Published in final edited form as:

Langmuir. 2014 January 21; 30(2): 631–641. doi:10.1021/la403943w.

Holistic assessment of covalently-labelled core-shell polymeric nanoparticles with fluorescent contrast agents towards theranostic applications

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Abstract

The successful development of degradable polymeric nanostructures as optical probes for use in nanotheranostic applications requires the intelligent design of materials such that their surface response, degradation, drug delivery and imaging properties are all optimized. In the case of imaging, optimization must result in materials that allow differentiation between unbound optical contrast agents and labeled polymeric materials as they undergo degradation. In this study, we have shown that use of traditional electrophoretic gel-plate assays for determination of the purity of dye-conjugated degradable nanoparticles is limited, due to polymer degradation characteristics. To overcome these limitations, we have outlined a holistic approach to evaluating dye- and peptide-polymer nanoparticle conjugation by utilizing steady-state fluorescence, anisotropy, and emission and anisotropy life-time decay profiles, through which nanoparticle-dye binding can be assessed independent of perturbations, such as those presented during the execution of electrolyte gel-based assays. This approach has been demonstrated to provide an overall understanding of the spectral signature-structure-function relationship, ascertaining key information on interactions between the fluorophore, polymer and solvent components that have a direct and measurable impact on the emissive properties of the optical probe. The use of these powerful techniques provides feedback that can be utilized to improve nanotheranostics by evaluating dye emissivity in degradable nanotheranostic systems, which has become increasingly important as modern platforms transition to architectures intentionally reliant on degradation and built-in environmental responses.

1. INTRODUCTION

Much current interest has been placed on the development of complex polymer-based nanoscopic platforms, such as polymeric micelles and shell crosslinked knedel-like nanoparticles (SCKs), as efficient imaging and nanotheranostic platforms.^{1–3} Recent

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interests.

Supporting Information. Additional experimental, characterization and spectroscopic information is supplied as Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

development of such platforms has shifted from the use of non-degradable polymeric materials towards biodegradable polymer components,^{2, 4–6} including poly(glucose carbonates),^{7–10} polyphosphoesters^{11–13} and poly(lactic acid).^{14–16} However, with this shift comes an intrinsic complexity to the preparation, characterization and utilization of degradable materials that must be considered with rigorous experimental investigation.

In the design of degradable polymeric nanomaterials as optical probes, such rigorous assessment is critical to the successful development of these platforms (both *in vitro* and *in vivo*), and requires that one is able to distinguish between free optical contrast agents and labeled polymers as they undergo degradation and excretion from the body. Traditional evaluation of non-degradable polymeric materials, generally having different uptake characteristics *in vitro* and long clearance times *in vivo* as compared to free dyes,¹⁷ has allowed for their use largely without concern for the efficiency of dye conjugation and the removal of free dye from the final polymeric contrast agent.^{18, 19} When evaluation of the efficiency of conjugation or binding events has been of importance, non-degradable materials allow for the use of typical gel-plate assays commonly employed to evaluate biomacromolecule (antibody, protein, *etc.*) dye conjugation.^{20, 21} Thus, methods for assessment of covalent dye coupling to degradable polymers have not typically been developed independent of those appropriate for non-degradable materials.^{17, 22, 23} We have found that applying the use of gel-plate assays to determine the purity of dye-conjugated degradable polymers does not provide a reliable answer as to the purity of the final polymeric contrast agent, unlike traditional non-degradable platforms. Herein, we demonstrate that such methods can cause degradation of the polymer itself, which leads to an inaccurate assessment of labeling efficiencies. To accurately follow the biodistribution and clearance of such materials *in vivo*, it is important to not only identify materials-based degradation characteristics, but to thoroughly evaluate the labeling efficiency for covalent coupling of optical contrast agents. The complications of premature degradation during initial characterization of labeled, degradable materials, and their subsequent extended degradation when applied *in vitro* and *in vivo* are especially challenging for rapidly degrading systems, including certain polyphosphoesters, where degradation has been confirmed in solution within 48 hours.²⁴ In such cases, the ability to track the biodistribution of unbound dyes (normally cleared within 24 h) *vs.* early degradation products (with unknown clearance rates) becomes increasingly difficult.

Within this body of work, we describe a series of nanoparticle systems whereby dye labeling is assessed in a holistic manner. Specifically, we propose a combination of steady state, anisotropy, and emission lifetime and anisotropy decay techniques in which polymeric nanoparticle-fluorophore conjugates can be assessed independent of “harsh” methods, including electrolyte gels that may lead to degradation.

2. EXPERIMENTAL SECTION

Materials and Methods

All chemicals and reagents were used as received from Sigma-Aldrich unless otherwise noted. Bis pentafluorophenol discrete poly(ethylene glycol)₄ (Bis dPEG4-PFP ester) was obtained from Quanta Biodesign Limited. Dichloromethane (DCM) was purified by passage through a solvent purification system (JC Meyer Solvent Systems) and used as dry solvent. Phosphate buffered saline (PBS) was purchased in a 10x solution from VWR and diluted to a concentration of 1x with nanopure water. Nanopure water (18 M Ω ·cm) was acquired by means of a Milli-Q water filtration system, Millipore Corp. Fluorescein isothiocyanate (FITC) labeled F3 scramble peptide at > 98% purity by RPHPLC was prepared by ChinaPeptides and has the sequence FITC-(aminohexanoic acid)-

KDEARALPSQRSRKPAAPPKPE PKEKKAPAKKC. Alexa 488 (A488) tetrafluorophenol (TFP) ester was purchased from Life Technologies.

UV/Vis measurements were acquired on a Shimadzu UV-2550 spectrophotometer. All steady state emission, excitation and anisotropy spectra were obtained with a Horiba Fluoro-Max4 with automatic polarizers. Time correlated single photon counting (TCSPC) was employed to obtain all fluorescence lifetime and fluorescence anisotropy decay spectra. Measurements were achieved *via* a Fluorotime 100 fluorometer with a 405 nm solid state ps diode laser source (PicoQuant) in matched quartz 0.7 mL cells (NSG Precision Cells). Instrument response functions (IRF) were determined from scatter signal with a solution of Ludox HS-40 colloidal silica (0.01% particles in water wt/wt). Steady state spectra were analyzed in FluorEssence (Horiba) and in Origin 9 Pro (Origin Labs). TCSPC analysis was performed on Fluorofit (PicoQuant) software and confirmed by tail-fitting in Origin 9 Pro. All IRF deconvolved exponential fits were performed with the number of exponents selected for completeness of fit as determined by boot-strap chi-squared analysis in Fluorofit.

