles for medical applications, whereby a PEG based shell shields the inorganic core. Examples include PEGylated Au nanoparticles for targeting kidney mesangium,⁴ as well as silica nanoparticles (SNPs).^{5–8}

Among ultrasmall nanoparticles, fluorescent core-shell SNPs termed Cornell dots (C dots⁵) and the more recent C' dots,⁸ which are synthesized in aqueous rather than in alcoholic media, have emerged as promising medical tools for imaging and cancer treatment. At the end of 2011, based on extensive preclinical studies,⁹ Cornell dots received FDA approval as an investigational new drug (IND) for first-in-human clinical trials, which suggested their safe use.¹⁰

The general synthesis route of C' dots is shown in Figure 1a;⁸ broadly, a fluorescent dye is covalently encapsulated in a silica core, with sizes tuned by temperature, pH, or other synthesis conditions, or by growing sequential silica shells around the core. The particle cores, often less than 5 nm in diameter, are then PEGylated for colloidal stability.¹¹ Targeting moieties or other functional groups, such as small peptides (e.g. cyclic Arg-Gly-Asp (cRGD) or biotin), can be introduced to this organic polymer brush coating.^{8,11,12} These multi-layered, multi-component particles, shown as a molecular rendering in Figure 1b, have demonstrated improved fluorescence brightness through dye confinement within the rigid silica matrix,^{5,13–15} making them excellent materials for bioimaging.^{16–18} Display of signaling and small peptide groups on the C or C' dot surface enables targeted sensing and imaging.^{19–22} Furthermore, the ultrasmall size (< 10 nm) of C and C' dots has shown promise for cancer diagnostics and treatment,^{23,24} addressing important safety concerns as particles demonstrate rapid renal clearance profiles leading to low off-target accumulation and high target-to-background ratios.^{10,17,25,26} In addition to medical applications, the combined particle brightness and ability to add reactive groups to the PEG shell, silica core,

or both, has led to their application as rigid tracer particles, e.g. for studying polymer (hydrogel) physics and diffusion^{27,28} and surface functionalization.²⁹

In the development of nanoparticles for medical applications, a critical step includes thorough particle characterization.³⁰ For example, many nanoparticle synthesis methods are plagued by dispersity and/or aggregation issues, occurring either during the synthesis or through subsequent particle processing steps. In particular, for ultrasmall targeted nanoparticles designed to lead to either targeting of specific biological environments (e.g. cancer tissues) or else efficient renal clearance (“target or clear” paradigm),³¹ broad particle size distributions would be detrimental, as part of the distribution could be beyond the cutoff for renal clearance, which is below 10 nm.^{10,17} This renders quantitative characterization of particle size distributions critical to understanding their use as effective theranostic nanoprobe and, based on this understanding, to the development of robust nanoparticle design criteria for their safe use, e.g. in nanomedicine applications in general and oncological applications in particular.²⁴

Several techniques have been developed for interrogating sizes and size distributions of nanoparticles. Dynamic light scattering (DLS) has been a workhorse method for particle size distribution characterization. However, the fluorescent core of the hybrid C' dots can generate problems with DLS measurements because the dye emission may interfere with the scattered signal if the laser and dye emission spectra overlap, which may be overcome only by irreversibly photobleaching the sample. Nevertheless, DLS of dye-free or irreversibly dye-bleached particles has suggested highly narrow size distributions of C' dots, enabling rapid renal clearance.^{8,10} Fluorescence correlation spectroscopy (FCS)^{32–34} has emerged as a powerful method for characterizing fluorescently tagged ultrasmall nanoparticles and, together with fluorescence cross correlation spectroscopy (FCCS), has been utilized to characterize not just final particle sizes, but also kinetics of C' dot PEGylation.^{11,12} Previous studies of C dots and C' dots with FCS have shown a single exponential decay in nearly all cases of optimized synthesis protocols, for a range of particles sizes and functionalities, leading to the description of these particles as narrowly dispersed. Example FCS autocorrelation curves for C' dots used in this study are shown in Figure 1d and also exhibit single exponential decay, indicating narrow dispersities. However, FCS is sensitive only to dye-containing particles. There may be a subpopulation of dye-free particles in the sample that form during synthesis and are invisible for the FCS technique. Recently, reversed-phase high performance liquid chromatography (HPLC) has suggested there may be some surface chemical heterogeneity in these particles,^{35,36} but whether this arises from the inorganic silica core or the organic, PEG-based coating has remained unclear. Examination of these particles by transmission electron microscopy (TEM), as shown in Figure 1c, corroborates suggestions of a low dispersity in particle size, but this method is predominately sensitive to the inorganic core and measures only a small sample size of particles. High resolution (HR-)TEM methods combined with electron energy loss spectroscopy (EELS) have been used to visualize and quantify both the organic and inorganic components of a PEG coated quantum dot,³⁷ but these methods require relatively high electron density in the particle composition for reasonable image contrast and many images to gain good statistics, which may degrade beam-sensitive silica. Capillary electrophoresis has also been used to determine size distributions of SNPs,³⁸ but this method

relies on the knowledge of the particle surface potential, and only provides information on the overall particle size. Thus, the ability to thoroughly characterize the particle dispersity, both of the inorganic core and of the organic (PEG) coating, for the entirety of a particle batch has not previously been demonstrated.

Small-angle X-ray scattering (SAXS) is a less utilized but promising technique for ultrasmall NP characterization. SAXS has previously been used to study SNP growth *in situ*,^{39,40} and to characterize other nanoparticles.^{41–43} Here, we demonstrate that SAXS is an effective tool to simultaneously measure properties of organic and inorganic components of a set of organic-inorganic hybrid core-shell nanoparticles in water, taking advantage of the scattering contrast between the silica core and the PEG shell. We show that the initial observation of x-ray beam induced particle damage can be overcome by working with set ups in which the aqueous particle solution is oscillated back and forth across the irradiated area, thereby minimizing exposure times of particular sample volumes to the beam. Moreover, using form factor modelling, the size dispersity of the inorganic core and the overall particle can be quantitatively assessed demonstrating particle size dispersities around 0.2. Applying this quantitative analysis to a set of chemically distinct particles highlights differences in particle architecture. We finally demonstrate that for same sized particles from two separate synthesis batches our SAXS analysis is sensitive to the skewness of the particle size distribution as revealed by comparison of results from gel permeation chromatography (GPC) elugrams of as-synthesized materials. We hope that results of such SAXS studies will help formulate robust particle design criteria for successful applications in areas including nanomedicine and oncology.

MATERIALS AND METHODS

Chemicals and Reagents.

All chemicals were used as received without further purification. Dimethyl sulfoxide (DMSO), 2.0 M ammonium in ethanol, ammonium hydroxide, (3-mercaptopropyl)trimethoxysilane (MPTMS), (3-aminopropyl)triethoxysilane (APTES), tetramethyl orthosilicate (TMOS), and tetraethyl orthosilicate (TEOS) were purchased from Sigma-Aldrich. Maleimide functionalized Cy5 and Cy5.5 fluorophores (Cy5-mal and Cy5.5-mal) were purchased from GE Healthcare. 2-[Methoxy-(polyethyleneoxy)_(6–9)propyl]trimethoxy silane (PEG-silane), with molar mass ca. 500 g/mol was purchased from Gelest. Heterobifunctional PEG with an NHS ester and maleimide (NHS-PEG-mal, molar mass ca. 870 g/mol) or with biotin and maleimide (biotin-PEG-mal, molar mass ca. 922 g/mol) were purchased from Quanta BioDesign. Cyclo(Arg-Gly-Asp-D-Tyr-Cys) peptide, c(RGDyC), was purchased from Peptide International. De-ionized (DI) water with a resistivity of 18.2 MΩ·cm was generated using a Millipore Milli-Q system.

