Triplet-triplet Annihilation-Based Photon Upconversion in Protein Biomaterials

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Triplet-triplet Annihilation-based Photon Upconversion in Protein Biomaterials

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B.S., University of Connecticut, 2020

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Triplet-triplet Annihilation-based Photon Upconversion in Protein Biomaterials

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Abstract

Not long ago, the implementation of heavy metals into everyday applications was a historically common practice. While much work has been done to replace old, inorganic technologies, the remediation of well-established inorganic materials has been an uphill battle. As a result, advanced biological materials (ABMs) have risen as environmentally friendly, biocompatible, and effective alternatives to inorganic materials. This work offers solutions to the problems of inorganic materials in solar and biomedical fields by commentating on the design of several bovine serum albumin (BSA) protein-based ABMs that can efficiently carry out photophysical phenomena. A completely organic BSA/sodium dodecyl sulfate (SDS) hydrogel was created to facilitate triplet-triplet annihilation-based photon upconversion (TTA-UC) under aerobic conditions, which achieved a high TTA-UC efficiency of 19.0 ± 0.7% using palladium octaethyl porphyrin (PdOEP) and 9,10-diphenylanthracene (DPA). Completely organic, spin-orbit charge-transfer intersystem crossing (SOCT-ISC), boron-dipyrrromethene (BODIPY) -based sensitizers were also tested within the BSA/SDS hydrogel, which returned TTA-UC efficiencies ranging from 1.0 ± 0.1% to 3.5 ± 0.3%. Through transient absorption and fluorescence spectrophotometry, it was determined that the multiphasic nature of the BSA/SDS hydrogel simultaneously protected against oxygen quenching, maximized collisional frequencies, and extended triplet excited state lifetimes, thus enhancing TTA-UC. Also, in this work, a millifluidic reactor was developed for the kilogram-scale, size-controlled production of GlowDot, an edible BSA nanoparticle. GlowDot was bound with sensitizers and annihilators, and TTA-UC was then observed in a purely protein-based system for the first time. Furthermore, the upconverting GlowDot produced emission that extended into the ultraviolet region, which can be utilized in TTA-UC bioimaging and photodynamic therapy applications.
CHAPTER 1: Introduction

1.1. Objectives

The purpose of this chapter is to introduce the reader to the photophysical concepts fundamental to understanding the phenomena presented in chapters 2 – 5. This introduction will summarize the nature of light and basic photophysical phenomena.

1.2. Motivation

During this modern era, the study of applied photophysics has become increasingly important, as solar energy conversion in solar devices can be improved by manipulating the properties of photoactive molecules. Maximizing the efficiency of solar devices is critical as humanity strives to reduce the consumption of fossil fuels, which are nonrenewable and harmful to the environment. Photoactive molecules also have a bright future in biomedical applications, where they can serve as organic imaging agents, drug delivery triggers, and cancer therapies. Therefore, the underlying study of photoactive molecules and their interaction with light is of key importance to the advancement of society.

1.3. The Nature of Light

In elementary school, students are taught Newton’s third law: for every action, there is an equal and opposite reaction. When light acts upon photoactive molecules, they too react, but in a subtle manner that cannot be fully appreciated without the help of modern technology. Galileo pioneered the telescope in the 1600s to observe the stars, thus confirming the heliocentric theory. Since then, modern technologies and optics have been developed to manipulate, measure, and harvest light, allowing chemists to act upon photoactive molecules in new ways. The field of
‘photophysics’ concerns the physical *reactions* that result from the interaction of light with photoactive molecules.

What is currently accepted as the ‘nature of light’ originates from a multifaceted research effort across time from history’s most renowned physicists. In fact, the effort was kickstarted by Newton’s study of optics in the 1700s, which reported that a single ray of light could be separated into different colors by a prism [1]. Thomas Young’s famous double-slit experiment in 1807 established that light exhibits wave-particle duality [2]. Therefore, it made sense for different colors of light to be unique based on the different properties of waves, such as wavelength (\( \lambda \)) and frequency (\( \nu \)). As seen in Figure 1.1, wavelength is the distance between the start and end of a sine wave, while frequency is the number of sine waves that transmit past a fixed point per second.

Only until the early 19th century was it established that light is not a single stimulus, but a collection of stimuli known as photons, which are particles of light with wave-like behavior, according to Einstein’s quantum theory of light [3]. Soon thereafter, Planck and Einstein developed a relationship between the energy of light and its frequency, 

\[
E = h\nu = \frac{hc}{\lambda},
\]

where \( E \) is the energy of the light, \( h \) is Planck’s constant, and \( c \) is the speed of light [4]. Based on Planck’s relationship, chemists can use particular “energies,” or wavelengths, of light to evoke certain, expected photophysical reactions from molecules. Photophysical reactions refer to processes that are induced by the absorption of light, which do not result in net changes to the chemical identity of the molecule.
Figure 1.1. The wavelike nature of light and its relationship to the electromagnetic spectrum.
1.4. Photophysical Processes

When a photon (hv) interacts with a molecule, and its energy is absorbed, the molecule is said to have been “excited” from the ground state (R) to an excited state (R*), as represented by R + hv → R*, where R* is higher in energy than R [5]. For absorption to occur, the photon must have a particular energy that is equivalent to the energy difference between the excited state and the ground state. Transitions from R to R* can be purely rotational, vibrational, or electronic (which are rare at room temperature), or a combination of one transition accompanied by the preceding transitions [4]. In addition to the energy selection rule, these transitions are usually governed by a number of other selection rules [4]. The absorption of light, as illustrated by the Jablonski diagram (Figure 1.2), assumes that the nuclear geometries of a molecule remain fixed during the timescale of absorption (~ 10^{-15} s), as the heavy nuclei can be perceived as motionless compared to the much lighter electron (Franck-Condon principle) [5]. As such, the ground state (S_0) and n^{th} singlet excited state (S_n) manifolds overlap in symmetry, and absorption (blue arrow) may occur.
Figure 1.2. Jablonski diagram showing photophysical processes. Radiative processes are represented by solid lines while nonradiative processes are represented by dashed lines. The bold, horizontal black lines correspond to electronic states, while the thinner, horizontal black lines correspond to vibrational states. Rotational states are not depicted within the vibrational levels.
Following the absorption of light, a number of photophysical processes may occur. Electronic transitions are accompanied by vibrational and rotational transitions, while vibrational transitions are accompanied by rotational transitions at room temperature [4]. According to Kasha’s rule, an electronically excited molecule must nonradiatively decay to the lowest vibrational energy level of S₁ via internal conversion and vibrational relaxation before fluorescence, intersystem crossing (ISC), or phosphorescence may occur [4,5].

1. **Internal Conversion**: A nonradiative transition between excited states of the same spin multiplicity that occurs within $10^{-15} - 10^{-12}$ s, typically followed by vibrational relaxation to the ground state [4,5].

2. **Vibrational Relaxation**: A nonradiative decay process that occurs within $10^{-12} - 10^{-10}$ s. The excited molecule will collide with other molecules in the sample, thus surrendering vibrational energy and descending vibrational levels until the first vibrational state within an electronic manifold is reached [4,5].

3. **Fluorescence**: A radiative decay process in which a S₁ state molecule relaxes to the S₀ state by emitting energy in the form of a Stokes-shifted photon, usually within $10^{-10} - 10^{-7}$ s. This so-called “Stokes-shift” in the wavelength of an emitted photon is a consequence of nonradiative energy loss via vibrational relaxation that occurs between absorption and emission events [4,5].