Dynamic light scattering (DLS) measurements were conducted using a Delsa Nano C from Beckman Coulter, Inc. equipped with a laser diode operating at 658 nm. Scattered light was detected at 165° to incident and analyzed using a log correlator over 70 accumulations for a 1.0 mL of sample in a PMMA size cell (3.0 mL capacity). The photomultiplier aperture and the attenuator were automatically adjusted to obtain a photon counting rate of *ca.* 10 kcps. Calculation of the particle size distribution and distribution averages was performed using CONTIN particle size distribution analysis routines using Delsa Nano 3.73 software. The peak averages of histograms from intensity, volume and number distributions after 70 accumulations were reported as the average diameter of the particles. All determinations were repeated 10 times.

Transmission electron microscopy (TEM) images were collected on a JEOL 1200EX operating at 100 kV and micrographs were recorded at calibrated magnifications using a SIA-15C CCD camera. Samples for TEM measurements were prepared as follows: 5 μ L of a 0.2 mg/mL solution of cationic shell-crosslinked knedel-like nanoparticles (cSCKs) was deposited onto a Formvar carbon film on 300 mesh copper grid for 1 min. Excess sample was wicked off using filter paper and the grids were allowed to dry in air. Subsequently, the grids were stained with 5 μ L of a 1 wt% phosphotungstic acid (polyphosphoester (PPE) cSCKs) or 1% uranyl acetate (poly(acrylamidoethylamine) (PAEA) cSCKs) aqueous solution. After 1 min, the excess staining solution was wicked away with a piece of filter paper and the samples were left to dry under ambient conditions overnight. Particle analysis was performed on 100+ particles using ImageJ (NIH) software, using both intensity thresholding and circularity filters. Atomic force microscopy was performed using a Multimode 8 system (Bruker) with a SA Fluid+ silicon probe (k 0.7 N/150 kHz, Bruker). For AFM preparation, samples were dissolved at 0.2 mg/mL in PBS pH 7.4 and 20 μ L of the sample was spin coated onto a glass coverslip. All AFM samples were stored at room temperature in a vacuum desiccator until use. AFM images were assessed with Nanoscope Analysis (Bruker).

Synthetic Protocols

Synthesis and characterization of cationic PPE₄₉-*b*-PLLA₄₄ (cPPE₄₉-*b*-PLLA₄₄) was performed as reported previously.¹⁶

Synthesis of 2,3,5,6-tetrafluorophenyl but-3-enoate—4-pentenoic acid (2.0 g, 0.020 mol), tetrafluorophenol (4.0 g, 0.024 mol) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (4.6 g, 0.024 mol) were dissolved in dry DCM (25 mL) under N₂ with stirring at room temperature. After 20 h the reaction was checked by thin

layer chromatography (TLC). The reaction was then washed with aqueous saturated sodium bicarbonate solution (50 mL) twice, extracted with DCM (25 mL), washed with brine (50 mL) and dried over anhydrous sodium sulfate. The organic layer was then removed under vacuum and the product was purified by column chromatography (SiO₂) eluted with a stepwise gradient of 0 to 20 % ethyl acetate in hexanes to yield the desired product (4.3 g, 0.017 mol, 87 % yield). ¹H NMR (500 MHz, CDCl₃) δ ppm 7.04 – 6.93 (m, 1H), 5.8 (ddt, *J* = 10.0, 6.43 Hz, 1H), 5.20 – 5.06 (m, 2H), 2.79 (t, *J* = 7.38 Hz, 2H), 4.28 (dd, *J* = 10.0, 4.6 Hz, 1H), 2.58 – 2.50 (m, 2H); ¹³C NMR (126 MHz, CDCl₃) δ ppm 168.98, 146.14 (dtd, *J* = 248.5, 11.9, 4.1 Hz), 140.73 (dddd, *J* = 250.1, 15.1, 4.6, 2.1 Hz), 135.59, 116.53, 103.27 (t, *J* = 22.8 Hz), 32.87, 28.7; ¹⁹F NMR (282 MHz, CDCl₃) δ ppm –154.98 – –154.80 (m, 2F), –141.14 – –140.92 (m, 2F).

Preparation of cationic shell crosslinked knedel-like nanoparticles (parent PPE cSCKs)—cPPE_{49-b}-PLLA₄₄ (80 mg, 0.31 mmol) was dissolved in PBS pH 7.4 at 1 mg/mL and sonicated for 45 min in ice water, formation of micelles was confirmed by DLS. 2,3,5,6-tetrafluorophenyl but-3-enoate (0.37 mg, 0.0015 mmol) in DMSO at 1 mg/mL was added to the micelle solution dropwise over 2 min. Subsequently, the crosslinker Bis pentafluorophenol discrete poly(ethylene glycol)₄ (Bis dPEG₄-PFP ester) (29 mg, 0.046 mmol, 100 mg/mL in DMSO) was added by syringe pump over 1 h with stirring at room temperature. After an additional 2 h, the resulting cSCKs were separated into a 20 mL fraction for A488 labeling and a 60 mL fraction of parent PPE cSCKs, the latter of which was allowed to continue reacting for 2 h and then purified in 20 mL fractions by dialysis (regenerated cellulose (RC) dialysis tubing, 50 kDa MWCO) against PBS pH 3.0 for 60 h, then nanopure water for 12 h (all at 4 °C). Following purification, a fraction was separated for attachment of the F3 scramble peptide. The remaining (~40 mL) of parent cSCKs were lyophilized in 0.1 and 0.5 mg fractions and stored at –20 °C.

Alexa488 (A488) labeled Parent cSCKs (A488 cPPE)—20 mL of the parent PPE cSCK solution was removed to a separate reaction vessel and Alexa488 tetrafluorophenyl ester (Alexa488-TFP) (0.34 mg, 0.037 μmol), dissolved in DMSO at 1 mg/mL, was added dropwise over 1 min. The reaction was left for 2 h with stirring at room temperature before purification by dialysis (RC dialysis tubing, 50 kDa MWCO) against PBS pH 3.0 for 60 h, then nanopure water for 12 h (all at 4 °C). Dialysis water was changed every 6 – 12 h. Dialysate was monitored by fluorescence spectroscopy for loss of A488. A488 cPPE were lyophilized in 0.1 and 0.5 mg fractions and stored at –20 °C.