Synthesis of Ultrasmall Fluorescent Core-Shell Silica Nanoparticles (C' dots).

Details of the C' dot synthesis are provided elsewhere.^{8,11} In brief, Cy5-mal or Cy5.5-mal (50.0 µl of 2 mg/ml in DMSO) was combined with MPTMS (0.57 µl) at molar ratio 1:25 in DMSO (5.0 ml) in a dark, inert atmosphere for 12 h to form the fluorescent, dye-silane conjugate (Cy5-silane or Cy5.5-silane). Separately, 1 ml of 0.02M aqueous ammonia

(diluted from 2.0M ammonia in ethanol with DI water) was added to DI water (4 ml) and stirred for 10 min to form a basic solution with pH ~9. 34 μ l TMOS (0.23 mmol) was added under vigorous stirring and immediately followed by the dye-silane conjugate solution. This mixture was then stirred overnight at room temperature (ca. 12h) to form fluorescent core particles. Silica core-shell particles (C' dot-4, C' dot-5) were formed by diluting the core particle (C' dot-3) solutions 5-fold with DI water. A mixture of TEOS and DMSO (1:4 v/v) was then dosed into the reaction solution at a dose level of 1 μ l per 5 ml of reaction mixture under vigorous stirring. To form a single, non-fluorescent shell around the core, 50 doses of TEOS/DMSO were added at 30 min intervals. For C' dot-4, this procedure was repeated once more to form two shells, and for C' dot-5, this procedure was repeated three times more to form four shells. To maintain solution conditions near neutral pH, 1–2 ml of 0.02M ammonium hydroxide (*aq*) was added after every two shells.

Cy5 fluorophore-containing silica core and core-shell particles were PEGylated (C' dot-3, C' dot-4, C' dot-5) by adding PEG-silane (0.021 mmol per ml of reaction mixture) and stirring at room temperature overnight (ca. 12h). The reaction was then heated to 80 °C and incubated 8–12h without stirring. PEGylation of Cy5.5 fluorophore-containing core particles with RGD-functionalized PEGs (C' dot-2, C' dot-6) was performed by first coupling NHS-PEG-mal to APTES at a 1:0.9 molar ratio in DMSO at a concentration of approximately 0.2M under inert conditions overnight to form mal-PEG-silane. c(RGDyC) peptide was then added to the mal-PEG-silane solution at 10% molar excess and stirred for one day to form c(RGDyC)-PEG-silane. PEGylation was performed as described above using a mixture of c(RGDyC)-PEG-silane (ca 25 mol%) and PEG-silane. PEGylation of Cy5 fluorophore-containing core particles with biotin-functionalized PEGs (C' dot-1) was performed by first conjugating biotin-PEG-mal (17.4 μ l of 100 mg/ml in DMSO) to MPTMS (0.70 μ l) at molar ratio 1:4 in DMSO (5.0 ml) for 12h in a dark, inert atmosphere to form biotin-PEG-silane. PEGylation was performed as described above using a mixture of biotin-PEG-silane (10–15 mol%) and PEG-silane. After PEGylation, all particles were cooled to room temperature and purified by dialysis (Pierce, MWCO 10,000) in DI water to remove unreacted reagents and side products, and syringe filtration (200 nm, Fisher) to remove any aggregates. Lastly, gel permeation chromatography (GPC) fractionation was performed on a Biologic LP system using a 275 nm UV detector and a Superdex200 resin from GE Healthcare to achieve clinical quality particle purity. GPC traces of all particles used in this study before and after purification can be found in the Supporting Information (Figure S1). The collected eluent fraction (in 0.9 w% NaCl from GPC elution) was spin-filtered to exchange to pure DI water and stored at 4 °C in the dark to maintain long term stability.

Particle Characterization by non X-ray methods.

Hydrodynamic particle sizes and size distributions were measured by DLS on a Malvern Zetasizer Nano-ZS at 20 °C. Samples were photobleached immediately prior to measurement to eliminate interference from the encapsulated dyes. Each sample was measured three times and averaged to produce final distributions, and number distributions were determined assuming a refractive index of silica of $n = 1.457$.

FCS data were measured on a home-built system using a 633 nm solid state laser to excite the Cy-5 and Cy-5.5 dyes. The hydrodynamic size, brightness per particle, and particle concentration were obtained from fits of the FCS autocorrelation curves.¹³ The measured autocorrelation curves and fits are provided in Figure 1d and the Supporting Information (Figure S2). The particle concentration as determined by FCS was used to determine the dilutions for appropriate concentrations for the SAXS measurements.

TEM images were collected using a FEI Tecnai T12 Spirit microscope operated at an accelerating voltage of 120 kV. The TEM samples were prepared by directly dropping one droplet of purified C' dot aqueous solution onto a copper mesh TEM grid coated with carbon film, removing excess liquid by blotting with filter paper and finally leaving the TEM grid at room temperature for about 10 min to dry.

Particle Characterization by Small-angle X-ray Scattering (SAXS).

C' dot solutions were diluted to concentrations of 0.25, 1.0, and 5.0 μM in deionized water. As the C' dot stock solutions had been filtered after synthesis, they were not re-filtered prior to SAXS measurement. (We note the PEGylated particles are very stable against aggregation;¹¹ this was corroborated here as no large aggregates were observed by SAXS in the initial solutions in these studies.)

Static SAXS measurements on C' dot solutions were carried out at Cornell High Energy Synchrotron Source (CHESS) G1 station using an X-ray beam of 9.83 keV. SAXS patterns were collected on a Pilatus 300K (Dectris) detector at sample-to-detector distance 1293 mm, yielding a q -range of 0.015–0.25 $\text{\AA}^{-1}$. Data reduction and analysis of the static measurements were carried out using NIKA.⁴⁴ SAXS measurements using an oscillating flow capillary cell were carried out at the MacCHESS BIOSAXS^{45,46} station (CHESS G1), details of which are described elsewhere.⁴⁷ SAXS patterns were generated using 9.95 keV X-rays with a $250 \mu\text{m} \times 250 \mu\text{m}$ beam size and flux of 5.91×10^{11} photons/second and were collected on two PILATUS 100K detectors at distances of 1518 mm and 433 mm from the sample, covering a q range of 0.0063–0.275 $\text{\AA}^{-1}$ and 0.252–0.81 $\text{\AA}^{-1}$, respectively. Samples were measured for 40–90 s, depending on the appearance of changes in the scattering profile, with frames collected every 1 s and then averaged over the stable timeframe. Background solutions (deionized water) were measured before and after each sample measurement for 90s (1 s frames) to confirm no changes in the deionized water background conditions between samples. All stable background frames were averaged and then subtracted from the measured sample scattering to produce the final scattering curves. Live monitoring of the measurements, 2d reduction, data merging and subtraction was carried out using BioXTSAS RAW (v. 1.2.3).⁴⁸ BIOXTAS RAW was also used to perform model independent analysis (*e.g.* Guinier fitting and maximum size estimation by GNOM^{49,50}).