4. **Intersystem Crossing**: A nonradiative, spin-forbidden transition from the S₁ state to the T₁ state that occurs within $10^{-10} - 10^{-8}$ s, during which the excited molecule undergoes a change in spin multiplicity [4,5]. This phenomenon can be triggered by a variety of mechanisms, some of which are discussed in section 2.5.
5. **Phosphorescence:** A radiative decay process in which a T₁ state relaxes to the S₀ state by releasing energy in the form of a Stokes photon, usually at longer wavelengths than fluorescence. Relative to fluorescence, phosphorescence is slower (10⁻⁶ – 10 s) and the quantum yield is lower because it is a spin-forbidden process [4,5]. However, the phosphorescence quantum yield can be enhanced in molecules with fast ISC rates.

1.5. **The Absorption Spectrum**

The relative measure of light absorbed by a molecule, termed absorbance, can be measured by an ultraviolet-visible (UV-Vis) spectrophotometer at wavelengths from 200 – 700 nm, resulting in an absorption spectrum. Since UV-Vis light sources emit photons that are suitable in energy for exciting π-π* transitions in organic molecules [4,5], the absorption spectrum will be a profile of photon energies at which electronic transitions, and their accompanying vibrational and rotational transitions, may occur. The absorption spectrum can be indicative of structural motifs in a molecule, as the energy gap between molecular orbitals is dictated by molecular structure and bonding [6]. For example, as the extent of conjugation increases in an organic molecule, its molecular orbitals will become progressively closer in energy, enabling the absorption of photons that are increasingly lower in energy [5,6]. The energy of a transition may also be influenced by external factors; the interaction that a solvent, solute, or binding environment has with a molecule may polarize its orbitals, alter the magnitude of the energy gap, and change the energy of the photon needed for absorption to occur [5-7]. The polarization of molecular orbitals may be read as a “peak shift” on the absorbance spectrum, while a change in the intensity of the absorption peak may correlate to a change in the dipole moment of the molecule [5-7]. A variety of photoactive molecules and their absorption spectra are measured herein to characterize their interaction with light in a given environment.
CHAPTER 2: PHOTON UPCONVERSION

2.1. Objectives

The goal of this chapter is to introduce the reader to the phenomenon of upconversion and provide an overview of the relevant theories that inform decision-making in the design of a highly efficient upconversion system. Furthermore, the theories behind common upconversion studies, such as Stern-Volmer quenching, nonlinear excitation intensity dependence, and the determination of upconversion efficiency are discussed.

2.2. Motivation

The global demand for energy is expanding rapidly as the most widespread source of energy, fossil fuels, is diminishing with each passing year. One solution to this problem is to generate energy using nonrenewable resources, and the fastest growing method for this is solar energy conversion. The sun bathes Earth’s surface with about 1.74 x 10^{17} W worth of energy per second, so even if the efficiency of solar energy conversion in solar devices is improved by a small percentage, this could translate to massive energy returns over time. As a consequence, billions of dollars and decades of time have been funneled into the improvement of commercial solar cells, which are currently only 20% efficient on average. According to Shockley and Queisser, the efficiency of a single bandgap solar cell under typical solar irradiance cannot surpass 32% due to the many pathways of energy loss during solar energy conversion [8].

For example, the photophysics of inorganic semiconductors dictate that only particular energies of light can be absorbed, and those photons with energies that fall above or below the bandgap of the semiconductor are wasted as heat [8]. Even different energies of light that fall
within the semiconductor bandgap are converted into electricity at varying efficiencies [8]. Silicon solar cells, in particular, are excellent for harvesting sunlight that transmits in the blue region, but their efficiency decreases at longer wavelengths, and drops off drastically in the ultraviolet region [8,9]. Therefore, a large percentage of sunlight that hits Earth is not efficiently converted by silicon-based solar cells. To address this problem, several methodologies are currently being explored, which aim to convert the sunlight that solar cells cannot harvest efficiently into light that they can harvest efficiently.

Fluorescence and singlet fission, a photophysical process in which two low-energy photons are produced from a single high-energy photon, have been exploited to ‘downconvert’ ultraviolet light into blue light for the improvement of solar cell efficiency [10,11]. However, only a small fraction of light that hits Earth is ultraviolet to begin with. A much larger fraction of light that is wasted by solar cells is low in energy, thus the unorthodox strategy of ‘upconversion,’ a photophysical phenomenon that produces one high-energy photon using two low-energy photons, is an excellent method for increasing solar cell efficiency and for surpassing the Shockley-Queisser theoretical limit [9].

2.3. Lanthanide-based Photon Upconversion

In lanthanide-based upconversion (LUC), low-energy light is absorbed by lanthanide ions, which undergo energy transfer or excited state absorption (ESA) events to produce one high-energy excited state that can emit an anti-Stokes photon (Figure 2.1.) [12]. Lanthanides are typically chosen because their f-electron transitions are characterized by long excited state lifetimes [13], during which energy transfer and ESA are more likely to occur. Unfortunately, very few lanthanide ions can efficiently undergo LUC, their transitions only cover a narrow absorption
range, and the transitions are low in intensity due to their parity-forbidden nature, thus limiting the LUC efficiency to high-power laser excitation sources (kW cm\(^{-2}\)) [12-14]. While these challenges deter the application of LUC to solar devices, LUC may still be viable in bioimaging applications because lanthanides benefit from high photostability, near-infrared to infrared absorption, and the potential to upconvert light over a large spectral range (> 100 nm) [14,15], as seen in Figure 2.2. However, the use of lanthanides and the inorganic host systems that support them are nonetheless toxic to human health at low dosages [15], and organic dyes provide safer and more effective alternatives.
Figure 2.1. Six processes responsible for LUC. The red arrows represent absorbance, the blue arrows represent the upconverted emission, and the dashed, black arrows represent the possible routes of energy flow. Processes b – f rely on the collision of two or more species, while a only requires one species. Image obtained from [12].
**Figure 2.2.** General depiction of a core-shell lanthanide-doped upconversion nanoparticle, (a), accompanied by pictures of NIR-to-blue, green, and yellow LUC, (b – c), respectively. Image obtained from [12].
2.4. Triplet-triplet Annihilation-based Photon Upconversion

An approach that is favored over LUC is triplet-triplet annihilation-based photon upconversion (TTA-UC), which utilizes organic molecules, termed sensitizer and annihilator (upconverters), rather than lanthanide ions. Not only does the use of organic molecules circumvent the enviro- and bio-toxicity of LUC, but TTA-UC sensitizers also benefit from broad absorption profiles in the visible to near-infrared regions, high absorption intensities, long T₁ state lifetimes, potential to upconvert photons over a large spectral range (> 100 nm), and they are amenable to modification [12,17,18]. TTA-UC is initiated upon the photoexcitation of a high triplet-yielding sensitizer, which then undergoes triplet energy transfer (TET) with an appropriate annihilator via the Dexter mechanism [12,17,18]. The collision of two sensitized acceptor triplets generates a high energy S₁ state annihilator, which will emit a delayed, anti-Stokes photon [12], as shown by Figure 2.3. Images of TTA-UC emission are shown in Figure 2.4.
**Figure 2.3.** Simplified Jablonski diagram showing the TTA-UC mechanism. Processes that lead to successful TTA-UC emission are colored green and those that quench TTA-UC are colored black.
Figure 2.4. Images of green-to-blue (a), red-to-blue (b), near-infrared-to-yellow (c), and red-to-yellow (d) TTA-UC emission from liquid-state upconverter solutions. Images obtained from [12].
2.4.1. Upconverter Requisites for Efficient TTA-UC

According to the mechanism of TTA-UC, the desired flow of energy starting with the photoexcited sensitizer depends upon four consecutive processes: sensitizer ISC, TET, TTA, and annihilator fluorescence, all of which must be optimal for efficient TTA-UC to occur. Therefore, a set of preconditions must be satisfied to ensure that the excitation energy cleanly flows through each step of the TTA-UC mechanism, and without energy loss via quenching pathways.