F3 scramble (F3 scr.) peptide conjugation to cSCKs (F3 scr. cPPE)—2,2-Dimethoxy-2-phenylacetophenone (DMPA) (0.19 mg, 0.75 μmol, 1 mg/mL in dimethyl sulfoxide (DMSO)) and F3 scr. peptide (1.14 μmol) in 200 μL DMSO was added to a 20 mL fraction of dialyzed parent cPPE cSCK and degassed under N₂ for 15–20 min. Ultraviolet (UV) light (365 nm) was applied for 4 h with stirring at room temperature. The particles were then purified by dialysis (cellulose ester (CE) tubing, 300 kDa MWCO) against PBS pH 3.0 for 60 h, then nanopure water for 12 h (all at 4 °C). Dialysis water was changed every 6 – 12 h. Dialysate was monitored by FL spectroscopy for loss of FITC labeled peptide. The product was checked at dialysis water changes by sodium dodecyl sulfate (SDS) poly(acrylamide) gel electrophoresis (PAGE) for removal of free fluorescein isothiocyanate (FITC) labeled F3 scr. peptide. F3 scr. cPPE were then lyophilized in 0.1 and 0.5 mg fractions and stored at –20 °C.

Gel electrophoresis (SDS-PAGE)—The cSCKs or control samples (A488, A488 cPPE, A488 PAEA, A488, F3 scr. cPPE, F3 scr. peptide and parent PPE cSCKs) were dissolved in PBS pH 7.4 such that the A488 or FITC concentration was 1 μM. When no dye was present,

the cSCKs were dissolved at the same weight/volume as the labeled particles. Each sample solution of 25 μ L was added to 3 μ L of Laemmli buffer and sonicated at room temperature for 10 min. Samples were then loaded into a well of a pre-cast 4–15 % gradient acrylamide gel (Bio-Rad) and electrophoresed in Tris-glycine-SDS running buffer for 20 – 25 minutes at 200 V. Each gel was then imaged using ChemiDoc XRS Imager (Bio-Rad). Emission profiles for each lane were obtained by ImageJ (NIH) analysis. Spectral deconvolution of the emission profiles for each lane of a gel was carried out in Origin 9.0 Pro using multi-peak Gaussian fitting.

3. RESULTS AND DISCUSSION

Polymer Composition and Assembly into Core-shell Nanoparticles

Hydrolytically-degradable cationic shell crosslinked knedel-like nanoparticles (cSCKs), having an amphiphilic core-shell morphology, were designed such that they are capable of acting as nanotheranostics having the following capabilities: (1) hydrophobic therapeutics can be encapsulated into the core; (2) siRNA or DNA can be loaded into the cationic shell; and (3) the amine functionalities in the shell can be conjugated to imaging agents and/or targeting moieties. The cSCKs utilized were prepared from the functionalized cationic diblock copolymer of polyphosphoester (PPE)-*block*-poly(L-lactide) (PLLA) (cPPE₄₉-*b*-PLLA₄₄) (Scheme 1), prepared as previously described.¹⁶

Self-assembly of cSCKs was carried out by direct dissolution of cPPE₄₉-*b*-PLLA₄₄ into PBS buffer at pH 7.4 at 1 mg/mL with sonication at 0 °C for 45 min, followed by crosslinking of nominally 30 % of the free amines within the shell by reaction with bis dPEG₄-PFP ester and simultaneous addition of 3-butenic acid 2,3,5,6-tetrafluorophenol ester (3-BA) for subsequent attachment of the F3 scramble peptide carrying a FITC label (F3 scr. peptide). Alexa 488 (A488) labeling of the cPPE₄₉-*b*-PLLA₄₄ cSCKs was carried out concurrently with crosslinking of the cPPE shell. All cSCKs were purified by dialysis at 4 °C against PBS buffer at pH 3.0 and then nanopure water to ensure the removal of unreacted reagents and free dyes while preventing premature degradation of the cPPE shell. Following dialysis, parent cPPE cSCKs were outfitted with FITC labeled F3 scr. peptide (FITC-(aminohexanoic acid)-KDEARALPSQRSRKPAPPKPEPKE-KKAPAKKC) *via* the C terminal cysteine moiety using an aqueous thiolene reaction (Scheme 2). Following synthesis, all particles were evaluated by conventional microscopic and light scattering methods.

Evaluation by Microscopic and Light Scattering Methods

Commonly, polymeric nanoparticle size distributions are assessed by one or more microscopic (transmission electron microscopy (TEM), high resolution scanning electron microscopy (SEM) after metalation, atomic force microscopy (AFM)), or light scattering methods (dynamic light scattering (DLS), static light scattering (SLS)). As each of these methods provides a different view of the particle state, we posit a multi-environment regime in which TEM, AFM and DLS are utilized to assess the physical structure of the particle. Together, this array of analysis provides *in vacuo* solid state imaging without metalation (profile, TEM), ambient at-surface imaging (height, AFM) and in suspension analysis (hydrodynamic diameter, DLS).

Figure 1 presents representative images and particle distributions for the unlabeled parent cPPE₄₉-*b*-PLLA₄₄ cSCKs (parent PPE cSCKs), the A488 conjugated cPPE₄₉-*b*-PLLA₄₄ cSCKs (A488 cPPE) and F3 scr. peptide conjugated cPPE₄₉-*b*-PLLA₄₄ cSCKs (F3 scr. cPPE), in comparison to the previously described^{6, 14} 5 % crosslinked poly(acrylamidoethylamine)-*block*-poly(DL-lactide) (PAEA-*b*-PLA) A488 labeled cSCKs (A488 PAEA) (Figure S.1). TEM image analysis (assuming contrast is sufficient) provides a

good indication of intrinsic, non-hydrated, fully collapsed particle size. TEM particle analysis of the three cPPE systems showed comparable collapsed sizes with number-average diameters (D_{av}) of 12 ± 3 nm for the parent PPE cSCKs, 13 ± 2 nm for the A488 cPPE conjugates and 17 ± 3 nm for the F3 scr. cPPE conjugates, as determined by particle analysis. A comparison to the DLS results, which demonstrate number-average hydrodynamic diameters ($D_{h(number)}$) that are comparable between systems, indicates the cSCKs have roughly 3 times larger diameter when swollen in aqueous solutions than the collapsed diameters observed by TEM ($D_{h(number)} = 28 \pm 8$, 28 ± 8 , and 23 ± 7 nm, respectively). This difference is reasonable given the extreme hydrophilicity of the cPPE shell, the low glass transition temperature of the polymer ($T_g = -7.49$) and the aqueous room temperature measurement conditions. We propose that comparison beyond this level, *via* these methods where quantitative measurements lead to a qualitative understanding that the system has not significantly changed, is unreasonable and will only convolute the efforts to assess dye conjugation. In short, these methods provide value to the assertion that the reactions performed have not caused a critical change to the cSCK morphology.