Model dependent fitting and analysis, including scattering length density calculation, were performed using SasView (v. 4.1.2).⁵¹ Form factor fits to the scattering data were minimized using Levenberg-Marquardt non-linear least squares regression. Further estimation of parameter uncertainty was carried out using the DREAM algorithm (Markov Chain Monte Carlo sampling).⁵²

RESULTS AND DISCUSSION

X-ray scattering results for aqueous C' dot solutions and beam induced particle damage

Synchrotron X-ray sources have been extremely valuable for the generation of high flux X-rays for high signal-to-noise, rapid data collection, which has enabled detailed structural characterization of nanoparticles, including studies of their formation and growth. For example, Tobler *et al.* have used *in-situ* SAXS to measure the early nucleation and growth of silica nanoparticles.^{39,40} However, the high fluxes generated by synchrotron sources have a potential drawback of rapid radical generation, which is particularly pernicious in aqueous media. This phenomenon, while well documented in protein scattering, has remained complicated to predictably quantify.⁵³ X-ray induced sample damage, typically resulting in aggregation, has received less attention in nanoparticle research, but is critical to consider in the examination of nanoparticle properties, particularly in estimations of size distributions. Shown in Figure 2 are scattering curves for a 5.0 μM solution of C' dot-3 (PEGylated Cy5 encapsulating C' dots; Figure 1a) measured under static conditions in a sealed capillary, in which a large number of radicals are generated in a small, localized sample volume with direct beam exposure (green and gray squares). Synchrotron X-ray exposure onto the C' dot solution under static conditions for 30 s (gray squares) leads to a significant change to the scattering profile relative to 1 s beam exposure (green squares), particularly at low q where the intensity increases sharply below $0.08 \text{ \AA}^{-1}$, indicating a large increase in particle size due to beam induced aggregation. It is worth noting that these PEGylated C' dot particles have been shown to be otherwise stable against aggregation for weeks to months.¹¹ While the mechanism of beam-induced aggregation is not understood for these organic-inorganic hybrid nanoparticles, we speculate that the irradiation induced hydroxy radicals (from water) attack the PEG corona, either along the backbone or more likely at the PEG-silyl interface, leading to changes in particle surface coating and charge, and hence stability. This beam damage occurs rapidly; indeed, even after the first 1 s exposure (green curve), some aggregation is already observed. The change in intensity at low q stabilizes as exposure is continued beyond 30 s, likely due to reaching an equilibrium between radical generation and diffusion of species out of the directly irradiated volume. It is worth noting that upturns in the scattering intensity at low q have been observed for other PEGylated ultrasmall inorganic nanoparticles and have been reasonably ascribed to the structure of the PEG (5 kDa) corona.⁵⁴ Thus, it is critical for researchers to identify whether upturns in low q intensity are signs of aggregation in these organic-inorganic core-shell nanoparticles, even when the sample appears to be in equilibrium, in order to accurately determine the structure.

To mitigate the effect of beam damage, an alternate setup using a flow system in which the sample solution is injected into a sample holder, oscillated during irradiation, and then discarded, yields consistent, reproducible measurements without feature evolution (Figure 2, red and blue curves). Even after 30 s of exposure (blue circles), the curves are identical, and the low q intensity shows no evidence of aggregation or changes to particle surface charge. This stability is due to the constant renewal of sample volume exposed to the beam. Comparing the substantial differences in static and oscillatory flow derived X-ray scattering results on C' dots in Figure 2, in particular at low q , strongly emphasizes the importance of beam damage effects on particle behavior in aqueous solutions. Based on these results, we

recommend that future studies of PEGylated and other hybrid particles, especially in aqueous solutions, should be carefully examined for signs of radiation-induced sample changes such as aggregation and should utilize a flow or oscillating setup whenever possible to eliminate such concerns.

In order to capture a wider range of possible feature sizes, SAXS were collected across two detectors and merged, covering a q range from 0.0065 to 0.8 Å⁻¹. A representative sample curve showing the scattering from 5 μM C' dot-2 dispersion in water [cyclic(arginine-glycine-aspartic acid-D-tyrosine-cysteine) (c(RGDyC)) functionalized and PEGylated Cy5.5 encapsulating C' dots; Figure 1a] is shown in Figure 3. Due to the increased contrast provided by the inorganic particle core and moderately high sample concentrations, reasonable signal to noise is achieved quickly and error bars are generally very small, becoming apparent only at larger q where the sample and subtracted background solution intensity are nearly identical. The scattering curve is, to first order, consistent with a smeared or dampened spherical particle and reminiscent of scattering from block copolymer micelles; the scattering intensity decreases sharply at lower q (< 0.25 Å⁻¹), commensurate with the dominant scattering object size, and has a weak but perceptible oscillation at mid q range (0.25–0.5 Å⁻¹), which is critical to dispersity estimation.

Quantitative modelling of C dot solution scattering data

At low concentrations where interparticle interactions are absent, the scattering profile, $I(q)$, can be described solely through the form factor of the scattering object. C' dots, with their rigid silica core coated in a PEG outer layer, may be most simply modelled by a core-shell sphere form factor, yielding:

$$I(q) = A \left[\left(\frac{r_{core}}{r_{tot}} \right)^3 (\rho_{core} - \rho_{shell}) \left(\frac{j_1(qr_{core})}{qr_{core}} \right) + (\rho_{shell} - \rho_{H2O}) \left(\frac{j_1(qr_{tot})}{qr_{tot}} \right) \right]^2 + B \quad (1)$$

where A is a scaling factor that incorporates sample concentration and beam flux, B is the background scattering, r_{core} is the radius of the particle core, r_{tot} is the total particle radius ($r_{tot} = r_{core} + r_{shell}$, where r_{shell} is the thickness of the PEG shell), j_1 is the first kind spherical Bessel function, and ρ_{core} , ρ_{shell} , and ρ_{H2O} are the scattering length densities (SLD) of the particle core, PEG shell, and solvent ($\rho_{H2O} = 9.45 \times 10^{-6}$ Å⁻²), respectively. Compared to crystalline quartz (2.65 g/cm³ H₂O) or bulk amorphous silica (2.2 g/cm³), amorphous silica nanoparticles have lower densities, which vary depending on synthesis method, but are generally in the range of 1.6–2.1 g/cm³.⁵⁵ Assuming an intermediate density of 1.9–1.95 g/cm³,⁵⁶ then $\rho_{core} = 16.5 \times 10^{-6}$ Å⁻² core. The SLD of the PEG shell, ρ_{shell} , must fall between the infinite dilution case of pure solvent (ρ_{H2O}) and the pure PEG melt ($\rho_{PEG, melt} = 10.5 \times 10^{-6}$ Å⁻²) and is fit for in the modelling (*vide infra*).

The best fit results of this monodisperse core-shell model, where the particles are assumed to be of uniform core and shell size, to the data in Figure 3 are shown as the dashed yellow curve. Whereas distinct features of local maxima and minima appear in the scattering fit for monodisperse systems due to the overlaid form factor extrema of the core and shell, the

measured scattering curve for the C' dots has substantially smoother features. This smearing of features in C' dot data (and other particles systems) results from dispersity in the size of the particles, which can be modelled as the sum of discrete subpopulations of monodisperse particles representing the entire distribution.