The first process, ISC, is typically ensured by choosing a sensitizer that has an enhanced rate of ISC, so a large population of T1 states form upon photoexcitation [19], which may then participate in TET. To maximize the TET efficiency, the sensitizer triplets should be relatively long-lived (> 10 µs), so that the large population of sensitizer triplets may actively diffuse over a longer timescale, during which there is a higher chance for collisional energy transfer via the Dexter mechanism [19]. In addition, the rate of collisional energy transfer events, or the TET and TTA efficiencies, may be enhanced by increasing the upconverter concentrations, but not so much as to the cause the self-quenching of TTA-UC [20]. The TTA process is also subject to energetic requirements, as the combined T1 state energies of the annihilator must not be less than the S1 state energy of the sensitizer, otherwise TTA-UC becomes trivial and a Stokes-shifted photon is produced [12-20]. Lastly, the energetically viable annihilators must have a high fluorescence quantum yield to ensure that most TTA events result in the emission of an anti-Stokes photon, rather than nonradiative decay [12-20].

Some additional requirements lie in the electronic structures of the sensitizer and the annihilator. For example, one may want to select an extensively conjugated sensitizer with high intensity absorption peaks in the green to near-infrared regions, which are sufficiently far away in wavelength from the S1 state absorption of the annihilator [12-20].
2.4.2. The Role of Spin Statistical Factors on TTA-UC Efficiency

Since TTA-UC consumes two low-energy photons to produce one high-energy photon, it is mistakenly thought that the theoretical TTA-UC efficiency is 50%. Rather, in the formation of the spin-allowed, annihilator triplet-triplet pair, spin statistical factors have a negative role in dictating the theoretical TTA-UC efficiency. In fact, the triplet-triplet pair is unstable, and it will have an equal chance of dissociating into one of nine existing spin states (1 S₁, 3 T₁, and 5 quintet excited states) [21,22]. Therefore, assuming an annihilator fluorescence quantum yield and TET efficiency of 100%, the theoretical TTA-UC efficiency only reaches 11% [21,22]. However, there have been many reports of the TTA-UC efficiency exceeding 11% [12-20], which could only mean that T₁ states are recycled from triplet and quintet pairs, thus permitting the theoretical TTA-UC efficiency to reach 40% [21,22]. At the time of writing this, there has not been any reported cases of TTA-UC that are 40% efficient or higher.

2.4.3. Dependence of TTA-UC on Excitation Intensity

Since TTA-UC relies on the absorption of two photons, unlike fluorescence, the upconverted emission exhibits a nonlinear dependence on the excitation intensity, rather than a linear dependence [5,23,24]. This nonlinear dependence of TTA-UC is typically evaluated by steady-state fluorescence spectrophotometry, using either coherent or noncoherent excitation sources with adjustable irradiance. However, noncoherent excitation sources have shown to be ineffective at reaching the high-annihilation kinetic limit [25], thus necessitating the use of high-power laser excitation sources in the determination of the maximum TTA-UC efficiency.

The nonlinear dependence of TTA-UC on the excitation intensity results from the combination of two different competing rates of annihilator triplet (T₁Δ) decay processes,
described by equation 1, where $k_T$ represents all of the unimolecular and pseudo-first order decay pathways of $T_1^A$, $k_{TTA}$ is the bimolecular rate constant of TTA, and $t$ is time [5].

$$\frac{d[T_1^A]}{dt} = -k_T[T_1^A]_t - k_{TTA}[T_1^A]_t^2$$ (1)

$$N_F = \int_0^\infty I_F(t) = \int_0^\infty \Phi_F k_{TTA}[T_1^A]_t^2 dt$$ (2)

The intensity of the TTA-UC emission ($N_F$) is proportional to $k_{TTA}$, the fluorescence quantum yield of the annihilator ($\Phi_F$), and the square of the $T_1^A$ population. The solution to equation 2 is given below, where $\beta$ is the fraction of initial decay that occurs via TTA [5].

$$[T_1^A]_t = [T_1^A]_0 \frac{1-\beta}{e^{k_T t} - \beta}$$ (3)

$$\beta = \frac{k_{TTA}[T_1^A]_0}{k_T + k_{TTA}[T_1^A]_0}$$ (4)

If TTA-UC is measured under the low excitation intensity regime, then the population of triplets will be small and TTA will be weak, so $k_T >> k_{TTA}[T_1^A]$, and first order decay will dominate the $T_1^A$ population. Assuming that $k_{TTA}[T_1^A]$ is significantly low under low excitation intensities, equation 3 can be simplified to equation 5, then substituted into equation 2 and solved to give equation 6, which states that the TTA-UC emission will be proportional to the square of the $T_1^A$ population [5]. Therefore, a quadratic dependence between TTA-UC emission and excitation intensity will be observed when the excitation intensity is low [5].

$$[T_1^A]_t = [T_1^A]_0 e^{k_T t}$$ (5)

$$N_F = \Phi_F k_{TTA}[T_1^A]_t^2 / 2k_T$$ (6)
On the other hand, when TTA-UC is measured under the high excitation intensity regime, the population of triplets will be large and TTA will be strong, so $k_T << k_{TTA}[T^{A}_1]$, and equation 3 can be simplified to equation 7. When substituted into equation 2 and solved, equation 8 is produced, which states that the TTA-UC emission will be proportional to the $T^{A}_1$ population in the strong annihilation limit; a linear dependence will be observed at high excitation intensities [5].

$$[T^{A}_1]_t = \frac{[T^{A}_1]_0}{1 + k_{TTA}[T^{A}_1]_0 t} \quad (7)$$

$$N_F = \Phi_F [T^{A}_1]_0 \quad (8)$$

The excitation-dependent kinetics of TTA serve as the theoretical basis for experimentally distinguishing whether an observed emission has been produced via TTA-UC or radiative, first-order processes. In a typical TTA-UC excitation-dependence experiment, a sample is pumped with continuous wave, coherent light at several different intensities and the resulting emission is measured. If the TTA-UC emission follows a quadratic dependence at low excitation intensities, which changes to a linear dependence under high excitation intensities, then the emission in question is indeed TTA-UC emission [23]. The TTA-UC emission intensity is typically plotted against the excitation intensity on a double logarithmic plot, where the quadratic dependence is depicted as a line with a slope equal to 2 and the linear dependence is a line with a slope equal to 1 [23-25].

2.4.4. Effects of O₂ and Upconverter Concentration on TTA-UC

Its standard practice that liquid-state TTA-UC samples should be deaerated prior to optical measurements, as molecular oxygen quenches the upconverter excited states (Figure 2.5.), effectively redirecting the desired flow of energy needed for TTA-UC to occur efficiently [26-28]. Since quenching by molecular oxygen is considered a pseudo-first order decay process, it is
factored into k_T [5]. Therefore, deaeration has the kinetic effect of minimizing k_T and increasing the fraction of upconverter T_1 states available for TTA-UC. Generally, the desired flow of energy through TTA can be ensured by taking any measure that maximizes the population of the T_1^A states.