Conventional AFM provides a slightly different set of information, and it is appropriate for supplemental analysis of the particle stability at an interface (height) and can readily observe polymeric structures that are not part of well-defined particles. To highlight this functionality, we present AFM images of the particles on a glass substrate after lyophilization, cold storage and re-suspension (Figure 1, images 1). It was observed that a combination of individual particles, dimers and larger aggregates are expressed in all systems. The peptide-functionalized particles possess circumferences occupied by a small amount of lower-lying materials bound to the particle, but not contributing structurally to it, a finding that is reasonable given that the peptide should be bound near the surface of the particles. Lastly, the height profiles of the parent particles, A488 and F3 scr. systems (7 ± 1 , 8 ± 2 , and 7 ± 1 nm respectively) were similar, supporting the claims that particle architecture is not extensively changed by the conjugation process.

Electrophoretic cSCK Analysis

Initial efforts to examine dye conjugation of the fully degradable A488 and F3 scr. cPPE conjugates were carried out *via* standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to estimate the ratio of bound and unbound dye (Figure 2A). A488 PAEA, having a degradable PLA core and a non-degradable cationic PAEA shell, was utilized as a control (Figure 2B).^{17, 27} The use of SDS (in the case of evaluating polymeric materials) should interrupt and obstruct non-covalent interactions in non-reactive systems (such as electrostatic, π - π stacking, *etc.*). After imaging, each band of the gel was identified as free A488, A488 cSCKs or free polymer conjugated to A488 (not in micellar or cSCK supramolecular structures).

In the case of the fully degradable A488 cPPE having 30% crosslinking, assignment of free fluorophore and labeled polymer or cSCKs in each well was possible, but left multiple unidentified bands attributed to polymer degradation and the resultant poorly defined assemblies (Figure 2A). Emission profiles for each lane were obtained by image analysis in ImageJ. Intensity deconvolution of these profiles identified up to 11 different emitting bands following electrophoresis. Using the area of each emission peak, it was calculated that the ratio of gel emission for polymer/cSCK conjugated A488:degraded polymer/cSCKs conjugated A488:free A488 is 0.41:1:0.05, clearly demonstrating the potential difficulty in evaluating dye conjugation to degradable polymeric materials *via* an electrophoretic technique. At best, it can be estimated that *ca.* 96% of the fluorophore remaining with the cSCKs following purification was covalently conjugated to the particles. Unfortunately, as

the degradation products cannot be identified there is no ready way to ascertain the original state of the dye-polymer relationship.

Alternatively, evaluation of the control A488 PAEA conjugates, having only 5% crosslinking within the non-degradable PAEA shell, revealed fluorescent bands corresponding only to the labeled cSCKs and polymers (Figure 2B). The low intensity of the cSCK band was attributed to the low crosslinking percent, which allowed for dynamic reordering of the polymer from micelles and/or cSCK into a free state. Addition of SDS to the sample favored free polymer by preventing intermolecular interactions, leading to polymer disassociation from supramolecular assemblies. The broader distribution of the fluorescent band assigned as labeled free polymer was attributable to the polydispersity of the PAEA-*b*-PLA diblock copolymer. Unlike the fully degradable cSCKs, SDS-PAGE of the control particles demonstrated the advantages in evaluating dye conjugation to polymeric materials with high stability under electrophoretic conditions.

The electrophoretic results clearly demonstrated that the nature of the polymeric material must be taken into account when applying characterization techniques to degradable materials. Previous studies of polyphosphoester-containing cSCKs indicated that cPPE particles undergo rapid and complete hydrolytic degradation within a matter of days in PBS pH 7.4,²⁸ whereas the PAEA particles display only 9% total core hydrolysis over 5 days in 0.1 M Tris-HCl buffer pH 7.4.²⁷ The increased rate of degradation for the cPPE block may be due in part to attack by deprotonated ammonium side chain groups onto the phosphoester backbone bonds. While both cSCKs contain a degradable polylactide core, differing shell stability, and degradation pathways, between the degradable cPPE and non-degradable PAEA leads to the *in situ* degradation of the cPPE shell during electrophoresis

Confirmation of covalent coupling of labels to nanoparticles is especially necessary in the development of nanotheranostics, where *in vivo* studies require accurate differentiation between unconjugated fluorophore and fluorophore attached to the degrading polymeric material. Such requirements make accurate evaluation of dye conjugation both more complex and essential. To provide a more accurate assessment of dye-polymer and dye-nanoparticle conjugates, we propose a holistic approach combining the use of traditional electrophoretic methods with spectroscopic techniques, including emission lifetime and anisotropy decay and steady state techniques. The use of such techniques has long been applied to evaluation of small molecule fluorophores and their conjugation to biological moieties (proteins, antibodies, *etc.*), but has been largely underutilized in the evaluation of nanoparticle- based optical contrast agents consisting of dyes conjugated to polymeric materials.^{25–28}

Steady State Spectroscopy

In many applications UV/Vis spectroscopy allows for quantitative assessment of the extent of dye conjugation to macromolecules, assuming that scattering is insignificant. The effects of Mie scattering are observed in the UV/Vis spectra of cSCK conjugates at a concentration of 0.1 mg/mL (A488 conjugates) or 1 mg/mL (F3 scr. conjugates) in water. Scattering is due to intrinsic particle size, particle clustering (Figure 3, top), as well as environmental/solvent media interactions for A488 cPPE in water, PBS pH 7.4 and 5% aqueous fetal bovine serum (FBS) (Figure 3, bottom). In cases where scattering is even more significant, due to both intrinsic and environmental effects on particle inhomogeneity, the absorbance signal of the dye becomes obscured. Conventional methods then become inaccurate in quantifying multiple parameters, including dye conjugation, fluorescence quantum yield (Φ_{FL}), the critical micelle concentration and the upper and lower critical limits.