The distribution of sizes in the C' dot silica cores arises from the distribution in silane and/or silica cluster condensation events during particle growth;^{8,57–59} hence the silica core size distribution may be well represented by the Schulz distribution, originally derived for polymer chain growth but frequently applied to polydisperse colloids and nanoparticles.^{60–62} The distribution of particle radius, r , is given by:

$$f(r) = K^{-1}(\beta) \left(\frac{r}{r_o}\right)^{\beta-1} \frac{\exp\left(-\frac{\beta r}{r_o}\right)}{r_o \Gamma(\beta)} \quad (2)$$

where K is a normalization factor, r_o is the mean of the distribution, and β is a representation of the distribution width and related to the polydispersity of the silica core, PD_{core} , by

$$\beta = PD_{core}^{-2} \quad (3)$$

Using eq (1) with eq (2) applied to the C' dot silica core radius, r_{core} , yields a minimized fit shown as the red curve in Figure 3, where $r_{core} = 13.7 \text{ \AA}$, $r_{shell} = 12.2 \text{ \AA}$, and $PD_{core} = 0.268$. While the inclusion of dispersity of the core eliminates the sharp features of the core-shell model, it is unable to accurately reproduce the weak oscillation of the data around $0.25\text{--}0.5 \text{ \AA}^{-1}$ and over-dampens the fitted curve (likewise over-estimating the polydispersity of the silica core). Since applying a dispersity to the silica core radius does not fully capture the measured scattering profile of the C' dot, the modelled structure of the PEG shell layer must be considered in further detail.

Understanding the structure of polymer brushes on curved surfaces has been of ongoing experimental and theoretical interest.^{63–66} Most typically, polymer brushes on spherical nanoparticles are assumed to behave according to the Daoud-Cotton (DC) model,⁶³ which combines straight-forward geometric considerations of a sphere with excluded volume effects to predict a power law scaling to the polymer brush density as a function of radial distance. In the original derivation for star shaped polymers, highly branched (or highly grafted) particles have a core region of constant polymer density, a concentrated regime where the polymer density, $\phi(r)$, scales as r^1 , and a semi-dilute region where $\phi(r) \sim r^{4/3}$. For polymer grafted nanoparticles, this scaling model is often reduced to two regions (concentrated and semi-dilute) or, in some cases, considers only one of these depending on grafting density and size of the core.^{67,68} For example, Grünewald *et al.* found that SAXS patterns of ultrasmall iron oxide nanoparticles grafted with PEG (3.55 chains/nm²) are well described by a core-shell structure with a two region DC model for the shell.⁵⁴ In comparison, the grafting density for PEG on our C' dots is 1.7–1.9 chains/nm².^{8,11} suggesting a concentrated region near the core may also exist. More recently, molecular

dynamics simulations of 20 monomer PEG grafted to ultrasmall gold ($r = 1\text{--}3\text{nm}$) found that the PEG density generally follows a concave decrease over the shell radius, approaching DC-like scaling particularly at high grafting densities, but can deviate depending on particle size. At lower grafting densities, their results did not predict a dense, constant PEG density region.⁶⁹

Most studies on spherical surfaces have utilized highly monodisperse polymer brushes, whereas the PEG chains on C' dots range in molar mass from 459 to 591 g/mol ($N=6\text{--}9$). Further complexity is introduced by surface functional groups like biotin or c(RGDyC) peptides inserted via longer PEG chains (C' dots-1, 2, and 6), varying in estimated chain fraction between 0.030.20, and with much larger molar masses of up to 1400 g/mol. Thus far, the effect of polydispersity on brush structure has been most thoroughly explored on flat surfaces, revealing that the brush density profiles are highly sensitive to molar mass distribution; for bidisperse systems, the shorter chains compress near the interface while the longer chains stretch away from the interface, deviating from the parabolic profiles expected in the monodisperse systems and leading to a more extended and concave density distribution.^{70,71} Monte Carlo simulations of polydisperse brushes on spherical surfaces suggest analogous effects and deviations from the DC model also occur.⁷² Thus, in order to account for the variety of possible brush behaviors of short but highly disperse chains on the C' dot core, we use a simplified two region model for the shell, such that the scattering length density of the PEG corona, ρ_{brush} , varies radially as:

$$\rho_{brush}(r) = \begin{cases} \rho_{shell} & r < r_{shell} \\ \rho_{shell} - (\rho_{shell} - \rho_{H2O}) \left(\frac{r - r_{shell}}{t_{brush}} \right)^{-a} & r > r_{shell} \end{cases} \quad (4)$$

where ρ_{shell} is the SLD of the PEG layer at the interface with silica, r_{shell} is the radius at which the PEG brush density is no longer uniform and becomes a semi-dilute region, a is the exponent that controls steepness of (concave) density decay, and t_{brush} is the thickness of the semi-dilute region, defined such that the SLD at $r = r_{shell} + t_{brush}$ is that of the solvent. This combined model of (1) with the Schulz dispersed core (2) and the polymer brush density decay in (4) is hereafter referred to as “SB”. Fitted parameters in the SB model are r_{core} , PD_{core} , r_{shell} , t_{brush} , ρ_{shell} , a , A (intensity scaling) and B (background).

As a simplified alternative to the SB model, which contains four fitted parameters in (4) for the PEG layer alone, we also consider the PEG as a uniformly dense, solvated polymer layer that is itself a polydisperse structure, encompassing the molar mass distribution in the PEG brushes, variation in grafting density, variation in functional group incorporation, and making no assumptions regarding the physics of individual polymer brush structure. A rudimentary schematic comparing these two approaches is shown in the top of Figure 3. A simple distribution for the PEG chains in C' dots, assuming molar masses near symmetrically distributed about a mean of ~ 520 g/mol, is a gaussian (normal) distribution:

$$f(r) = (2\pi)^{-1/2} (PD_{shell} \cdot r_{shell}) \exp\left(\frac{-(r - r_{shell})^2}{2(PD_{shell} \cdot r_{shell})^2}\right) \quad (5)$$

where the dispersity of the shell, PD_{shell} , is about a mean size, r_{shell} . The model comprising the core-shell form factor (1), Schulz distributed core (2) and gaussian distributed shell (5) in the following discussion is termed “SG”. Fitted parameters in the SG model are r_{core} , PD_{core} , r_{shell} , PD_{shell} , ρ_{shell} , A (intensity scaling) and B (background).

The minimized fits of SG and SB models to the X-ray scattering data are shown as the middle green and bottom blue curves, respectively, in Figure 3. Both models are exceptionally effective at reproducing the measured scattering curve. Compared to the model that only considers the polydispersity of the core (red curve), both models accurately recapitulate the mid-q weakly oscillating features, evidenced visually and by the residuals of the fits shown at the bottom of Figure 3. Qualitatively, the fits look extremely similar to each other, and, quantitatively they also produce almost equal results for some parameters. The SG model produces a silica core with a radius of 15.0 Å and a dispersity of 0.19, with a PEG shell average thickness of 9.5 Å and a dispersity of 0.65, see Table 1. The DC-like SB model suggests a similar silica core size, with radius 14.6 Å and a dispersity of 0.21, surrounded by a concentrated PEG layer of 7.7 Å that decays over ~30 Å, see Table 2. ρ_{shell} was found to be $10.14 \times 10^{-6} \text{ Å}^{-2}$ for SG and $10.00 \times 10^{-6} \text{ Å}^{-2}$ for SB; assuming a 100% PEG melt to have a SLD of $10.5 \times 10^{-6} \text{ Å}^{-2}$, this suggests the PEG volume fraction is 0.52–0.66 at the interface with the silica core. This is in excellent agreement with recent simulations of PEG on $r = 2$ nm gold particles, where the PEG fraction at the interface was predicted to be 0.5–0.6 for grafting densities between 1.5 and 2.2 chains/nm².⁶⁹