Therefore, increasing the annihilator concentration and the TET efficiency also have the effects of enhancing TTA-UC [26-28]. The TET efficiency can be increased by using a sensitizer with high absorption intensity at the excitation wavelength and a medium that ensures high collisional frequencies [28]. In fact, less viscous media facilitate high upconverter mobility, frequent collisions, and consequently enable strong annihilation and linear TTA-UC behaviors at even lower laser power densities [29,30]. Therefore, one goal in the field of solar energy conversion is to design a TTA-UC medium that simultaneously protects against oxygen quenching and facilitates high upconverter mobility, so as to decrease the threshold excitation intensity at which strong TTA behavior is observed, ideally below the intensity of sunlight (100 mW cm^{-2}) [9]. It’s worth noting that, even if the threshold intensity of TTA-UC in a particular system is greater than the intensity of sunlight, strong annihilation behavior and high TTA-UC efficiencies are still achievable in everyday settings if the sunlight is focused using lenses.
Figure 2.5. Mechanism of excited state quenching by molecular oxygen.
2.4.5. **Diffusion-Controlled Energy Transfer in TTA-UC**

The TTA-UC emission is generally delayed because the TET and TTA processes are limited by the diffusion of upconverters in the medium, as shown by Figure 2.6 [31]. Upon the diffusion-controlled collision of upconverters in TET and TTA, resonant energy transfer takes place via exchange interaction, called Dexter energy transfer, if the relevant energy levels of the sensitizer and annihilator are a good match [31]. Unlike the sixth-power dependence of distance on Förster energy transfer, a long-range mechanism [7], Dexter energy transfer requires spatial overlap between the sensitizer and annihilator wave functions [31], as described by equation 9,

\[ k_{ET}(R) = \frac{hK^2}{\pi L} \int F_S(E)G_A(E)dE \]  

(9)

where \( F_S \) and \( G_A \) are the normalized sensitizer (S) and annihilator (A) fluorescence and absorption spectra, respectively, \( K \) is a constant with units of energy, and \( L \) is a measure of overlap between the sensitizer and annihilator wave functions [5].

If the Perrin approximation is applied, which assumes that there is an “active quenching spherical radius \( (R_0) \)” for each isolated upconverter pair under static conditions, then the rate of Dexter energy transfer is fast at short distances \( (R << L) \) and negligible at long distances \( (R > L) \) [5]. The Perrin approximation allows for the Dexter radius to be determined from photoluminescence (PL) measurements, according to the equations below [5].

\[
\frac{l}{l_0} = e^{\left(\frac{c_A}{c_0}\right)} \quad \text{or} \quad ln\left(\frac{l_0}{l}\right) = K_p c_A
\]  

(10)

\[
K_p = \frac{1}{c_0} = \frac{4}{3}\pi R_0^3N_A
\]  

(12)
where $I$ is the PL intensity of the sensitizer in the presence of annihilator, $I_0$ is the PL intensity of the sensitizer in the absence of annihilator, $C_A$ is the annihilator concentration, and $C_0$ is a constant critical transfer concentration, equal to the inverse of the Perrin constant ($K_p$) [5]. To experimentally prove this theory, Pt(II)-octaethylporphyrin (PtOEP) sensitizer and 9,10-diphenylanthracene (DPA) annihilator were frozen in a solution of 1,1,1-trichloroethane at 77 K and the PL measurements fitted perfectly with equations 10-12. The Dexter radius for collision overlap was calculated to be 26.5 Å [32], supporting that energy transfer during TET takes place via the Dexter mechanism.
Figure 2.6. A schematic illustrating the role of diffusion in the TTA-UC mechanism. Image obtained from [32].
Meanwhile, the kinetics of photophysical biomolecular quenching in the fluid phase can be observed using the Stern-Volmer relationship, equation 13, which provides information on the dynamic behavior of energy transfer between molecules [33,34].

\[ \frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + K_{SV} C_A \rightarrow \frac{1}{\tau} = k_q [Q] + \frac{1}{\tau_0} \]  

\[ K_{SV} = k_q \tau_0 \]  

where \( k_q \) is the bimolecular quenching rate constant, \( I \) is PL intensity, \( \tau \) is the lifetime of the sensitizer T1 state, \( K_{SV} \) is the Stern-Volmer constant, and \( C_A \) is the annihilator concentration. The subscript ‘0’ denotes the absence of annihilator. While Dexter energy transfer was previously discussed from the perspective of static quenching, the model for diffusion-controlled Dexter energy transfer can be derived from the diffusion-dependent quenching rate constant obtained via Stern-Volmer analysis [7].

\[ k_q = 4\pi DR_0 = \frac{2k_BT_0}{3\eta(R_{ms}+R_{mA})} \]  

where \( D \) is the summed diffusion coefficients of the sensitizer and annihilator, \( k_B \) is Boltzmann’s constant, \( T \) is temperature, \( \eta \) is viscosity, and \( R_m \) is the effective molecular radius. Its worth noting that, according to equation 15, Dexter energy transfer should become more efficient as temperature increases [7]. Afterall, the enhancement of molecular diffusion in bimolecular systems is a key strategy for improving the TTA-UC efficiency. Therefore, to ensure a high probability of collisions between upconverters, their diffusion lengths should be orders of magnitude larger than the Dexter radius, typically achieved by extending the T1 state lifetime. Furthermore, local upconverter mobility can be improved by selecting upconverters with small Dexter radii and embedding them in a low viscosity medium [29].
Since $k_q$ is related to diffusion and energy transfer in a dynamic environment, an expression that describes the TET efficiency ($\Phi_{\text{TET}}$) between the sensitizer and annihilator may be constructed [5].

$$\Phi_{\text{TET}} = \frac{k_q c_A}{\frac{1}{\tau_0} + k_q c_A} \quad (16)$$

### 2.4.6. TTA-UC Efficiency

The TTA-UC efficiency ($\Phi_{\text{UC}} /\%$) depends on the quantum yield of all the processes involved in TTA-UC, multiplied by a factor of 2 to normalize for the absorption of two photons, as shown by equation 17 [5].

$$\Phi_{\text{UC}} = 2 \times \Phi_{\text{ISC}} \Phi_{\text{TET}} \Phi_{\text{TTA}} \Phi_{\text{F}} \times 100 \quad (17)$$

where $\Phi_{\text{ISC}}$ is the ISC quantum yield of the sensitizer, $\Phi_{\text{TET}}$ is the TET quantum yield, $\Phi_{\text{TTA}}$ is the TTA quantum yield, and $\Phi_{\text{F}}$ is the fluorescence quantum yield. However, the determination of absolute quantum yields in TTA-UC is difficult, as the $\Phi_{\text{UC}}$ scales nonlinearly with excitation intensity and typical integrating sphere setups are not compatible with high-power laser excitation sources, without challenges [35]. Instead, using equation 18, the $\Phi_{\text{UC}}$ can be calculated relative to the known PL quantum yield ($\Phi_{\text{PL}}$) of a reference [12-32],

$$\Phi_{\text{UC}} = 2 \times \Phi_{\text{PL ref}} \left( \frac{A_{\text{ref}}}{A_{\text{UC}}} \right) \left( \frac{I_{\text{ref}}}{I_{\text{UC}}} \right) \left( \frac{\int F_{\text{UC}}}{\int F_{\text{ref}}} \right) \left( \frac{n_{\text{UC}}}{n_{\text{ref}}} \right)^2 \times 100 \quad (18)$$

where $\Phi$, $A$, $I$, and $n$ denote the quantum yield, absorbance, area of the emission curve, and refractive index of the medium, respectively, at a given excitation wavelength. The subscripts ‘UC’
and ‘ref’ represent the TTA-UC sample in question and the reference sample, respectively, which
the $\Phi_{UC}$ is calculated relative to.