Within the polymer structure there are abundant sites, including oxygen, nitrogen and other electron deficient regions, where energy transfer (collisional quenching) can take place. Therefore, it is expected that the Φ_{FL} should decrease upon conjugation to the cSCKs. In the simplest systems, such as A488 cSCK conjugates in water, where scattering is least significant, the Φ_{FL} follows the expected trend (Table 1). Alternatively, in systems with increased inhomogeneity due to significant scattering effects, calculated Φ_{FL} increases significantly. Such an increase is in part due to the ambiguity of extracting meaningful molar extinction data from the scattering laden absorption spectra, as well as the effect of increased light scattering during emission quantification.

Unlike UV/Vis, the use of steady state fluorescence anisotropy has the ability to provide information on the dynamic behavior of molecules, including molecular orientation and rotational diffusion, which typically change in a dramatic fashion upon conjugation to macromolecules and nanoparticles.²¹ Emission anisotropy (r) is useful as a measurement of the average angular displacement of a fluorophore occurring during the time between excitation and emission. Angular displacement is dependent upon the rate and extent of rotational diffusion during the excited state and can be described by the Perrin equation (1). Being dependent upon rotational diffusion and emission lifetime means that anisotropy measurements are independent of total sample absorbance and, thus, the scattering effects observed in UV/Vis measurements have less influence.

$$r = r_0 \frac{\theta}{\theta + \tau} \quad (1)$$

Where r_0 is the limiting anisotropy (0.4 as an ensemble average for one-photon excitation^{21, 29} for the ideal fluorophore), τ is the fluorescence lifetime and θ is the rotational correlation time of the fluorophore as described by equation 2.

$$\theta = \frac{\eta V}{RT} \quad (2)$$

Where η is the viscosity, T is the temperature in °K, R is the gas constant and V is the volume of the rotating unit.

With all experimental parameters except V held constant, changes in the rotational diffusion of a fluorophore will be solely dependent upon V . In a low-viscosity solution, small molecule fluorophores, such as A488 and FITC, exhibit rates of rotational diffusion that are significantly faster than the rate of emission, leading to depolarized emission and an observed r near zero (Table 1). Upon conjugation to cSCKs, the larger nanoparticles dictate the diffusion rate, with rotational diffusion slowing such that reorientation occurs on time scales at or below the emission lifetime. The effect of slower diffusion is an increase in polarized emission and a resultant increase in the observed r . Similar changes in rotational diffusion should also take place following conjugation of a dye-labeled peptide to a much larger cSCK. Observation of these changes will be dependent upon whether the fluorophore experiences a significant increase in volume of the rotating unit upon conjugation.

Anisotropy measurements were obtained in water, PBS pH 7.4 and 5% FBS (Table 1). The rationale behind this selection is the importance of assessing the conjugate in multiple aqueous environments to ensure the results are due to actual dye-nanoparticle conjugation and not hydrophobic, electrostatic or other interactions between the unconjugated dye and polymeric nanoparticle. Results in Table 1 indicate that both A488 and the FITC labeled F3 scr. peptide are conjugated to cPPE and PAEA cSCKs. In water, a significant increase in r is

observed between the free fluorophore, dye-labeled peptide and their conjugated equivalents. The same trend is seen in PBS, where many hydrophobic and electrostatic interactions are dispersed as a result of the increased rate of ion exchange, providing additional evidence towards conjugation of the dye and peptide to the cSCKs.

In 5% FBS the opposite trend is observed for only free dye (but not free peptide), which displays a higher r than both A488 cPPE and A488 PAEA. The increase in r for free dye is not unexpected, as bovine serum albumin (BSA), a major component of FBS, is known to have non-specific binding interactions with many hydrophobic fluorophores, to include dyes with molecular structures similar to A488 and FITC.^{32–34} Therefore, particle conjugated A488 is partly protected from BSA binding by the polymeric cSCK, leading to lower r than the free dye that takes part in non-specific binding to the bulky and aggregation-prone BSA protein. Unlike the free dye, a higher anisotropy is observed for the F3 scr. cPPE when referenced to the free peptide. In this example, while the peptide protects the FITC label from interactions with BSA to an extent, the dye is not free to readily dissociate once covalently bound to the particle through the peptide. Therefore, the physical tethering of the dye to the particle through covalent conjugation allows for both dye-BSA and particle-BSA interaction to decrease the rotational rate, resulting in a more conventional r trend than that observed in the free dye system. While these findings strongly support covalently bound dye-particle and dye-peptide-particle systems, additional confirmation can be obtained through the use of time-domain spectroscopy.

Fluorescence Lifetime and Anisotropy Decays

In the evaluation of dye-conjugated polymeric materials, nanoparticles and nanocapsules, time-domain measurements are under-utilized by the translational medicine community, despite having the potential to be extremely powerful tools. Time-domain spectroscopy's increased sensitivity to differences in both the electronic state and molecular interactions, as compared to steady state measurements, allows emission decay-based measurements to provide valuable information on the supramolecular environment of the fluorophore and, in turn, the nanoparticle itself. As such, the information derived from time-domain fluorescence measurements offers a venue for further validation of successful conjugation, as well as insight into the surface response of the particle in various solvent environments. For the purpose of assessing emission lifetime and anisotropy decay change in dye-polymer conjugate systems, solution-state time correlated single-photon counting (TCSPC) was performed with a pulsed 405 nm diode laser (135 ps fwhm) to obtain emission and anisotropy decay profiles for both A488 and F3 scr. cSCK conjugates (Figures 5 and 6, respectively). Corresponding kinetic information can be found in Table 2. TCSPC spectra were obtained in water, PBS pH 7.4 and 5% FBS, with a similar motivation to that forwarded in the steady state assessment above. Lifetime decay profiles (Figure 4) illustrate a decrease of the fluorescence lifetime (τ) of A488 and the F3 scr. peptide following conjugation to the cSCKs. The average intensity weighted lifetime (τ_i), along with its individual components, were obtained by exponential fitting after deconvolution of the instrument response function (IRF) (Table 2).

A488, A488 PBYP and A488 PAEA emission decay—In the case of A488, in both water and PBS, only a single lifetime component is observed. Conjugation introduces a fast (τ_1) component, due to the introduction of interactions with the polymer of the cSCK. Introduction of τ_1 is in addition to a longer lived (τ_2) component from fluorophore solvent interactions, which occur less frequently due to the collisional protection afforded by the proximate polymer. Interestingly, conjugation of A488 to the PAEA cSCK appears to have less of an effect on τ_i than when conjugated to the cPPE cSCK, despite both showing similar trends in the amplitude (population) ratios of the excited states decaying *via* the τ_1 and τ_2

pathways (*ca.* 0.35), a metric often not discussed in the assessment of time resolved studies that displays a statistical similarity of states between the two systems. Three marked differences in the particle systems may account for the divergence in decay profiles.