Whereas the PEG density as a function of radius, r , is given directly in the SB model through eq (4), the overall PEG density profile in the SG model can be derived explicitly as the complement of the cumulative distribution function of the Gaussian, i.e.:

$$\varphi_{PEG}(z) = \kappa \left[1 - \frac{1}{2} \operatorname{erfc} \left(\frac{r_{shell} - z}{\sqrt{2} PD_{shell} \cdot r_{shell}} \right) \right] \varphi_{0,PEG} \quad (6)$$

and

$$\varphi_{0,PEG} = \frac{\rho_{shell} - \rho_{H2O}}{\rho_{PEG,melt} - \rho_{H2O}} \quad (7)$$

where $z = r - r_{core}$ and κ is a correction factor near unity such that $\varphi_{PEG}(0) = \varphi_{0,PEG}$. A comparison of the PEG density profiles derived from the SG and SB model fits from Figure 3 are shown in Figure 4. While the SB model does predict a dense brush phase not permitted in the SG model, the SG model predicts a higher density near the shell. Overall, density

distributions are broadly similar, with both reaching PEG fractions ~0.25 around 12 Å away from the core. Average brush height, H_{av} , can be determined from the 2nd moment of the PEG density distribution with respect to volume:^{65,66}

$$H_{av}^2 = \frac{\int_0^\infty z^2 (z + r_{core})^2 \varphi_{PEG}(z) dz}{\int_0^\infty (z + r_{core})^2 \varphi_{PEG}(z) dz} \quad (8)$$

The two PEG density profiles are in good agreement: $H_{av}(SB) = 10.7$ Å and $H_{av}(SG) = 10.4$ Å. For comparison, assuming ideal chain properties for low MW PEG (persistence length, $l_p = 3.8$ Å),^{73,74} the radius of gyration, R_g , of a 500 g/mol chain is estimated as 7.1 Å, the mean squared end-to-end length as 18.4 Å, and the contour length as 40 Å. We note that the fitting parameters in the SB model (r_{shell} , t_{brush} , ρ_{shell} , and a) are strongly correlated (see Supporting Information Figures S3), and the regression does not guarantee a global minimum. Therefore, it is possible a PEG brush without a concentrated inner layer and more similar in profile to the SG model is equally valid. However, the ability to probe this is obfuscated by the stronger scattering of the silica core and beyond the scope of this work. We find that both SB and SG models serve to describe the experimental SAXS data well and predict reasonable sizes and distributions of the C' dot inorganic core and PEG shell. The above in-depth analysis has been shown and discussed in relation to C' dot-2, selected because of the high number of SAXS frames collected providing superior data quality relative to other data sets; however, this analysis has been equivalently performed and is applicable to the other C' dots, as discussed below.

Comparison of SAXS results from a range of different C dots

In order to compare the size dispersity across a range of particles, SAXS patterns were collected for five different types of C' dots: the smallest, C' dot-1, is a 5.3 nm diameter (by FCS), simple core particle with a biotin end-functionalized PEG brush; C' dot-2, as previously discussed, is a slightly larger (6.3 nm diameter) simple core particle with a cRGD end-functionalized PEG brush; and C dot-3 is the largest simple core particle (6.6 nm diameter) with a PEG brush containing no targeting moieties. C' dot-4 and C' dot-5 are larger particles (7.3 nm and 8.8 nm diameter) that have been synthesized by sequentially growing 2 shells and 4 shells of silica, respectively, on to the core of C' dot-3. The scattering curves for these five nanoparticles are compared in Figure 5a, shown in order of size as determined by FCS. Of first note is that the size differences are immediately apparent, as the scattering features consistently shift to lower q (larger real space size) and show steeper drop offs.

The size of the C' dots was first estimated by determining the radius of gyration, R_g , from Guinier approximation of the low q region of the scattering curves.⁷⁵ Since this low q region is highly sensitive to aggregation and interparticle interactions, Guinier fits (see Supporting Information Figure S4 and Table S1) were performed on the lowest sample concentrations (0.25 µM), although we note generally that in our samples R_g varied < 5% between lowest (0.25 µM) and highest (5.0 µM) concentrations studied (see Supporting Information Figure

S5). The overall particle size was then calculated by assuming a uniformly dense sphere, where:

$$r_{sphere}^2 = \frac{5}{3} R_g^2 \quad (9)$$

Resulting r_{sphere} values are listed in Table 1. In all cases, the C' dot radius estimated from the Guinier approximated R_g is 1225% smaller than as determined by FCS. Since FCS measures the hydrodynamic radius, it is expected to overestimate the true particle size given the increased drag created by strongly associated water molecules. Likewise, the Guinier approximated size from the SAXS data may under-predict size since there is greater contribution to the scattering from the electron rich core. The two methods generally follow the same size trend which may make using the Guinier approximation a valuable alternative in cases where sample concentrations are too low or samples are too disperse for accurate form factor modelling.

The SG and SB models were used to analyze the scattering curves of various synthesized C' dots, and the SG minimized fits are shown with the data in Figure 5a. Plot comparisons of minimized fits to the data using the SB model are also provided in the SI (Figure S6). As discussed above with the Guinier approximation, small amounts of aggregation or interparticle attraction/repulsion can strongly affect the scattering intensity at low q . In fact, in contrast to the particle aggregation induced by beam damage, some of the C' dots are natively slightly repulsive. This is most prevalent for C' dot-2, where a down turn in the scattering can be seen at very low q (and more clearly in the Guinier plot in SI Figure S4a), suggesting that the c(RGDyC) ligands may alter the surface charge of the particles. However, these particle-particle interactions are highly concentration dependent, and, as concentration of these samples is estimated by FCS, which is insensitive to any dye-free particles, identically matching concentrations between different samples is very challenging. In order to compare the model parameters between C' dots, any differences between samples due to interparticle interaction effects must be accounted for or eliminated. Rather than bias the modelling away from the low q region by weighting the fitting as a function of q , a low q fitting boundary was established for each particle based upon the maximum particle size dimension, D_{max} , which is determined from the particle size distribution using DATGNOM⁵⁰ (see Supplementary Information Table S1) and was found to be independent of sample concentration. Accordingly, scattering intensity from q ranges that correspond to sizes larger than D_{max} (i.e. $q < 2\pi/D_{max}$) do not provide any additional information about a scattering object that must be smaller than D_{max} . Generally, these D_{max} values agree strongly with the Guinier determined sizes and are roughly 150% larger than r_{sphere} .