2.5. Strategies of Triplet Sensitization

2.5.1. Spin-Orbit Coupling

The phenomenon of spin-orbit coupling is one of the underlying causes of spin-forbidden
transitions, such as ISC; one of the processes that must be maximized to ensure efficient TTA-UC.
Spin-orbit coupling arises from the magnetic interaction of the nucleus with an orbiting electron
[5]. The nucleus induces a magnetic field, $B$, which is proportional to the angular momentum, $L$
[5]. Meanwhile, the spin, $S$, of the electron produces a magnetic dipole moment, $\mu_s$, that is subject
to coupling with the magnetic field of the nucleus [4,5]. The interaction energy, $H$, of the magnetic
field with the magnetic moment is given by equation 19, which can be simplified to equation 20
since $B$ and $\mu_s$ are proportional to $L$ and $S$ [4,5], respectively.

$$H = -\mu_B \times B$$  \hspace{2cm} (19)

$$\hat{H}_{SO} = \zeta_{SO} L \times S$$ \hspace{2cm} (20)

where $\zeta_{SO}$ is proportional to the fourth power of the nuclear charge. A higher nuclear charge will
generate a larger magnetic field, therefore increasing the interaction energy and inducing stronger
spin-orbit coupling. The centripetal force of an orbit also tends to be greater as the attraction
between the nucleus and the electron increases, causing the electron to orbit closer to the nucleus;
a consequence among many heavy atoms that exhibit strong spin-orbit coupling [4,5].

The spin-orbit coupling between $L$ and $S$ enables the conversion of a $S_1$ state to a $T_1$ state,
or vice versa, by providing enough angular momentum to an electron, in addition to its own, to
cause an electron spin flip [4,5]. Therefore, spin-orbit coupling is one of the primary mechanisms responsible for ISC, and ISC will occur more readily in molecules containing atoms that exhibit strong spin-orbit coupling; one of the key concepts that must be considered in the selection of a sensitizer for TTA-UC [19].

Heavy, paramagnetic metal (Pd$^{2+}$, Pt$^{2+}$, Ir$^{2+}$, Ru$^{2+}$, etc.)-containing molecules that also satisfy the ideal absorption and long T$_1$ state lifetime preconditions, such as metallo-porphyrins, benzoporphyrins, phthalocyanines, tris(bipyridine) complexes, and their derivatives are typical high-triplet yielding TTA-UC sensitizers [19]. The spin-orbit coupling that is associated with the metal leads to high rates of ISC in the sensitizer that are beneficial to increasing the TTA-UC efficiency. However, in applications where expensive metals are not desired, high ISC quantum yields can still be achieved in organic molecules by the incorporation of a heavy halogen, such as iodine or bromine, but this has the unwanted effect of reducing the T$_1$ state lifetime [36].

2.5.2. Spin-Orbit Charge-Transfer Intersystem Crossing

Spin-orbit charge-transfer intersystem crossing (SOCT-ISC) is another strategy by which the triplet quantum yield of a molecule may be enhanced to better sensitize TTA-UC, except the presence of a heavy, paramagnetic metal or a heavy halogen is not required [37-40]. Rather, SOCT-ISC sensitizers are donor-acceptor (D-A) dyads that can be completely organic [37], making them great alternatives in applications where environmental impact, biocompatibility, and low cost are of utmost importance. Photoexcitation of a SOCT-ISC sensitizer, with strong electronic coupling between donor and acceptor components, results in the formation of a spin-correlated singlet excited state ($^{1}S_{1}(A^*)$), which undergoes charge separation to form the singlet charge-separated excited state $^{1}(D^+\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot
a change in orbital angular momentum to compensate for the change of electron spin angular momentum during ISC [37-40]. This effect greatly enhances ISC and leads to the formation of a spin-correlated triplet state ($T_1A^*$), as shown in Figure 2.7.
Figure 2.7. Simplified Jablonski diagram of SOCT-ISC in a D-A molecule. Image adapted from [37].
3.1. Introduction

The development of photofunctional systems capable of facilitating triplet-triplet annihilation-based photon upconversion (TTA-UC) has stimulated considerable growth in the fields of renewable energy, biomedicine, and photocatalysis [12,41-45]. TTA-UC is initiated upon the photoexcitation of a high triplet-yielding sensitizer, which then undergoes triplet energy transfer (TET) with an appropriate annihilator via the Dexter mechanism [12,41-45]. The collision of two sensitized acceptor triplets generates a high-energy singlet excited state, which will emit a delayed anti-Stokes photon (Figure 2.3) [12,41-43]. The application of TTA-UC to solar devices is therefore a desired strategy for surpassing the Shockley-Queisser theoretical limit [8,9,46]. Computations show that the energy output of wide-bandgap, single-junction solar cells may increase by 1-6% as a result of TTA-UC technology, depending on its efficiency [8,9,46]. Unfortunately, this result has not been achieved because, while high TTA-UC efficiencies (>15%) are attainable under an inert atmosphere [29,47], a TTA-UC system that is both, efficient under aerobic conditions and environmentally conscious, has yet to be invented.

Herein, we focus on the three major challenges that previous TTA-UC systems have faced: 1) quenching of the triplet state by molecular oxygen and the concomitant photooxidation of upconverters (sensitizer and annihilator), 2) low collisional frequencies between upconverters due to the dynamics of the system, and 3) environmental aspects [30,48-52]. The first challenge necessitates deaeration in liquid-state TTA-UC systems. Since deaeration is burdensome in industrial settings, the study of TTA-UC under aerobic conditions is critical for it to be successfully applied to solar devices. Although TTA-UC can be efficient in supramolecularly crowded and
ionic liquids without deaeration [53,54], soft matter and solid-state systems may possess relative oxygen impermeability due to their high viscosity [55-60]. These states of matter can also be molded into films for convenient integration into solar devices, whereas liquids cannot [12,53,54].

The disadvantage of soft matter and solid-state systems, however, is that their high viscosity hampers upconverter mobility [61,62], thus inflicting penalties to the TET and TTA-UC efficiencies. Correspondingly, countless low-efficiency soft matter systems exist in which the upconverters are embedded in a network, scaffold, or polymer chain that impairs their mobility [60,62-67]. This negative effect on TTA-UC is even more significant for solid-state systems in which the upconverters are immobile, but it is negligible for liquid-state systems [53-60]. Therefore, an ultimatum between upconverter mobility and protection against oxygen presents itself in soft matter and solid-state systems, which has prevented them from reaching their full potential to upconvert light efficiently in recent studies.