1. *Size*: from DLS data, the A488 PAEA system has an average volume that is *ca.* 115 times larger than that of the A488 cPPE system.
2. *Shell rigidity*: from DSC, the PAEA-*b*-PLA block copolymer has a glass transition that is 55.5 °C higher than that of the cPPE-*b*-PLLA block copolymer and well above room temperature ($T_g = 48\text{ }^{\circ}\text{C}^{27}$ vs. $T_g = -7.49\text{ }^{\circ}\text{C}^{16}$).
3. *Shell chemistry/ionic character*: while both systems possess cationic terminal ammonium functionalities, the ammonium group populations differ between the PAEA-*b*-PLA and the cPPE-*b*-PLLA. Additionally, the side chain functionalization of cPPE uses a thiol coupling strategy that incorporates thioether moieties, which may alter the charge density/hydrophilicity of the shell.

In accounting for the divergence in decay profiles, size alone can be reasonably discounted given steady state trends of the anisotropy of the two systems. For both PBS and aqueous solutions of the two systems, τ_1 was faster for A488 PAEA, implying a greater interaction between A488 and the particle, and strongly suggesting that non-ionic forces dictate the extent of additional interaction (beyond that enforced by the covalent linkage). Such interactions are supported by a further decrease in τ_1 for this system upon exposure to the highly ionic PBS environment, as the dye interacts more strongly with the shell, and by a τ_1 increase for A488 cPPE where differing charge density of the shell results in stronger interactions with the media and lower non-covalent interactions with the fluorophore. To confirm these observations, the rotational correlation times (from equation 1) were calculated both in water and in PBS from the steady state and emission decay values. For A488 cPPE, a marked increase (46%) was observed when a transition from water to PBS was made, indicating the cPPE particles are strongly interacting with the more ionic media. Alternatively, the A488 PAEA showed only nominal change (4%), largely attributed to the increased viscosity of PBS in relation to water (*ca.* +5%). While a lower glass transition may contribute to the volumetric response of cPPE cSCKs to a more ionic media, the steady state anisotropy does not support this.

On transition to a protein rich environment, 5% FBS, both the A488 cPPE and A488 PAEA conjugates possess longer component and average lifetimes, including a transition to a state where the τ_1 decay pathway, associated with dye-macromolecular interactions, dominates relaxation. These findings are not unexpected, as non-specific interactions with BSA^{36–38} create an environment where solvent interactions, in part, mimic those of non-conjugated polymer-dye interactions (*e.g.* dye-biomacromolecular interactions). With increased viscosity and additional protection from collisions with solvent molecules, both the overall increase in lifetime and a shift away from a solvent-collision dominated emission decay environment are in keeping with the composition of FBS media as compared to either water or PBS.

F3 scr. peptide and F3 scr. cPPE emission decay—In the case of the FITC labeled F3 scr. peptide, biexponential decay is observed in solution, consistent with both a fast lifetime related to dye-macromolecule (τ_1) interactions and a longer decay associated with solvent effects (τ_2). On transition to a more ionic PBS environment, no significant change of the emission components (τ_1 , A_1 , τ_2 , A_2) or τ_i are observed, indicating that ionic interactions between the solvent and the peptide are not affecting either the peptide-dye non-covalent interaction or the dye-solvent collision rate. Coupling this result to the steady state anisotropy, it is reasonable that the peptide has equal solubility and similar solvent interactions in both systems.

Upon cSCK conjugation, there was a shift in the population of excited states decaying *via* each of these pathways, expressed as a tripling of A_1 in water and a doubling of A_1 in PBS. Given the affinity of the cPPE shell for increased solvent interactions in PBS, it is not surprising that the dye-macromolecular interaction relaxation pathway shows dominance in the aqueous solution for the F3 scr. cPPE conjugate, and is much smaller in PBS. The observed effect in PBS, where ionic insertion/competition at the shell surface is strong enough to severely restrict non-covalent dye conjugate interactions, is not sufficient to return the emission properties to the F3 scr. peptide profile. In total, these findings offer further evidence that covalent linkage has occurred and that electrostatic forces, which have a trivial effect on the peptide-dye interactions and dye-repulsive effects for cSCKs in PBS, are not responsible. Similar to the A488 conjugate systems, an attribution to BSA binding can be made for the observed results of the F3 scr. cPPE system in 5% FBS, where by both the F3 scr. peptide and the cSCK conjugate systems see increased emission lifetimes and an increased population of dyes relaxing by the τ_1 pathway.

Emission anisotropy decay—While the observed emission lifetimes, decay components and steady state anisotropies indicate successful conjugation of both fluorophore species to the cSCKs, anisotropy decay profiles are also capable of supporting these findings. Anisotropy decay lifetimes (τ_r) probe the rotational diffusion of the fluorophore as a function of lifetime and are sensitive to both changes in emission lifetime and the local environment of the dye molecule, which is useful for systems where inter- and intramolecular interactions are expected.

In water and PBS A488 cPPE and A488 PAEA cSCKs display a significantly longer τ_r than the free dye (Figure 5, Table 2). However, the assertion that the cPPE system interacts strongly with PBS, having greatly decreased non-covalent interactions with the dye, relative to when in water, is strongly supported by a decrease in τ_r . Such results indicate a dye state that is not static on the surface of the particle, but that acts as a tethered species having significant freedom of movement. In the PAEA system, where a less pronounced particle-PBS interaction exists, the reverse occurs. An increase in the τ_r is observed when comparing its PBS response to that of water. In PBS, additional freedom of movement at the particle interface is suppressed as the dye interacts more strongly with the particle surface in the more ionic media. The τ_r values in 5% FBS follow the emission decay trends, with both the PAEA and cPPE systems expressing an increase in τ_r due to nonspecific BSA binding and an increase in solution viscosity.

On the other hand, τ_r of the FITC label on the F3 scr. peptide displayed almost no difference between the free peptide and the cSCK conjugate in water and in PBS (Figure 5, Table 2). The lack of difference indicates that the peptide affects rotation as to minimize the effect on anisotropy decay from association with the particle, regardless of the ionic character of the media. While this result is marginal in terms of supporting covalent linkage on its own, it is consistent with a decreased conjugation effect on lifetime for the F3 scr. cPPE conjugate when compared to the A488 cPPE and A488 PAEA conjugates.

