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Photophysical and biological assessment of coumarin-6 loaded polymeric nanoparticles as a cancer imaging agent.

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Materials and Methods

Materials

Pluronic F-127 was purchased from Sigma-Aldrich (St. Louis, MO., U.S.A), coumarin 6 (MW 350.43) from Santa Cruz Biotechnology (Dallas,TX, USA) and D-Alpha Tocopheryl Polyethelene Glycol 1000 Succinate (Vitamin E-TPGS) from Eastman Chemical Company (Kingsport,TN, USA). Acetone (ACS grade) and Dichloromethane anhydrous (purity ≥ 99.8 %) were purchased from Sigma-Aldrich and Dimethyl Sulfoxide (DMSO), extra pure (99.9%) from Scharlau chemicals (Barcelona, Spain). Phosphate-Buffered Saline (PBS) tablets were purchased from gibco by life technologies (Grant Island, NY, USA).

Cell culture and Reagents

MCF-7, MDA-MB-231 and MCF-10A cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). MCF10CA1a (MIV) breast cancer cells expressing the luciferase gene (MIV-Luc) [1] were obtained from the Karmanos Cancer Institute (Detroit, MI, USA) and were used as a negative control in all experiments since they do not display any green autofluorescence. MCF-7 and MDA-MB-231 breast cancer cell lines were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS) and 1% antibiotic/antimycotic. MCF-10A immortalized breast cell line and MIV-Luc cell line were cultured in DMEM F12 supplemented with 20 ng/ml EGF, 100 ng/ml Cholera Toxin, 500 ng/ml Hydrocortizone, 10 µg/ml Insulin, 5 % Horse Serum (HS) and 1% antibiotic/antimycotic. Sub culturing of the cells was performed using 0.25% trypsin. The DMEM, FBS, antibiotics and trypsin used in cell culture were purchased from Gibco, Invitrogen (Carlsbad, California, USA).

Nanoparticle synthesis

Coumarin 6-loaded nanoparticles were prepared by the single-emulsion method and as previously described [2]. Briefly, Pluronic-F127 was used as the polymer carrier, D-alpha-tocopheryl polyethylene glycol 1000 succinate (Vitamin E-TPGS) as the emulsifier and Coumarin 6 as the encapsulant. Pluronic-F127 and Coumarin 6 were mixed in dichloromethane at a ratio of 1:40 by mass and the resulting polymer/encapsulant solution was added to Vitamin E-TPGS at a ratio of 1:2 by volume on high vortex. The resulting emulsion was ultrasonicated (Misonix, Ultrasonic Liquid Processors) and magnetically stirred in 45 mL Vitamin E-TPGS solution for 3 hrs to evaporate the solvent, washed three times with distilled water by centrifugation (17,000 rcf for 15 min at 22°C (Cientec CT-15000R centrifuge)) to remove unentrapped drug and unused polymer. The supernatant from the washes was collected and further used in the determination of the drug encapsulation efficiency (EE). The resulting nanoparticles in water were freeze-dried and stored until further use. For all experiments, the nanoparticles were resuspended in relevant media prior to use in biological assays by sonication in a water bath sonicator and subsequently filtered through a 0.45 µm syringe filter ((PTFE, Hydrophilic, Dissolution Accessories, (Oosterhout, The Netherlands)) to remove aggregates.

Physical Characterization

AFM For AFM imaging, the nanoparticles were diluted 1:100 from original solution (3.1 mg/ml) with PBS and were filtered through a 0.45 µm syringe filter. The nanoparticle suspension was sonicated at room temperature for 15 min and 30 µl was deposited onto pretreated mica with 3-aminoproply-trietoxy silane (APTES) (Pelco Mica Disc, V1, Ted Pella) for 5 min. The sample was rinsed with 1 ml deionized water and the surface was dried using a stream of argon. AFM images were captured using an Asylum Research MFP-3D-BIO (Oxford Instruments) microscope in tapping mode at room temperature AFM probe with resonance frequencies of approximately 70 kHz and spring constant of approximately 2 N/m was used for imaging. Images were collected at a speed of 1 Hz with an image size of 2 x 2 µm or 0.5 x 0.5 µm at 512 x 512 pixels resolution. The images were processed with Igor software.