In other words, for a TTA-UC system to be as efficient as possible, its design must grant protection against quenching by molecular oxygen without the introduction of unfavorable dynamics that result in low collisional frequencies between upconverters. Fortunately, we unintentionally stumbled upon a solution to this issue upon the analysis of a multiphasic hydrogel, which consisted of bovine serum albumin (BSA) protein, sodium dodecyl sulfate (SDS) surfactant, and water. The BSA/SDS hydrogel can be envisioned as having co-assembled BSA network and SDS micelle-like domains, which have mutually exclusive functionalities in ensuring efficient TTA-UC (Figure 3.1). In line with the spectroscopic data presented herein, the dense protein matrix that comprises the hydrogel is thought to behave like an oxygen-impermeable, protective mesh, with micellar environments lodged between its pores. Meanwhile, the hydrophobic, fluid-phase interior of these micellar environments is thought to behave as a cage in which the
upconverters may collide with high mobility. Due to the multiphasic nature of our system, TTA-UC may be protected from oxygen without consequence to the upconverter mobility, unlike other systems which are forced to sacrifice one for the other by design [60,62-67].
Figure 3.1. A cartoon of the upconverting BSA/SDS hydrogel accompanied by definitions of the involved reagents. The upconverters are housed within micellar SDS environments. Although hundreds of micellar environments per 2.5 ± 0.4 μm pore may exist as a distribution of sizes and shapes, they are depicted above as a single sphere per pore for ease of illustration. The hydrogel skeleton consists of non-covalently associated BSA molecules, which creates a three-dimensional soft matter maze that becomes increasingly difficult to navigate, thus protecting TTA-UC from quenching by oxygen while preserving upconverter mobility in the micellar environment.
It is worth nothing that, while the BSA/SDS hydrogel happened to address a fundamental problem in the TTA-UC field, our prime intention was always to design an environmentally conscious and low-cost system, thus advocating for our group’s mission to replace inorganic technologies with organic, bioinspired alternatives. However, a majority of reported TTA-UC systems utilize metal-containing sensitizers [8,9,12,30,41-67], so we extended this study to completely organic sensitizers as well. Although traditional TTA-UC sensitizers, such as metallo-porphyrin derivatives and tris-bipyridines, benefit from broad absorption profiles, high absorption intensities, and long triplet excited state lifetimes, their high triplet yields are due to the presence of heavy, paramagnetic metals (e.g. Pd$^{2+}$, Pt$^{2+}$, Zn$^{2+}$, Ru$^{2+}$, Ir$^{2+}$) [68-71]. Metal-containing sensitizers are not ideal because of their high-cost, high-temperature synthesis, which is only complicated by the production of heavy metal waste products that are toxic and difficult to remove [68-75]. Much research has been dedicated to the development of high triplet-yielding sensitizers that do not contain heavy metals, typically achieved by the incorporation of heavy halogens (Br & I) into chromophores that absorb light beyond 500 nm, such as boron dipyrromethene (bodipy) derivatives [72-75]. Unfortunately, the inclusion of halogens increases the cost of synthesis and decreases the sensitizer triplet state lifetime [72-75]. To avoid these problems, completely organic, Bodipy-based, orthogonal donor-acceptor dyads that efficiently access the triplet state via spin-orbit, charge-transfer intersystem crossing (SOCT-ISC) are currently desired for applications in TTA-UC [37,39,40,76-78]. However, as the TTA-UC community moves toward the elimination of heavy atom sensitizers, the photodynamics of SOCT-ISC sensitizers in soft matter have not been well-studied.

To the best of our knowledge, we accomplish TTA-UC in soft matter using bodipy-based, SOCT-ISC sensitizers for the first time, which is the second reported instance of SOCT-ISC
sensitized TTA-UC in soft matter overall. Traditional sensitizers, palladium octaethylporphyrin (PdOEP) and diiodo-Bodipy, are also studied to elucidate the effects that our unorthodox multiphasic soft matter system has on the three strategies of triplet formation and on the TTA-UC efficiency. Because of the simultaneous protection from oxygen quenching and preservation of upconverter mobility afforded by our multiphasic system, we hope to show that SOCT-ISC sensitizers have the potential to sensitize TTA-UC under aerobic conditions more efficiently than other reported systems, which utilize metal-containing and heavy atom sensitizers. We show that our system was somewhat successful in achieving this goal, despite the design limits imposed by low-cost, biocompatible, industrial, and easy-preparation requisites.

3.2.  Experimental

Materials. All reagents were used as-received. Palladium(II) octaethylporphyrin (PdOEP), 9,10-diphenylanthracene (DPA), perylene (Pery), and tetrahydro-furan (THF) were purchased from Sigma-Aldrich (St. Louis, MO). Fatty acid free bovine serum albumin was purchased from Equitech-Bio, Inc. (Kerrville, TX). The Bodipy-based sensitizers were synthesized and purified as part of previous work [37].
Figure 3.2. (A) Scheme depicting the preparation of the BSA/SDS/upconverter hydrogel. For a BSA/SDS hydrogel, containing no upconverters, plain THF was added in the second step. (B) Image of BSA/SDS hydrogel that showcases its transparency.
**Preparation of Upconverting BSA/SDS Hydrogel.** The BSA/SDS/upconverter hydrogel was prepared in three steps (Figure 3.2.). First, solid BSA was dissolved at room temperature in a 513 mM SDS solution, which consisted of 74 vol% deionized water (dH₂O) and 26 vol% THF. Second, THF/upconverter solution was added to the mixture while stirring rapidly, so as to avoid the precipitation of BSA by undispersed organic solvent. The stirring was discontinued once a homogenous BSA/SDS/upconverter solution was obtained, which consisted of 64 vol% dH₂O and 36 vol% THF. Finally, this solution was heated at 72.5 °C in a 1 cm x 1 cm glass cuvette and thermally-induced gelation occurred after 30 min. The final BSA/SDS/upconverter hydrogel consisted of 693 mM SDS in ~100 vol% water. The BSA and upconverter concentrations were varied depending on the experiment.

**Absorption Spectroscopy.** Absorbance spectra were recorded by a HP 8450 diode array spectrophotometer (Varian Inc.). For each absorption measurement, the baseline between 700 nm and 900 nm was averaged and then subtracted over the range. All samples were measured under aerobic conditions in a 1 cm x 1 cm glass cuvette at room temperature.

**Fluorescence Spectroscopy.** Steady state fluorescence spectra were recorded by a FLS1000 fluorescence spectrophotometer (Edinburgh Instruments) under lamp excitation unless noted otherwise. Absolute photoluminescence quantum yields (Φ₉) were measured using an integrating sphere. The Φ₉ data was processed using Fluoracle® software (Edinburgh Instruments). All of the measurements were conducted under aerobic conditions in a 1 cm x 1 cm glass cuvette at room temperature.

**Determination of TTA-UC Efficiency.** The normalized TTA-UC efficiency (Φ₉) was calculated by equation 18, relative to the sensitizer. The terms for absorbance, intensity, and refractive index were omitted because these quantities were constant between the UC and reference
samples. The TTA-UC emission and sensitizer photoluminescence spectra were recorded using the FLS1000 fluorescence spectrophotometer, which was outfitted with a 532 nm, 50 mW continuous wave laser (Edmund Optics) excitation source (beam diameter = 1.2 mm). An excitation intensity of 7.4 W cm\(^{-2}\) was applied for measurements pertaining to the determination of \(\Phi_{\text{UC}}\). The timescale of the measurement was adjusted to avoid photobleaching. The emission spectra were integrated using Fluoracle\textsuperscript{®} software. The \(\Phi_{\text{PL ref}}\) was determined at the same sensitizer absorbance as the UC sample.

**TTA-UC Emission Dependence.** The FLS1000 fluorescence spectrophotometer and laser setup, described above, was also used here to measure the TTA-UC emission as a function of external stimuli. The dependence of TTA-UC on the excitation intensity was studied by recording the TTA-UC emission spectrum of a BSA/SDS/upconverter hydrogel several times, each time employing a different excitation intensity. The excitation intensity was varied from 142 mW cm\(^{-2}\) to 1,177 mW cm\(^{-2}\) by placing combinations of neutral density filters (set NEK01, Thorlabs) in front of the laser path. For temperature dependence studies, the sample holder and BSA/SDS/upconverter hydrogel were heated from 20 °C to 70 °C by a QNW TC 1 temperature controller (Quantum Northwest), upon which the TTA-UC emission was measured. For each measurement, the sample was given 15 min to equilibrate to each temperature change and the laser excitation intensity was 7.4 W cm\(^{-2}\).