Physical blends of cSCKs and dyes/dye-conjugates—For all systems, control samples were generated with physical mixtures of dye or dye-labeled peptide with the relevant cSCKs in nanopure water, PBS and 5% FBS (denoted as “dye(A488)/dye-conjugate (F3 scr.) + cSCK (cPPE or PAEA)”, Table 1, Table 2). Detailed analyses are provided in the supporting information. In brief, the response of each control varied with solvent differently. For A488 + cPPE, control anisotropy and lifetime results indicate a largely cSCK-internalized state in both water and PBS, with emission properties indicating a free dye state in 5% FBS. In the case of A488 + PAEA, free dye behavior was observed in water and PBS, with mixed behavior in 5% FBS. In the third control, F3 scr. + cPPE, the F3 scr. conjugate

exhibited free conjugate response in water and mixed response in both PBS and 5% FBS. Taken collectively, these controls highlight the need to utilize several aqueous solvent systems in conjunction with multiple spectroscopic metrics for complete assessment of dye-cSCK and dye-biomacromolecule-cSCK complexes.

4. CONCLUSIONS

Successful development of degradable polymeric nanostructures as nanotheranostics that can be realistically translated from the laboratory to *in vivo* performance requires the ability to fully characterize such materials before, during and after degradation. In regards to imaging, it is of particular importance to guarantee that any observed changes and/or distribution profiles visualized during *in vitro* or *in vivo* studies are a result of degradation rather than impurities. Therefore, confirmation of conjugation is critical, followed by observations of release of active (targeting, therapeutic, diagnostic, etc.) sub-units during nanoparticle degradation.

Within this body of work, we have proposed that the assessment of dye/contrast agent labeling of polymeric materials and nanoparticles for theranostic applications should be carried out in a holistic manner. The application of multiple techniques provides the greatest breadth of data and the strongest position for determination of the extent of coupling. We have demonstrated that while some methods, such as gel electrophoresis, can be appropriate for use with non-degradable materials, they may interfere with the critical assessment of innovative new materials intentionally reliant on built-in degradability. Our results indicated that such interference is especially true in the case of our intelligently-designed, rapidly-degrading polyphosphoester systems, where electrophoretic techniques trigger premature destabilization, making evaluation of contrast agent conjugation more complex. To overcome the complexity introduced in evaluating degradable materials, we have applied the use of rapid, more reliable and less destructive methods of evaluation, employing a combination of spectroscopic and microscopic techniques to obtain an understanding of coupling efficiencies and of the dynamic interactions between contrast agents and their local environments.

Overall, the time domain measurements of fluorescence life-time and anisotropy decays strongly support conjugation of A488 and FITC-labeled F3 scr. peptide to the polymeric cSCKs, reasserting the need to consider conjugation outcomes based upon the known polymeric structures and the electronic interactions they may facilitate, specifically with the solvent environment. By obtaining a comprehensive understanding of the time domain and steady state measurements, a more complete assessment is obtained, allowing for confirmation of fluorophore conjugation, while assessing how the non-covalent interactions between the particle and the dye manifest as a function of solvent system and the use of a peptide-based biomolecule as part of the conjugate.

The use of this holistic approach has confirmed the covalent attachment of the desired contrast agents and demonstrated the significance of understanding such conjugation in terms of the spectral signature-structure-function relationships that this regime of assessment enables. Such an approach has the potential to guide more informed studies on the *in vitro* and *in vivo* biodegradation characteristics of polymeric optical imaging platforms, providing invaluable information that can guide the intelligent design of the next generation of nanotheranostic materials.. In developing new materials, with intrinsic and tunable degradation properties, these techniques are currently being utilized to evaluate dye-polymer conjugates for *in vivo* studies along with the effects of drug loading on particle assembly and emissive properties. Ongoing investigations include a detailed evaluation of a series of rapidly- and slowly-degrading nanoparticle materials, with differential degradation profiles

within the shells and core domains, to create a family of materials that will be applicable for a variety of biomedical applications that require different routes of administration and timeframes for the materials to remain intact.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge financial support from the National Heart Lung and Blood Institute of the National Institutes of Health as a Program of Excellence in Nanotechnology (HHSN268601000046C), the National Science Foundation (CHE-1057441), and the Welch Foundation through the W. T. Doherty-Welch Chair in Chemistry (A-0001).

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Figure 1. Particle size characterization of (A) parent cPPE cSCKs, (B) A488 cPPE and (C) F3 scr. cPPE. With (1) AFM, (2) TEM and (3) DLS.

Figure 2. SDS PAGE of A488 cSCKs. (A) lane 1 contains A488 and lane 2 A488 cPPE and (B) lane 1 contains A488 and lane 2 the control A488 PAEA.

Figure 3. UV/Vis absorption spectra of (top) A488 and F3 scr. cPPE conjugates in water and (bottom) A488 cPPE in water, PBS pH 7.4 and 5% aqueous FBS. The inset depicts the spectra of unbound A488 in all three solvents.

Figure 4.

Emission decay profiles for A488 as compared to 5-FITC (top), A488 cPPE and A488 PAEA (middle) and for 5-FITC compared to F3 scr. Peptide and F3 scr. cPPE (bottom) obtained in PBS pH 7.4. Fitting performed with one, two or three decay components with IRF deconvolution.

Figure 5.

Anisotropy decay profiles for free A488 as compared to 5-FITC (top), A488 cPPE and A488 PAEA (middle) and for 5-FITC compared to F3 scr. Peptide and F3 scr. cPPE (bottom) obtained in PBS pH 7.4. Decays were assessed with tail fitting after 95% of IRF decay.

Scheme 1.
Synthesis of parent and A488 labeled cPPE-*b*-PLLA cSCKs.

Scheme 2.

Conjugation of FITC labeled F3 scr. peptide to cPPE₄₉-*b*-PLLA₄₄ cSCKs.

Table 1

Steady state characteristics of A488 and F3 scr. cPPE and A488 PAEA nanoparticle conjugates.