Based on D_{max} , the models were fitted over the q range $[2\pi/D_{max}, 0.75 \text{ \AA}^{-1}]$ and are shown on top of the data in Figure 5a only over these ranges. The resulting values of the SG fits and their fit quality as measured by χ^2/N_{pts} are listed in Table 1. For comparison, the resulting parameters from the SB model are listed in Table 2. For the simple core particles (i.e. 0 shell SNPs) C' dot-1, C' dot-2, and C' dot-3, the silica core sizes are very similar, differing in average radius by 1.5 Å or less, and are ordered in size consistent with FCS results. The

polydispersity of the silica core for the SG model remains at or below 0.20, indicating that the silica condensation and covalent-encapsulation of the organic dye is very reproducibly controlled in these various optimized synthesis conditions. Core size and dispersity in the SB model are also similar, and nearly identical for C' dots 1 & 3. The PEG density at the surface of the silica core is estimated to be 54–66% of melt PEG (eq (7), *vide supra*), with an average PEG shell thickness (r_{shell}) of ~9.5–10 Å by the SG model. The dispersity of the shell is quite high in all cases. However, this is expected as the uniformly dense, discrete shell in the SG model attempts to replicate the polymer chain dispersity and realistic shape of a polymer brush decaying density profile. For comparison, in the SB model the PEG density is slightly lower, 39–52% with a 7.7–8.1 Å dense PEG layer (r_{shell}) followed by a power law decay over nanometers. Unlike C' dot-1 and C' dot-2, C' dot-3 contains no long PEG chains with surface functional groups (see Figure 1a), and thus would be expected to have a narrower corona dispersity (PD_{shell}) and overall shorter average brush height (H_{av}). Instead, our results suggest C' dot-3 has the largest and/or most disperse PEG corona, with a brush height estimated to be 1.8 Å larger than that of the C' dot-1. While polymer brushes of the same grafting density exhibit larger brush heights when the core radius is increased,⁶⁷ this would only account for ~2% difference in brush height between C' dot-1 and C' dot-3. Perhaps PEGylation in the absence of long, functionalized chains leads to higher grafting densities, and thus taller brushes. However, this has not been observed in previous studies of C' dot PEGylation, and the polymer densities at the particle surface estimated here are all similar. An alternative interpretation of these results may arise from the fact that functional groups like biotin and c(RGDyC) may have higher affinity for the silica core surface as compared to PEG.¹¹ At least a portion of these peptides may not end up on the outside of the PEG layer but may be immobilized on the silica core, and thus the picture of a bidisperse brush with extended long chains may not be accurate when peptides and other chemistries are attached to the longer brushes. Ultimately, a systematic study of PEG grafting density and dispersity of peptide-functionalized and non-functionalized PEGs needs to be performed in the future on otherwise identical C' dot cores to further understand the PEG shell structure in more detail.

As shells are grown onto the particle, the scattering profile in Figure 5a clearly starts to change compared to the simple core; the most apparent difference is the shift in features to smaller q (larger sizes). For C' dot-4, where two shells of silica have been grown on to the core of C' dot-3, the sphere radius by Guinier analysis (r_{sphere} , Table 1 column 3) increases by 7 Å from 25.4 to 32.4 Å, and then by another 5.3 Å when an additional two shells have been grown onto C' dot-4 to make C' dot-5. Both the SB and SG models indicate a large increase in the silica core size (r_{core} , Table 1 column 4), from 15.5 to ~21.3 Å for the first shell growth, with a slight increase in the size dispersity from 0.20 to 0.22–0.23. While the concentrated polymer brush region is also predicted to increase in size considerably, this may be a correlated artifact arising from the extremely low PEG p_{shell} density as determined by the fitting. p_{shell} from the SG and SB fits estimates the PEG density at the core to be 11–18%, which would only be reasonable for grafting densities less than 0.8 nm⁻². However, it is well established in the synthesis of C' dots and observed in DLS measurements that particles with PEG densities below 1.0–1.2 chains/nm² will aggregate quickly.¹¹ Thus, the predicted shell of C' dot-4, although apparently good by fitting metrics, is not reasonable.

Comparison of scattering profile and fit for C' dot-5 (4 shells) clearly shows that these particles are not well described by the SG or SB model, and hence the resulting fit values are denoted with an asterisk. In both models, the core particle size, r_{core} , is estimated to be smaller than the initial particle (C' dot-4) onto which the shells were grown, decreasing from ~ 21 Å to ~ 17 Å, and the large, systematic residuals from the best fit in Figure 5a strongly signal that key features of the form factor are not accounted for. Some of this deviation may be due to roughening of the silica surface upon shell growth, made evident by the TEM images in Figure 5b, which show the typical shape of C' dots with 0, 2, and 4, shells. For 0 shells, the silica cores appear round; in contrast, by the fourth shell growth, not only are the particles larger (as desired), but they become rougher and more irregular. One would expect roughness, if aperiodic, to be decently approximated by a polydisperse sphere and not to result in an apparent decrease in silica core size. Some indication of how the C' dot structure is changing with shell growth is provided by the C' dot-5 SG and SB fitted parameters, where the ρ_{shell} is determined to be greater than that of $\rho_{PEG, melt}$. This suggests a significant density change is occurring at the core interface, which is likely due to the differences in growth conditions between the silica core (TMOS condensation) and the subsequent silica shells (TEOS condensation). TEOS hydrolysis in water is slower, allowing for condensation onto the existing particle cores, preventing nucleation of new particles, and maintaining well defined size distributions. The reduction in condensation rate generally leads to a denser network structure, and additionally, unlike in the original core particle, there are no organic dyes encapsulated in the silica shells. Thus, we hypothesized that the change in scattering profile in C' dot-4 and C' dot-5 could be attributed to a dense shell layer between the core and PEG formed by the TEOS condensation.

Multi-shell particle models and comparison of results to FCS and DLS data sets

To test the validity of a dense silica shell layer, C' dot-4 and C' dot-5 data sets were fit with a multi-shell model by a simple extension of the brush profile described in eq (4) to include an additional dense, uniform "TEOS" shell layer between the Schulz distributed silica core and concentrated polymer layer. To reduce the number of free parameters, the particle core size and dispersity were assumed to be the same as for C' dot-3 (since the shells were grown on this core), $r_{core} = 15.6$ Å and $PD_{core} = 0.20$. Likewise, it was assumed that the PEG shell size and shape should be similar to the PEG shell on C' dot-3, thereby fixing these parameters to $r_{shell} = 7.86$ Å and $\rho_{shell} = 9.93 \times 10^{-6}$ Å⁻². A Schulz dispersity was also included on the TEOS layer to mimic the condensation process of shell growth. Using this revised model, new fits to C' dot-4 and C' dot-5 are shown in Figure 6.

While the residuals are still large (χ^2/N is 2.94 and 12.7 for C' dot-4 and C' dot-5, respectively) because most parameters were held to the conditions of C' dot-3, the fits are an improvement over both the SG and SB minimized fits, track the mid-q features better, and have resulting parameters that are reasonable. For C' dot-4, the TEOS layer is 4.6 Å with a SLD of 18.1×10^{-6} Å⁻², corresponding to a 10% increase in density over the dye encapsulating core grown from TMOS. The dispersity of the TEOS shell is estimated at $PD_{TEOS} = 0.55$, much larger than the dispersity of the core but consistent with patchy growth of a thin layer. C' dot-5 has a 9.5 Å silica shell, slightly more than double the thickness of the two shells on C' dot-4, and the SLD of the layer is 18.8×10^{-6} Å⁻², or 15%

more dense than the core. This dense layer is consistent with C' dot rigidification by shell growth previously observed by other methods and is credited with improving fluorescent brightness in C' dots.⁸ The dispersity of four shells was found to be similar to that of two shells, suggesting a fairly uniform and controlled rate of condensation across shell growth number. However, the fitting for C' dot-5 is not as good as for C' dot-4, likely due to other factors such as shape change and increasing roughness, and as such the fit quality is not readily dependent on small changes in dispersity.