DLS The NP solution was characterized for hydrodynamic diameter and surface zeta potential in H2O and saline. DLS experiments were performed using a Malvern 500 instrument to measure the mean diameter and polydispersity index (PDI) of the nanoparticles. Settings included a 632. 8 nm incident laser wavelength for the 10 mW He-Ne laser. Measurements were performed within 10 mm path length polystyrene cuvettes. Zeta potential was measured at room temperature on the same instrument using electrophoretic light scattering (ELS) to approximate the surface charge of the nanoparticles. The measurement settings were consistent with the DLS settings. For stability studies the nanoparticle in saline was kept in a 37°C incubator for the duration of the study. Where appropriate the samples were filtered through a 0.45 µm syringe filter prior to measurement.

Encapsulation efficiency The percent Encapsulation Efficiency (% EE) of Coumarin-6 loaded nanoparticles was determined by UV-VIS spectrophotometry. Two methods were utilized to verify the % EE: method 1, by indirect analysis of the supernatant obtained from the washes during synthesis (un-encapsulated drug) and method 2, by direct analysis of the nanoparticle (encapsulated drug). For method 1, the supernatant collected from the washes during synthesis was centrifuged at 10000 rpm for 5 min at 20°C to remove any free polymer and Vitamin E-TPGS. Absorption of the supernatant was recorded on a spectrophotometer to determine the absorption coefficient of coumarin-6 and thus the concentration of

drug that was not encapsulated in the nanoparticle. This concentration was subtracted from the one added during synthesis to determine the amount of drug encapsulated (C1) in the nanoparticle. For method 2, 1 mg of lyophilized nanoparticle in 1mL DMSO was sonicated for 15 min to break up the nanoparticles and ensure complete release of the encapsulated C6. The solution was then centrifuged at 10000 rpm for 10 min at 20°C to remove any polymer and vitamin E-TPGS in the solution and the supernatant was analysed on a UV-Vis spectrophotometer. The concentration of coumarin-6 was calculated at a maximum absorbance of 465 nm by using the Beer-Lambert law and its predetermined molar extinction coefficient (ε). All experiments were performed in six replicates, each from a different fabrication batch.

The % entrapment efficiency (EE%) was calculated from equation (1)

$$\mathbf{EE}(\%) = \frac{\mathbf{C1}}{\mathbf{C2}} \times \mathbf{100}\%\,, \qquad \text{eq. 1}$$

where C1 was the amount of Coumarin 6 encapsulated in the NPs and C2 is the total amount of Coumarin 6 used during synthesis.

Drug Release Study The release of Coumarin-6 from the nanoparticle was investigated at pH 7.3, pH 6.0 and pH 4.5 to mimic physiological pH, tumor pH and lysosomal pH, respectively. Stock solution of nanoparticle was dispersed in 3 ml phosphate-buffered saline (PBS) and placed in the dialysis cassette (MWCO 3500, Thermo Scientific). The dialysis cassettes were immersed in 500 mL of PBS (pH 7.3, pH 6.0 and pH 4.5) and the samples were maintained in an incubator shaker (climo-shaker ISFI-X) at 37° C and shaken at 60 rpm for the duration of the experiment. 2 mL samples were collected from outside the cassettes at each time point. Sink conditions were maintained by substituting an equal volume of buffer at each sampling point. The fluorescence of C6 was measured on a multimodal plate reader (Tecan Spark 20M) (excitation wavelength of 460 nm and an emission wavelength of 530 nm).