**Time-Resolved Studies.** The transient absorption setup was based on Edinburgh Instruments LP920. The excitation pulse at 532 nm was provided by a Nd:YAG-pumped laser (Continuum Surelite I) with a 10 Hz repetition rate and 5-7 ns pulse width. The energy of the pump beam was adjusted between 1 mJ and 25 mJ depending on the samples. The probe beam was supplied by a Xe Model 920, 450 W xenon arc lamp. A monochromator, with a P928
photomultiplier detector (Hamamatsu), was used for decay measurements. An iCCD camera (Andor Technology) was used for spectral measurements. The triplet excited state lifetimes of sensitizers were determined by measuring the triplet absorption decay of BSA/SDS/sensitizer hydrogels. The triplet excited state lifetimes were also obtained in the presence of several annihilator concentrations, which could be treated with the Stern-Volmer relationship (equation 13) to obtain $k_0$, and consequently, the TET efficiency ($\Phi_{\text{TET}}$) using equation 16. The TTA-UC emission lifetimes were determined by measuring the emission decay from BSA/SDS/upconverter hydrogels. The time constants were extracted from exponential decay fits, performed by L900 and Kaleidagraph data processing programs. The spectral absorption and emission spectra were also recorded, with time delays depending on the sample. All decay curves and spectra consisted of at least 40 averaged measurements. All hydrogel samples were measured under aerobic conditions in 1 cm x 1 cm glass cuvette at room temperature.

**Scanning Electron Microscopy.** Scanning electron microscopy (SEM) images were taken using a Teneo LVSEM. A thin cross-section of the BSA/SDS hydrogel was lyophilized with liquid nitrogen overnight. The freeze dried, sliced BSA/SDS hydrogel was then affixed to the SEM stub with carbon tape, spin-coated with Au/Pd (5 nm, 80% Au, 20% Pd) the next day, and then imaged immediately afterwards. Powder X-Ray Diffraction (XRD) studies of the same hydrogel that was previously lyophilized for the SEM measurements were performed using a Rigaku Ultima IV X-ray diffractometer.

### 3.3. Formation of BSA/SDS Hydrogel.

In the design of our multiphasic soft matter system, SDS was selected as the surfactant because of its high aqueous solubility (693 mM), low critical micelle concentration (8.3 mM) [79],
and ability to form micelles large enough to contain at least a few upconverters (~3.5 nm in diameter) [80]. Proteins were considered for the network component because of their biocompatibility, low toxicity, and low cost. A variety of proteins fit the above criteria, but BSA in particular, was chosen for its high molecular weight (66,463 Da) and aqueous solubility (6.0 mM) [81,82]; qualities that are conducive to forming dense hydrogel networks, which might hinder oxygen diffusion effectively. While BSA can be covalently crosslinked at high concentrations to form hydrogels [83], we did not pursue this option because carbodiimide crosslinking reagents are expensive, unstable at room temperature, and leave behind unremovable urea-based byproducts [83]. The method of thermally-induced gelation, on the other hand, does not suffer from these drawbacks [84]. According to previous reports, a hydrogel will form when a concentrated BSA solution is heated, predominately because of noncovalent interactions between thermally denatured protein domains [84]. However, it was also reported that the thermally-induced gelation of BSA was completely inhibited at SDS concentrations above 50 mM because the SDS molecules compete for noncovalent protein-protein interactions [85]. This maximum allowed SDS concentration of 50 mM was, unfortunately, too low to dissolve the millimolar concentrations of upconverters required for efficient TTA-UC.

To workaround this problem, we employed an organic solvent to both, solvate the upconverters and temporarily negate the interaction between SDS and BSA [86], thus enabling the noncovalent protein-protein interactions fundamental to thermally-induced gelation. The ideal organic solvent was THF because its boiling point was significantly below that of dH2O and it did not cause the precipitation of BSA when mixed thoroughly. By heating a solution of BSA, SDS, THF, and upconverters, a thermal BSA/SDS/upconverter hydrogel was able to form (Figure 3.2), which contained the maximum SDS concentration for improved upconverter solubility. During the
thermally-induced gelation, it is believed that most of the THF evaporated, the upconverters were infused into micellar SDS environments, and the BSA associated into a three-dimensional network simultaneously. Since millimolar concentrations of upconverters were soluble in the end, which were not soluble in the absence of SDS, we believe that a distribution of SDS micelle shapes and sizes existed within the BSA/SDS hydrogel. A fraction of the SDS molecules may be bound to the surfaces of the BSA network [87], but the extent of this is unknown, as the nature of hydrogels thwarts many methods of study. At least, several spectroscopic clues appear throughout this study, which indicated that the upconverters reside in a micellar environment.

All of the BSA/SDS hydrogels tested here passed the gel inversion test, as indicated by a resistance to gravity upon being turned upside down for an indefinite period of time. Without upconverters, the BSA/SDS hydrogel was tan-colored and completely translucent (Figure 3.2), which enabled the use of a variety of spectroscopic methods. The sol-gel transition was irreversible, as the BSA/SDS hydrogel also passed the gel inversion test at room temperature. The BSA/SDS hydrogel was stable up until the boiling point of water (100 °C), which highlighted the robustness of our system under high-temperature solar conditions. Interestingly, the gelation even took place at room temperature upon the evaporation of THF. Since the gelation of BSA/SDS/THF solution was thermodynamically favored at room temperature, we believe that the noncovalent interactions between chemically denatured BSA, rather than thermally denatured BSA, was the root cause of gelation. Contrary to this phenomenon, only thermal BSA/SDS hydrogels are reported here because quick gelation (~30 min) was preferred.
3.4. Characterization of TTA-UC in BSA/SDS Hydrogel

In working with a complex multiphasic system, it was necessary to determine which component facilitated TTA-UC: BSA or SDS. Afterall, BSA has binding sites for a variety of organic dyes, metal ions, and biomolecules [87,88]. The structures of the upconverters used in this study can be found in Figure 3.3. PdOEP (1) did not bind to BSA in aqueous solution, but DPA, Pery, and all of the Bodipy-based sensitizers (2-6), did bind, as shown by the shifted absorbance spectra of their complexes. The absorption and steady-state fluorescence spectra of DPA, Pery, and 1-6 within the individual BSA/SDS/hydrogel, SDS micelle, THF, and BSA environments can be found in Figure 3.4. The binding of 2-6 to BSA was characterized by a severe bathochromic shift in the absorbance, while the binding of DPA and Pery to BSA was characterized by a slight red-shift in the absorbance; both effects represented a change in the substrate’s environment from the solvent to a binding site of BSA [89,90].
Figure 3.3. The combinations of upconverters used in the BSA/SDS hydrogel, accompanied by pictures of the hydrogels under white light and under 532 nm, 5 mW laser excitation.
Figure 3.4. Absorbance and fluorescence spectra of upconverters in BSA/SDS hydrogel, SDS micelle, THF, and BSA complex.
Similar bathochromic shifts, however, were not observed in the absorbance and fluorescence spectra of the BSA/SDS/upconverter hydrogels. The spectral features of the BSA/SDS/upconverter hydrogels were analogous to those of the SDS/upconverter micelles, which implied that a majority of the upconverters were solvated by the SDS micelles rather than by BSA. In fact, the preferred binding of upconverters to SDS micelles within the BSA/SDS hydrogel may be explained by the denaturation of structural features that constitute the binding sites of BSA upon thermal gelation, as well as the higher affinity that SDS micelles have for nonpolar molecules [91]. Furthermore, TTA-UC was not observed from deaerated BSA/upconverter complexes, even under 532 nm laser excitation, probably because Dexter energy transfer cannot occur between upconverters that are restricted to distant binding sites and because BSA is known to bind only one polycyclic aromatic molecule [91], or annihilator, which is not enough to appease the TTA-UC mechanism. Conversely, TTA-UC was observed from a deaerated solution of SDS/upconverter micelles. These control tests, in addition to the fact that millimolar concentrations of upconverters were not soluble in the absence of SDS, confirmed that the upconverters were primarily situated in SDS micelle-like environments within the BSA/SDS hydrogel, and that these environments were the sites of TTA-UC, rather than unlikely BSA/upconverter complexes.