Solvent	Sample	λ_{ex} (nm)	λ_{em} (nm)	r	Φ_{FL}
Nanopure H ₂ O	A488	495	516	0.015 ± 0.003	0.92^{30}
	A488 + cPPE	496	519	0.125 ± 0.009	-
	A488 cPPE	498	520	0.114 ± 0.004	0.53
	A488 + PAEA	493	516	0.133 ± 0.004	-
	A488 PAEA	497	517	0.129 ± 0.006	0.28
	5-FITC*	490	517	0.012 ± 0.002	0.93^{31}
	F3 scr. Peptide	497	517	0.055 ± 0.003	1.14
	F3 scr. + cPPE	495	518	0.065 ± 0.004	-
	F3 scr. cPPE	487	517	0.153 ± 0.004	5.73
PBS pH 7.4	A488	493	514	0.013 ± 0.002	-
	A488 + cPPE	490	515	0.063 ± 0.003	-
	A488 cPPE	498	520	0.089 ± 0.002	0.36
	A488 + PAEA	490	515	0.044 ± 0.005	-
	A488 PAEA	497	518	0.132 ± 0.008	-
	5-FITC*	493	518	0.003 ± 0.000	-
	F3 scr. Peptide	496	516	0.048 ± 0.003	-
	F3 scr. + cPPE	493	515	0.063 ± 0.004	-
	F3 scr. cPPE	495	517	0.096 ± 0.004	-
5% FBS in H ₂ O	A488	498	517	0.259 ± 0.006	-
	A488 + cPPE	498	516	0.327 ± 0.009	-
	A488 cPPE	499	521	0.185 ± 0.005	1.93
	A488 + PAEA	497	517	0.253 ± 0.008	-
	A488 PAEA	497	518	0.217 ± 0.012	-
	5-FITC*	502	518	0.261 ± 0.005	-
	F3 scr. Peptide	499	516	0.112 ± 0.002	-
	F3 scr. + cPPE	497	516	0.141 ± 0.005	-
	F3 scr. cPPE	500	517	0.181 ± 0.005	-

r = steady state anisotropy $\lambda_{\text{ex}} = 430 \text{ nm}$, $\lambda_{\text{em}} = 580 \text{ nm}$

* Fluorescein-5-isothiocyanate

Table 2

Fluorescence lifetime and anisotropy decay kinetics of A488 and F3 scr. cPPE and A488 PAEA nanoparticle conjugates.

Solvent	Sample	A _{UF}	τ_1 (ns)	A ₁ (%)	τ_2 (ns)	A ₂ (%)	τ_1 (ns)	τ_2 (ns)
Nanopure H ₂ O	A488	-	4.22 ± 0.01	100.0	-	-	4.22	0.40 ± 0.01
	A488 + cPPE	-	1.66 ± 0.04	33.4	3.30 ± 0.02	66.6	2.97	1.64 ± 0.08
	A488 cPPE	-	1.15 ± 0.05	26.8	3.28 ± 0.01	73.2	3.04	1.58 ± 0.04
	A488 + PAEA	-	4.11 ± 0.03	100.0	-	-	4.20	3.21 ± 0.09*
	A488 PAEA	-	1.05 ± 0.05	29.5	4.15 ± 0.02	70.5	3.86	2.35 ± 0.09
PBS pH 7.4	5-FITC	-	3.10 ± 0.01	100.0	-	-	3.10	0.22 ± 0.02
	F3 scr. Peptide	-	1.08 ± 0.07	19.3	3.93 ± 0.01	80.7	3.75	0.83 ± 0.04
	F3 scr. + cPPE	-	1.46 ± 0.09	20.0	4.00 ± 0.02	80.0	3.79	1.09 ± 0.04
	F3 scr. cPPE	56.8	1.49 ± 0.02	64.2	3.92 ± 0.03	35.8	2.94	0.88 ± 0.02
	A488	-	4.08 ± 0.02	100.0	-	-	4.08	0.42 ± 0.02
5% Serum in H ₂ O	A488 + cPPE	-	1.68 ± 0.09	20.5	4.05 ± 0.02	79.5	3.82	3.11 ± 0.44*
	A488 cPPE	-	1.42 ± 0.05	28.4	3.55 ± 0.02	71.6	3.26	1.31 ± 0.04*
	A488 + PAEA	-	4.20 ± 0.02	100.0	-	-	4.15	3.39 ± 0.53
	A488 PAEA	-	0.79 ± 0.06	21.1	4.22 ± 0.02	78.9	4.06	2.71 ± 0.12
	5-FITC	-	1.17 ± 0.10	15.9	3.87 ± 0.02	84.2	3.73	0.29 ± 0.02
	F3 scr. Peptide	-	0.99 ± 0.08	16.9	3.88 ± 0.01	83.1	3.74	0.90 ± 0.02
	F3 scr. + cPPE	-	1.02 ± 0.04	36.0	3.81 ± 0.02	63.0	3.44	0.86 ± 0.03
	F3 scr. cPPE	-	0.96 ± 0.04	32.2	3.87 ± 0.02	67.8	3.56	0.98 ± 0.02
	A488	42.5	2.84 ± 0.03	68.4	5.82 ± 0.05	31.6	4.29	1.73 ± 0.05
	A488 + cPPE	48.8	2.83 ± 0.03	76.5	6.39 ± 0.06	23.5	4.29	3.45 ± 0.22*
	A488 cPPE	42.8	2.65 ± 0.02	84.0	5.98 ± 0.07	16.0	3.65	1.68 ± 0.03*
	A488 + PAEA	44.0	3.02 ± 0.03	75.6	6.39 ± 0.06	24.4	4.39	2.73 ± 0.22
	A488 PAEA	52.2	2.78 ± 0.03	80.4	7.15 ± 0.08	19.6	4.46	4.64 ± 0.40
	5-FITC	-	0.93 ± 0.04	31.1	4.21 ± 0.02	68.9	3.92	< 100 ps
	F3 scr. Peptide	-	0.99 ± 0.06	22.9	4.06 ± 0.01	77.1	3.85	0.90 ± 0.03
	F3 scr. + cPPE	31.0	3.28 ± 0.02	78.1	5.46 ± 0.06	21.9	3.97	1.51 ± 0.32*
	F3 scr. cPPE	-	-	-	-	-	-	-

Solvent	Sample	A _{UF}	τ_1 (ns)	A ₁ (%)	τ_2 (ns)	A ₂ (%)	τ_1 (ns)	τ_2 (ns)
	F3 scr. cPPE	35.5	2.44 ± 0.03	48.5	4.74 ± 0.02	51.5	3.99	0.92 ± 0.02

τ_r = anisotropy decay λ_{ex} 635 nm, τ_1 = intensity weighted lifetime = $\Sigma[(a_1\tau_1^2)/(a_1\tau_1)]$ τ_1 = fast component, τ_2 = slow component, A = absolute # of excited states that decay *via* this emission pathway, A_{UF} = contribution of total intensity that decays in the ultrafast region (< 100 ps),

* intensity weighted τ_r as determined from the 2 exponential decay