Photoluminescence Optical absorption was recorded by a Perkin Elmer Lamda 1050 spectrophotometer equipped with a three-detector module covering the 300-3000 nm spectral range. Steady-state photoluminescence (PL) experiments were carried out on a 0.35 m FluoroLog FL3 Horiba Jobin Yvon spectrofluorimeter. The samples were excited via an OXXIUS 375nm cw laser diode module at 4mW. PL quantum yield (QY) experiments were executed according to reference (J. C. de Mello; H. F.Wittmann; R. H. Friend, An Improved Experimental Determination of External Photoluminescence Quantum Efficiency, Adv. Mater., 1997, pp. 230–232.) using a 4 inches Labsphere integrating sphere. The light was collected via a fiber bundle and recorded via a 0.75 m Acton750i Princeton spectrometer equipped with a 1024x256 pixels PIXIS charge-coupled device (CCD) camera in the range of 250-950 nm. The samples were excited via the same oxxius, 375 nm, 4 mW laser diode used for the steady state PL measurements.

Stability studies C6NPs were suspended in solvent and vortexed at medium speed for 1-2 minutes. For size and composition characterization using FCS and DLS, Millipore water (ddH_2O) and 1x phosphate buffered saline (1x PBS) were used to resuspend the nanoparticles (NPs). For stability tests, solvents used to resuspend the NPs were ddH_2O, 1x PBS, and 0.05x fetal bovine serum (0.05x FBS). The particle suspension was then ultrasonicated (Branson 1510 Ultrasonic Bath Cleaner) for three rounds of 15 minutes each, with 2 minutes in between. After sonication, the particle suspension was filtered using a 0.45 µm syringe filter (PTFE, Hydrophilic, Dissolution Accessories, Oosterhout, The Netherlands). The filtered NP solution was monitored using fluorescence correlation spectroscopy (FCS) for a total of three hours (180 minutes). During this time, 30-second FCS runs were taken periodically to monitor particle concentration, brightness, and size distribution within the solution.

FCS methodology and instrumentation has been thoroughly described elsewhere [3, 4]. In the FCS instrumental setup, a 82 MHz 100 fs pulsed laser at a wavelength of 774 nm with an attenuated power measured at 30 mW is directed into the back aperture of a 40x water-immersion objective lens. The objective lens focuses this laser irradiation into an aqueous sample within a chamber slide, where a two-photon excitation process occurs to excite the analyte fluorophores in a focal volume on the order of one femtolitre. Fluorescence emission from the fluorophores is redirected to a network of detectors in a way that separates the emitted light spectrally and allows for the separate monitoring of red and green fluorescence. Fluctuations in fluorescent intensity signal are detected within the focal volume as the result of fluorophores' Brownian motion in and out of the focus of the laser. These fluctuations are temporally autocorrelated over time and result in an autocorrelation curve (Figure S3) which is fitted with the following function:

$$\mathbf{G}(\boldsymbol{\tau}) = \mathbf{G}(\mathbf{0})\left(\mathbf{1} + \frac{\mathbf{8D\tau}}{\mathbf{r_0}^2}\right)^{-\mathbf{1}}\left(\mathbf{1} + \frac{\mathbf{8D\tau}}{\mathbf{z_0}^2}\right)^{-\frac{\mathbf{1}}{\mathbf{2}}} \qquad \text{eq. 2}$$

where r0 is the laser beam waist radius, τ is the lag time, D is the diffusion coefficient, and z0 is the excitation focal volume depth. The autocorrelation amplitude, G(0), is influenced by brightness (η) and concentration (C) of emitters as shown in equation 2 below, where NA is Avogadro's number and V is the focal volume.

$$\mathbf{G(0)} = \frac{\sum \mathbf{i}\, \eta_i^2 C_i}{(\sum \mathbf{i}\, \eta_i^2 C_i)^2 V N_A} \qquad \text{eq. 3}$$