By convoluting the size distribution of the silica core with the size distributions of the TEOS shell, an overall distribution of the overall silica particle sizes as a function of shell growth can be compared. Figure 6b shows the number density of silica core particle sizes for the various C' dots in the shell growth series as un-shaded traces. As seen in Figure 6b, there is a steady increase in size of the silica core, with the most probable radius increasing from 1.5 to 1.8 to 2.5 nm, and a slight widening of the distribution as more shells are added. The total dispersity of the silica cores after shell growth is calculated to be 0.22 for C' dot-4 and 0.23 for C' dot-5, compared to 0.20 for the original particle. Overall, while the TEOS growth may be patchy and irregular, the dispersity of the particle remains relatively low (Note: dispersity $PD = \text{mean}/\text{variance}$, so as the particle sizes are increasing, the variance does increase from ~ 3 , to ~ 4 , to ~ 5.5).

Shaded curves shown in Figure 6b represent the estimated total hybrid particle size distributions from convolving the gaussian dispersed PEG shells determined from the SG models with the silica core particles (un-shaded curves in Figure 6b). The total C' dot size distributions also increase in width as more shells are grown, however the overall calculated dispersity decreases, due to the simultaneous overall increase in average size. The size distributions are compared with the radii determined by FCS and Guinier approximation as marked in Figure 6b. While the Guinier determined sizes agree well with the distributions, as expected (*vide supra*) the FCS determined radii are consistently larger.

Core and particle size distributions were quantified by the same method for C' dot-1, -2, and -6, and compared to the Guinier approximated particle radius (r_{sphere}), FCS radius, and number distribution as determined by DLS. Comparison of results with those of four shell C' dot-5 are displayed in Figure 7.

The total particle size as determined by the convolution of the SG model individual distributions (thick blue curve), agrees reasonably well with the particle size determined by FCS, considering that FCS is expected to somewhat overestimate physical particle size (*vide supra*). It is important to consider that the distribution used here to describe the PEG layer was a model Gaussian distribution (SG). It thus has unreal aspects to it, including predicted negative radii (as a result of wide tails of Gaussian distributions) and not allowing for variations in the polymer density through the shell layer, that are resulting in quite large estimated shell dispersities. As a result, the SG distribution may be thought of then as an upper limit to the width of the distribution. At the opposite end, the lower limit to the dispersity is the consideration that the PEG layer, while having some density scaling in the corona, overall has uniform shell thickness across the sample (*i.e.* is monodisperse). Thus, there would be no size distribution to the corona, and the overall C' dot size distribution

would simply be the silica core distribution shifted in radius, r , by the PEG brush radius, i.e. H_{av} . This is the SB model. The distribution for this lower limiting case is also shown in Figure 7 as the thin blue trace. These distributions are much narrower than those from the SG model (upper limit), and the most probable radius is shifted to larger sizes. Table 3 lists the overall C' dot size properties determined using these two methods, including the peak (mode) of the overall distributions and the dispersities, which are estimated by fitting a log-normal distribution to the convoluted total particle distributions in order to capture the underlying skewness of the silica cores. At the lower limit, the total C' dot dispersity, $PD_{C' dot}$ is 0.11–0.15, increasing with increasing shell growth; at the upper limit, $PD_{C' dot}$ is 0.24–0.31. For the upper limit case, the convolution of the gaussian shell with the core leads to an overall decrease in dispersity with increasing shell growth (however, variance is still increasing). The real structure of the hybrid particle likely falls between these two limiting cases; and indeed, the DLS data often lies within this range. For C' dot-5, which has four TEOS grown shells, DLS results indicate a broader and larger size, possibly due to artifacts introduced by the roughening of the particles. For comparison, the radius determined by FCS and the spherical radius calculated by Guinier analysis are indicated above the curves. For all particles, the r_{sphere} agrees quite well with the center of the distribution, while the values measured by FCS indicate a ~5–10% larger particle than the SAXS determined average.

Lastly, to examine how SAXS might provide additional information on the particles beyond typical characterization techniques, SAXS scattering profiles were compared and analyzed across two samples synthesized using the same protocol providing clinical use quality materials (c(RGDyC)-PEG-Cy5 C' dot-2 and C' dot-6, see Figure 1a) that have near identical properties by FCS and DLS (including dyes/particle). Their syntheses were months apart, however. Shown in Figure 8a are the scattering curves for each particle and the associated fits. Guinier fitting of the low q region is shown in the inset; both particle scattering curves show a slight downturn at very low q , consistent with weak repulsion. The DLS traces for the two C' dots are overlaid in Figure 8b and are indistinguishable in distribution. Likewise, the FCS correlation curves are shown in Figure 8c and are also nearly identical, with radii of 31.5 Å and 31.0 Å for C' dot-2 and C' dot-6, respectively.¹ While the SAXS profiles of both particles also look qualitatively very similar, detailed analysis revealed marked differences. The steeper slope of C' dot-6 in the Guinier analysis is indicative of a larger object. This is corroborated by fitting SG and SB models to the data supporting a larger silica core in C' dot-6 (15.7–15.9 Å) as compared to C' dot-2 (14.6–15.0 Å). The origin of this size discrepancy can be traced back to details of the initial particle synthesis process. Displayed in Figure 8d and 8e are the comparisons of GPC chromatographs for both C' dot-2 and C' dot-6 before and after the GPC based fractionation step. After size separation by GPC fractionation, in which particle product is separated from aggregates and precursors,⁸ the GPC traces (measured using a UV absorbance detector) are virtually identical. In contrast, prior to fractionation, the GPC elugram of synthesized C' dot-6 is slightly skewed towards shorter elution times, which

¹It is important to note that the two FCS autocorrelation curves in Figure 8c were collected under slightly different alignment conditions of the FCS setup on different days. Thus, these two FCS curves should not be compared directly, but r_{FCS} , which is determined from these curves, can be compared in Table 1

correspond to larger particle sizes. This initial wider distribution, fractionated down so that differences become immeasurable by GPC, DLS, and FCS, still leaves a footprint in SAXS. Whereas FCS and DLS are dependent on the interactions between the solvent and polymer brush on the silica core surface, only SAXS can decouple contributions to overall particle size from inorganic core and polymer shell providing detailed information on internal particle structure.