Equations 2 and 3 allow for independently diffusing entities in the solution to be characterized in terms of diffusion coefficients, concentration, and fluorescent brightness based on autocorrelation analysis. The functions can be adapted for two diffusers, in which case eq. 2 becomes:

$$\mathbf{G(\tau)} = \mathbf{G(0)_1}\left(\mathbf{1} + \frac{8D_1\tau}{r_0^2}\right)^{-1}\left(\mathbf{1} + \frac{8D_1\tau}{z_0^2}\right)^{-\frac{1}{2}} + \mathbf{G(0)_2}\left(\mathbf{1} + \frac{8D_2\tau}{r_0^2}\right)^{-1}\left(\mathbf{1} + \frac{8D_2\tau}{r_0^2}\right)^{-\frac{1}{2}} \quad \text{eq. 4}$$

which allows two entities to be distinguished based on diffusion coefficients (D_i) and their respective contributions to the overall signal as established by the fraction of the total G(0) amplitude contributed by each individual component ($G(0)_i$). Once diffusion coefficients are measured, these can be used to calculate hydrodynamic radii and diameters using the Stokes-Einstein equation shown below, where kb is the Boltzmann constant, T is temperature in Kelvin, η is viscosity of the solution at temperature T, and R_H is the hydrodynamic radius.

$$\mathbf{D} = \frac{k_b T}{6\pi\eta R_H} \qquad \text{eq. 5}$$

During stability tests, diffusion coefficients and G(0) values from autocorrelation curves were recorded over time to calculate hydrodynamic diameter values and to approximate the fractional composition of the NPs in solution. This was done by taking 35 to 50 15-second FCS autocorrelation measurements over the span of three hours. Data from these stability tests were used to calculate fractional composition and average hydrodynamic diameters, if the samples were established to be stable.

In vitro characterization

Fluorescence microscopy To evaluate the cellular uptake of NPs in cancer cells, MIV-Luc cells were incubated with the nanoparticle for 4 h. Cells were seeded in 6-well plates at a density of $2x10^5$ cells per well with coverslips and incubated overnight to allow for cell attachment. Cells were treated with 250 µg/mL of C6 NP and incubated for 4 hours. At the end of the incubation, cells were washed 3 times with PBS, fixed with 4% paraformaldehyde (PFA) for 10 minutes. Cells were washed 4 times with PBS. To quench auto-fluorescence coverslips were incubated with 1M ammonium chloride for 15 minutes. Next, the coverslips were washed 3 times with PBS and mounted with DAPI mounting medium (Sigma).

Flow cytometry Coumarin-6 loaded nanoparticles were incubated with MCF-7, MDA-MB-231, MCF-10A cells and MIV-Luc. MCF-7, MDA-MB-231, MCF-10A and MIV-Luc cells were seeded in 6-well plates (2x105 /well) and incubated with 100, 250 and 500 µg/mL of C6 nanoparticles for 30, 60 and 240 minutes. At the end of each incubation time point, cells were washed 3 times with PBS, harvested with trypsin and centrifuged for 5 minutes at 1500 rpm. Following supernatant aspiration, the cells were resuspended in 500 µl of PBS prior to FACs analysis. Data were acquired on a Bio-Rad S3e Cell Sorter flow cytometer and analyzed using FlowJo software (Treestar). The experiment was repeated three times on separate days for MCF-7, MDA-MB-231, MCF-10A, and only once for the MIV-Luc cell line.

In vivo experiments

Zebrafish housing and breeding Zebrafish (*Danio rerio*; Wik strain) were grown from bleached eggs acquired by the European Zebrafish Resource Centre Institute of Biological and Chemical Systems (Germany) and housed/maintained at a dedicated zebrafish facility licensed by the Veterinary Services of the Ministry of Agriculture, Rural Development and Environment of Cyprus of the Republic of Cyprus (license CY/EXP/PR.L8/2018). Zebrafish were maintained in an automated ZebTEC Stand-Alone system with an 'Active Blue' Technology of recirculating water system (Techniplast, Italy), maintaining optimum living conditions (28.5°C, 7.5 pH, and 550 µS conductivity). Photoperiod was set to 14:10 hours of light/dark cycles. Fish were fed twice daily (morning & afternoon) with a high percentage of soluble hydrolyzed marine proteins, HUFAs, phospholipids and algae (GEMMA 300micro for adult fish and GEMMA 75micro for larvae, Skrettting Ltd). Adult fish were also fed with instant baby brine shrimp (Artemia nauplii, Ocean nutrition Ltd) as additional portions, for the two days prior to breeding events.

Breeding events were performed in appropriate on-bench breeding tanks (Techniplast, Italy) using system water at 28°C. Eggs were collected at 1-2 h after first-light in the morning and fertilized eggs were selected and placed in open beakers with embryo medium (E3 medium; 13.7 mM NaCl, 0.54 mM KCl, 1.0 mM MgSO4, 1.3 mM CaCl2, 0.025 mM Na2HPO4, 0.044 mM KH2PO4, 4.2 mM NaHCO3), complemented with 0.00005% methylene blue, for ~72 h until all the larvae were

hatched. After hatching, the larvae were kept in E3 with ~50% medium changed on a daily basis. At day 5 post-fertilization (5 dpf) the larvae were transferred in a beaker with fresh system water that was changed daily prior a twice-a-day feeding with GEMMA 75micro. At 10dpf the larvae were in the ZebTEC system with a small stream of cycling water (1-2 drops per second) and were continued to be fed twice-a-day with GEMMA 75micro until the initiation of exposure experiments.

Results

Drug release

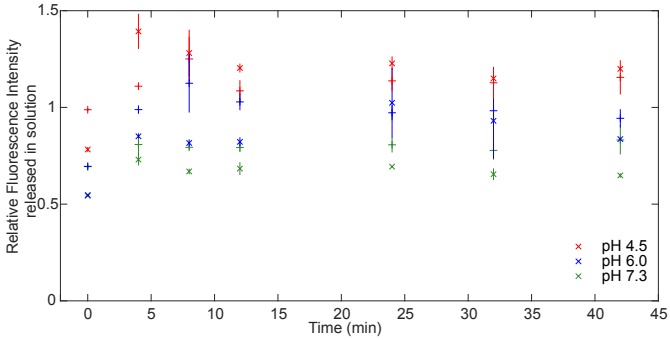


Figure S1. pH dependent C6 release. The relative fluorescence intensity released from C6-NPs suspended in PBS was measured by collecting solution through a dialysis membrane. Lower pH shows a faster release of the dye.