CONCLUSION

We have demonstrated that SAXS is a powerful tool to characterize structural details of ultrasmall organic-inorganic hybrid core-shell silica nanoparticles referred to as C' dots once X-ray beam induced particle damage is overcome, *e.g.* via oscillatory flow conditions minimizing beam exposure to the same sample volume. SAXS then allows one to robustly measure the inorganic core size and to provide reasonable estimates of polymer brush height. Quantitative SAXS data analysis therefore not only enables the assessment of total particle size dispersity, but also the quantification of the size and dispersity of the internal silica core of the C' dots. By utilizing form factor analysis, the distribution of silica cores was found to follow a Schulz-like distribution with reproducibly narrow dispersities $PD_{core} \sim 0.19\text{--}0.21$. The polymer brush layer was modelled using two different approaches (SG and SB models) to account for PEG chain polydispersity, incorporation of functional groups on the surface, and general polymer physics scaling of brushes on curved surfaces. These models serve as upper and lower bounds to the overall C' dot dispersity, agreeing well with DLS and FCS measurements. Decoupling the molar mass dispersity from brush density and curvature considerations remains an active challenge in polymer science. Recent studies of organic shell -inorganic core nanoparticles have begun to provide new methods and models to assess these structures,^{76,77} but polydispersity is thus far under-examined. Nevertheless, we find our simplified model to be an excellent approach for extracting key structure information of hybrid organic-inorganic nanoparticles that can be adapted to look at variations in inorganic core structure, such as additional inorganic shell growth. This information of the internal core structure is inaccessible by methods typically employed to measure particle size. This was highlighted by comparing two particles with identical size characteristics after GPC purification as determined by GPC, DLS and FCS, but with varying degrees of skewness in the as-made particle size distributions as revealed by original particle GPC elugrams on as-made materials (*i.e.* before GPC purification steps). In contrast to all other particle characterization methods employed, SAXS was able to detect differences in the final particle architectures between those two particles. This may be critical information when assessing the performance of these particles *e.g.* in biological environments in order to derive robust design criteria for their successful and safe application in nanomedicine and oncology fields.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Synthesis route to silica-based hybrid nanoparticles, termed C' dots. Multiple pathways and outcomes are shown, including core particles terminated by functionalized PEG-silane (C' dot-1, -2, and -6) or PEG-silane (C' dot-3) and particles with TEOS grown shells terminated with PEG-silane (C' dot-4 and -5). (b) *left*: Molecular-graphics rendering of C' dot structure with a PEG-brush surface (e.g. C' dot-3) and *right*: wedge cut of C' dot containing c(RGDyC) targeting peptides (e.g. C' dot-2) showing the inner silica core and encapsulated Cy5.5 dye. (c) C' dot representative TEM images at two different magnifications of C' dots with diameter ~6 nm. (d) FCS autocorrelation curves (points) and their fits for a selection of C' dots.

Figure 2. Changes in SAXS patterns due to radiation sensitivity of particles (C' dot-3, 5.0 μM) under a static exposure (squares) from a sample sealed in a capillary as compared to the same C' dot sample in an oscillating flow setup (circles).

Figure 3. Fitting C' dot SAXS patterns (C' dot-2, 5.0 μM) from simplest to more complex models: monodisperse silica spheres with a monodisperse, uniform PEG shell (orange dashed curve); silica core with PEG shell, with size dispersity (Schulz) only of the silica core (red curve); core-shell structure with polydisperse (Schulz) silica cores and polydisperse (Gaussian) shell of melt-like PEG (green curve; SG model); and polydisperse silica core (Schulz) with a PEG “brush” like structure (blue curve; SB model). The SAXS trace for C' dot-2 has been plotted three times for ease of comparing the models and these same data sets are shifted vertically for ease of view. Residuals for the three models using dispersity (red, green, blue) are shown at the bottom in the corresponding color. Simplified schematics of the model type are shown in the upper right outlined in the corresponding color, where silica cores are shown in dark gray and PEG shells in light blue.

Figure 4. PEG density profiles for C' dot-2 derived from the two models of the PEG shell, a gaussian distributed shell (SG) and a power law polymer brush (SB). $z = r - r_{core}$ (r_{core} is core radius).

Figure 5.

(a) Scattering curves and form factor fits of C' dots using SG model, with depictions of the particle type shown to the right of each curve. Residuals of the fitting shown below, matched in color to the scattering data. Fits correspond to parameters in Table 1. (b) TEM images of C' dots after 0, 2, and 4 shells grown.

Figure 6.

(a) SAXS profiles of C' dot-4 and C' dot-5 fit with a multi-shell, polymer brush model, depicted by cartoons in the upper right. (b) Calculated size distributions of the silica particle (line) and fully PEGylated particle for C' dots with 0 (C' dot-3), 2 (C' dot-4) and 4 shells (C' dot-5) and compared to other measures of size. Distributions are scaled to equal peak heights.

Figure 7. Comparison of particles sizes and size distributions determined by SAXS, FCS, and DLS for C' dot-1, -2, -5, and -6. Gray curve - distribution of silica cores; thick blue curve - total particle size distribution by SG model (upper dispersity limit); thin blue curve-total particle size distribution by SB model (lower dispersity limit). Red curve - DLS spectra. Closed blue triangle: r_{sphere} (Guinier approximation). Open red triangle: r_{FCS} .

Figure 8.

(a) Comparison of cRGDyC/Cy5.5 C' dot clinical particles made in separate batches. (a) SAXS profiles and SG fits of final purified particles. Inset: low q Guinier region with Guinier approximation fit. (b) size distributions by DLS and (c) FCS autocorrelation curves of the purified particles. (d) GPC elugrams of clinical particles before and after purification by size exclusion fractionation.

Table 1.

C' dot size properties determined by FCS and SAXS using Guinier approximation and SG model.

Sample	$R_{FCS}(\text{\AA})$	r_{sphere}^a (Å)	r_{core} (Å)	PD_{core}	$\rho_{shell} \cdot 10^6 \text{ \AA}^2$	r_{shell} (Å)	PD_{shell}	H_{av} (Å)	χ^2/N_{pts}
C' dot-1	26.5	22.2	14.0	0.20	10.02	9.86	0.63	10.8	1.08
C' dot-2	31.5	23.5	15.0	0.19	10.14	9.48	0.65	10.4	1.09
C' dot-3	33.0	25.4	15.5	0.20	10.12	10.1	0.71	11.8	1.15
C' dot-4	36.5	32.4	21.3	0.23	9.63	13.7	0.63	14.3	2.21
C' dot-5	44.0	37.7	17.1*	0.21	10.59*	13.7	0.23	16.5	32.7
C' dot-6	31.0	27.4	15.7	0.20	10.20	9.79	0.81	12.4	1.17

^a determined from Guinier Rg at lowest concentration (0.25 μM)

Table 2b.

C' dot size properties determined using SB model.

Sample	r_{core} (Å)	PD_{core}	$\rho_{shell} \cdot 10^6$ Å ²	r_{shell} (Å)	t_{brush} (Å)	a	H_{av} (Å)	χ^2/N_{pts}
C' dot-1	14.1	0.20	9.86	8.11	30.2	5.57	10.9	1.01
C' dot-2	14.6	0.21	10.00	7.73	29.6	5.37	10.7	1.20
C' dot-3	15.6	0.20	9.93	7.86	57.6	10.06	12.7	1.08
C' dot-4	21.7	0.22	9.56	11.9	63.4	6.04	20.0	1.25
C' dot-5	16.5*	0.22	10.54*	15.1	18.3	1.63	16.8	14.9
C' dot-6	15.9	0.21	9.96	7.72	51.2	6.99	13.8	1.15

Table 3.

Quantified polydispersities of C' dot hybrid core-shell particles

Sample	Lower limit (monodisperse brush)		upper limit (polydisperse shell)	
	$r_{C'dot}$ (Å) (mode)	$PD_{C'dot}$	$r_{C'dot}$ (Å) (mode)	$PD_{C'dot}$
C' dot-1	23.4	0.11(1)	23.9	0.27(7)
C' dot-2	24.0	0.11(7)	24.1	0.26(7)
C' dot-3	27.6	0.10(7)	25.5	0.29(2)
C' dot-4	31.7	0.13(1)	29.9	0.26(6)
C' dot-5	38.0	0.15(1)	36.5	0.24(3)
C' dot-6	24.9	0.12(2)	25.3	0.30(7)