Stability studies

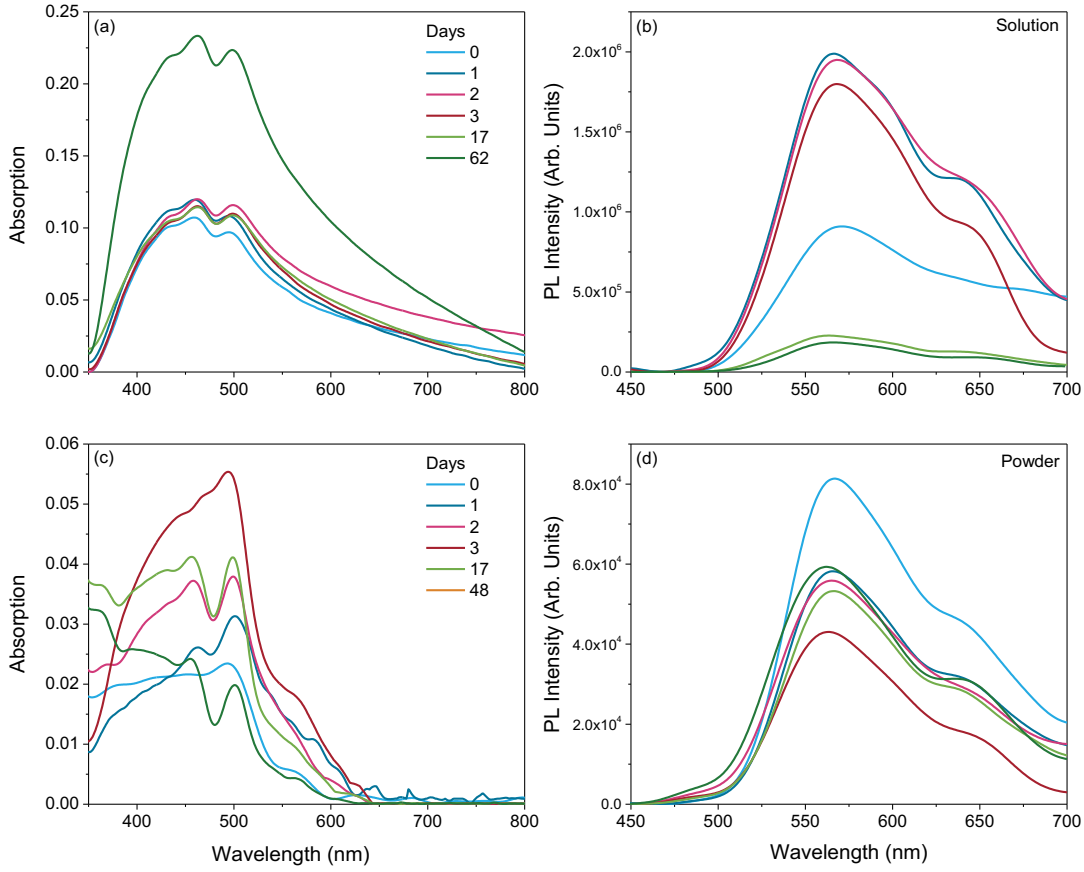


Figure S2: Nanoparticle absorption and emission spectra over a period of 62 days. A. Absorption spectra of the nanoparticle in solution (water). B. Emission spectra of the nanoparticle in solution. C. Absorption spectra of the nanoparticle in powder form. B. Emission spectra of the nanoparticle in powder form.

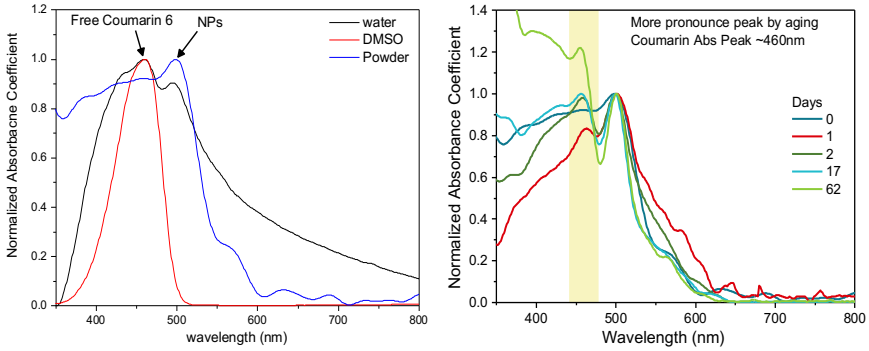
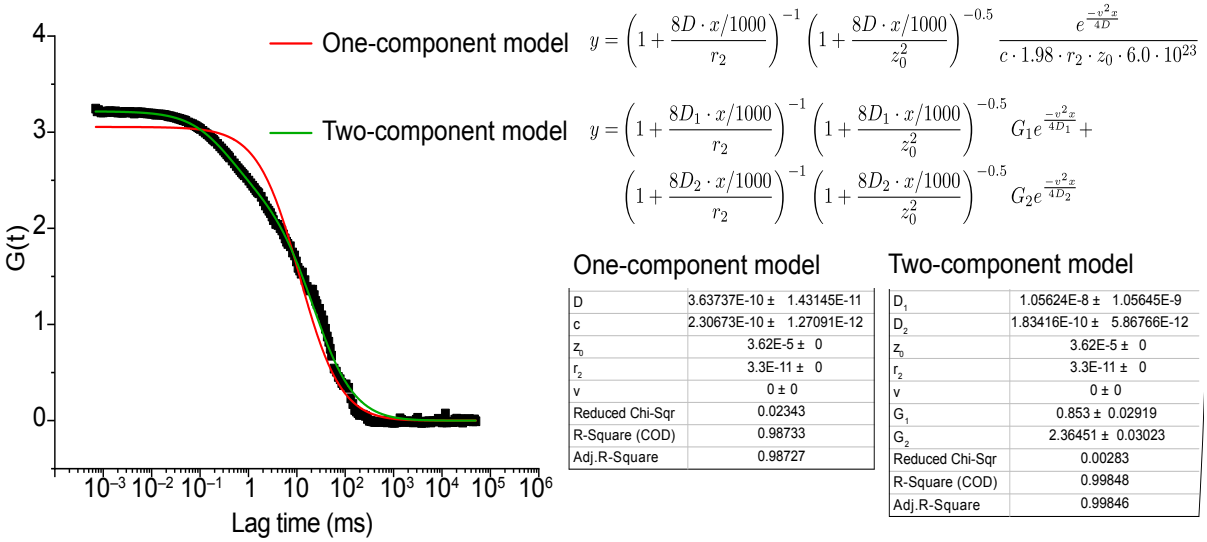


Figure S3: A. Absorption profile of water, DMSO and nanoparticle in powder form. B. Absorption spectra of the nanoparticle in powder form over a period of 62 days. The peak at 460 nm due to coumarin-6 gets more prominent over time.



$$y = \left(1 + \frac{8D \cdot x/1000}{r_2}\right)^{-1} \left(1 + \frac{8D \cdot x/1000}{z_0^2}\right)^{-0.5} \frac{e^{\frac{-v^2 x}{4D}}}{c \cdot 1.98 \cdot r_2 \cdot z_0 \cdot 6.0 \cdot 10^{23}}$$

$$y = \left(1 + \frac{8D_1 \cdot x/1000}{r_2}\right)^{-1} \left(1 + \frac{8D_1 \cdot x/1000}{z_0^2}\right)^{-0.5} G_1 e^{\frac{-v^2 x}{4D_1}} + \left(1 + \frac{8D_2 \cdot x/1000}{r_2}\right)^{-1} \left(1 + \frac{8D_2 \cdot x/1000}{z_0^2}\right)^{-0.5} G_2 e^{\frac{-v^2 x}{4D_2}}$$

One-component model

D	3.63737E-10 ± 1.43145E-11
c	2.30673E-10 ± 1.27091E-12
z_0	3.62E-5 ± 0
r_2	3.3E-11 ± 0
v	0 ± 0
Reduced Chi-Sqr	0.02343
R-Square (COD)	0.98733
Adj.R-Square	0.98727

Two-component model

D_1	1.05624E-8 ± 1.05645E-9
D_2	1.83416E-10 ± 5.86766E-12
z_0	3.62E-5 ± 0
r_2	3.3E-11 ± 0
v	0 ± 0
G_1	0.853 ± 0.02919
G_2	2.36451 ± 0.03023
Reduced Chi-Sqr	0.00283
R-Square (COD)	0.99848
Adj.R-Square	0.99846

Figure S4: Autocorrelation curve for C6NPs in ddH_2O, showing curve-fitting using a one-component diffusion model (in red) and a two-component diffusion model (in green).

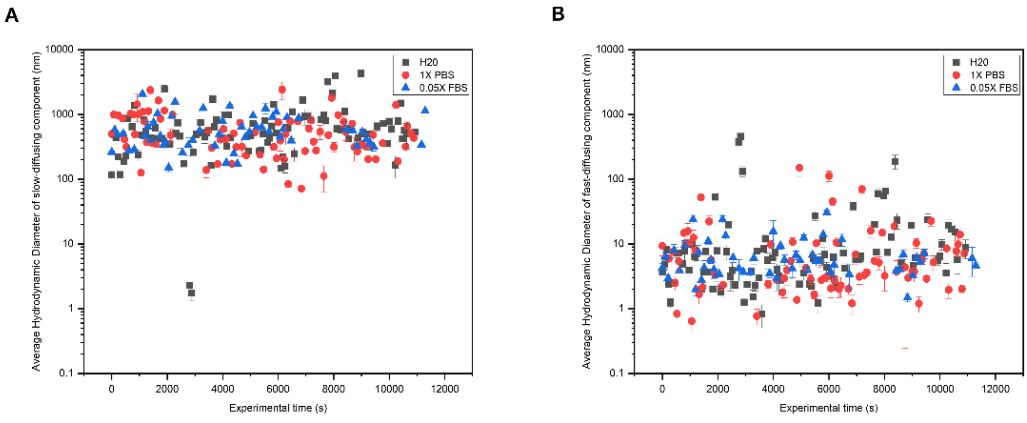


Figure S5: Example plots of hydrodynamic diameters over a three-hour measurement window, calculated for C6NPs, for A) fast-diffusing or small components in Millipore water, b) slow-diffusing or large components in Millipore water, c) fast-diffusing or small components in 1x PBS, d) slow-diffusing or large components in 1x PBS, e) fast-diffusing or small

components in 0.05x FBS, and f) slow-diffusing or large components in 0.05x FBS. Errors are from curve fitting error propagated through calculations to convert diffusion coefficient to hydrodynamic diameter.

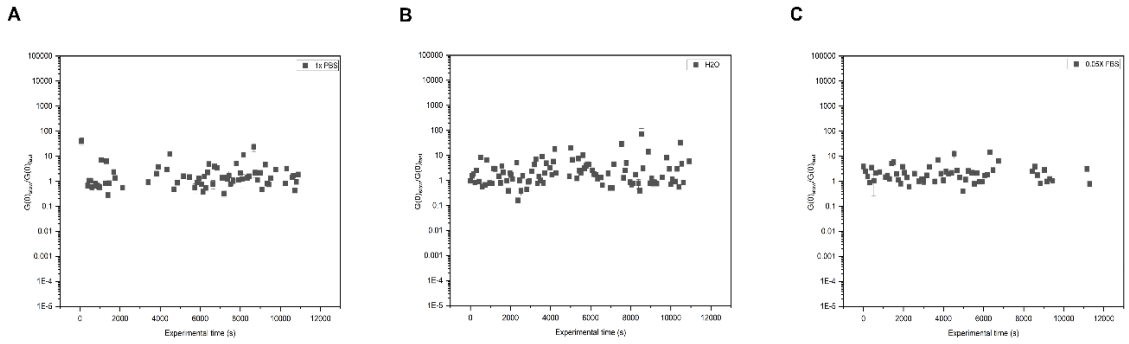


Figure S6: Plots of $G(0)_{slow}/G(0)_{fast}$ over a three hour measurement window, calculated for C6NPs, for A) slow-diffusing /fast-diffusing components in 1XPBS, B) slow-diffusing /fast-diffusing components in Millipore water and C) slow-diffusing /fast-diffusing components in 0.05XFBS. Errors are from curve fitting error.

References

1. Papageorgis, P.; Ozturk, S.; Lambert, A. W.; Neophytou, C. M.; Tzatsos, A.; Wong, C. K.; Thiagalingam, S.; Constantinou, A. I., Targeting IL13Ralpha2 activates STAT6-TP63 pathway to suppress breast cancer lung metastasis. *Breast cancer research : BCR* **2015,** *17* (1), 98.
2. Chrysafis Andreou, Y. G., Akbar Ali, Suchetan Pal, *SERS for Point-of-care and Clinical Applications*. Elsevier: 2021.
3. Clancy, A. A.; Gregoriou, Y.; Yaehne, K.; Cramb, D. T., Measuring properties of nanoparticles in embryonic blood vessels: Towards a physicochemical basis for nanotoxicity. In *Chemical Physics Letters*, 2010; Vol. 488, pp 99-111.
4. Yaehne, K.; Tekrony, A.; Clancy, A.; Gregoriou, Y.; Walker, J.; Dean, K.; Nguyen, T.; Doiron, A.; Rinker, K.; Jiang, X. Y.; Childs, S.; Cramb, D., Nanoparticle accumulation in angiogenic tissues: towards predictable pharmacokinetics. *Small (Weinheim an der Bergstrasse, Germany)* **2013,** *9* (18), 3118-27.