Upon photoexcitation of the BSA/SDS/upconverter hydrogels with a 5 mW, 532 nm laser, green-to-blue TTA-UC between 1 and DPA, as well as between 2-6 and Pery, was observed (Figure 3.3). The TTA-UC emission maxima of the BSA/SDS/1/DPA and BSA/SDS/2-6/Pery hydrogels were located at 434 nm and 476 nm, respectively (Figure 3.5). No TTA-UC emission was detected upon the 532 nm photoexcitation of the BSA/SDS/DPA and BSA/SDS/Pery hydrogels; that is, in the absence of sensitizer. These results implied that the blue emission was
not background fluorescence caused by photoexcitation of the annihilators, but that it was most likely TTA-UC emission.
Figure 3.5. The TTA-UC emission spectra of the BSA/SDS/1/DPA and BSA/SDS/2-6/Pery hydrogels measured upon 532 nm, lamp excitation.
The annihilator emission was also confirmed to arise from TTA-UC by observing its nonlinear dependence on the excitation intensity (Figure 3.6A). This nonlinear dependence was further quantified with double logarithmic plots of TTA-UC emission against excitation intensity (Figure 3.7). We observed both, quadratic and linear, TTA-UC dependencies on the excitation intensity at low and high excitation intensity regimes, respectively. As is typical of TTA-UC, the slopes of the double logarithmic plots were approximately 2 under low excitation intensities and 1 under high excitation intensities for all BSA/SDS/upconverter hydrogels. Surprisingly, the threshold intensity ($I_{th}$) of TTA-UC was lower when 2-6 were used as the sensitizers than when 1 was used. In other words, the heavy atom-containing and SOCT-ISC sensitizers approached steady state TTA-UC behavior at lower excitation intensities (~400 mW cm$^{-2}$) than the heavy metal-containing sensitizers (~700 mW cm$^{-2}$) in the BSA/SDS/upconverter hydrogel. While these $I_{th}$ values were higher than the intensity of sunlight (~100 mW cm$^{-2}$), significant industrial efforts have been made to focus sunlight, as is apparent by the availability commercial solar concentrator-enhanced solar panels [92].
Figure 3.6. (A) TTA-UC emission spectra of BSA/SDS/1/DPA, which exhibit a nonlinear trend with respect to the excitation intensity, (B) Double logarithmic plot of TTA-UC emission intensity as a function of excitation intensity for the BSA/SDS/1/DPA hydrogel, (C) Table of $I_{th}$ values and slopes of double logarithmic plots for all of the BSA/SDS/upconverter hydrogels.
Figure 3.8. Double logarithmic plots for all of the BSA/SDS/upconverter hydrogels.
3.5. Optimization of TTA-UC Efficiency

The optimization of the $\Phi_{UC}$ was conducted by measuring the $\Phi_{PL}$ and $\Phi_{UC}$ of several BSA/SDS/upconverter hydrogels in which the concentration of one reagent at a time was systematically varied while those of the other reagents were kept constant. First, the $\Phi_{PL}$ of the BSA/SDS/1 hydrogel, which contained constant $[1] = 21.2 \mu$M and varying $[\text{BSA}] = 2.6-6.0$ mM, was measured (Figure 3.9). Since the triplet yield of 1 is virtually unity [68-71], the $\Phi_{PL}$ of the BSA/SDS/1 hydrogel consisted purely of phosphorescence. Therefore, the $\Phi_{PL}$ of the BSA/SDS/1 hydrogel was regarded as a measure of oxygen protection afforded by varying degrees of BSA network density. The $\Phi_{PL}$ of the BSA/SDS/1 hydrogel decreased with lower $[\text{BSA}]$ because it was easier for oxygen to pass through the hydrogel at lower network densities. The $\Phi_{PL}$ of the BSA/SDS/1 hydrogel reached a maximum of $19.1 \pm 0.2\%$ at $[\text{BSA}] = 4.5$ mM, which indicated that 1 was most protected from quenching by molecular oxygen. Thus, $[\text{BSA}] = 4.5$ mM was assumed to be the optimal concentration for the BSA/SDS/2-6 hydrogels as well; an assumption justified by the fact that the BSA network functions independently from the micellar SDS environments, the sites of TTA-UC. In accordance with our hypothesis that a denser BSA network should hinder oxygen diffusion more effectively, the $\Phi_{PL}$ of BSA/SDS/1 was expected to continue increasing, but a decrease was actually measured at $[\text{BSA}] > 4.5$ mM due to the precipitation of BSA.
Figure 3.9. The phosphorescence quantum yield of the BSA/SDS/1 hydrogel as a function of [BSA]. [1] = 21.2 μM.
The $\Phi_{UC}$ of several BSA/SDS/1/DPA hydrogels, made using constant [BSA] = 4.5 mM, [1] = 21.2 µM, and varying [DPA] = 2.0-9.0 mM, was then measured (Figure 3.10). The $\Phi_{UC}$ of the BSA/SDS/1/DPA hydrogel was found to increase with increasing [DPA], and reached a maximum of 24.0 ± 1.0% at [DPA] = 9.0 mM. However, this result was set aside because the precipitation of DPA was observed, which produced light scattering in the measurement. The highest annihilator concentration at which no precipitation could be observed gave the optimal $\Phi_{UC}$, which was [DPA] = 7.5 mM and [Pery] = 3.0 mM. Decreases in the [SDS] caused the annihilator to precipitate at lower concentrations than usual, so [SDS] = 693 mM, the maximum aqueous solubility, was kept constant for all BSA/SDS hydrogels tested here. The sensitizer concentrations in the BSA/SDS/1/DPA and BSA/SDS/2-6/Pery hydrogels were then varied at constant [BSA] = 4.5 mM, [DPA] = 7.5 mM, and [Pery] = 3.0 mM to obtain the optimized $\Phi_{UC}$ for all upconverter pairs (Figure 3.11 and Table 3.1).
Figure 3.10. TTA-UC efficiency as a function of [DPA] in the BSA/SDS/1/DPA hydrogel. [I] = 21.2 μM and [BSA] = 4.5 mM.
Figure 3.11. TTA-UC efficiency as a function of sensitizer concentration for all of the BSA/SDS/upconverter hydrogels.
Table 3.1. The sensitizer and annihilator concentrations that have the highest $\Phi_{UC}$ values, accompanied by the $\Phi_{PL}$ value that the $\Phi_{UC}$ was calculated relative to.

<table>
<thead>
<tr>
<th>BSA/SDS/... Gel</th>
<th>[Sensitizer] (µM)</th>
<th>[Annihilator] (µM)</th>
<th>Abs. @ 532 nm (a.u.)</th>
<th>Sensitizer $\Phi_{PL}$ (%)</th>
<th>$\Phi_{UC}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/DPA</td>
<td>21.2</td>
<td>7.5</td>
<td>0.27</td>
<td>19.1 ± 0.7</td>
<td>19.0 ± 0.7</td>
</tr>
<tr>
<td>2/Pery</td>
<td>43.5</td>
<td>3</td>
<td>1.02</td>
<td>2.8 ± 0.3</td>
<td>10.7 ± 0.4</td>
</tr>
<tr>
<td>3/Pery</td>
<td>14.7</td>
<td>3</td>
<td>0.32</td>
<td>23.3 ± 0.1</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>4/Pery</td>
<td>27.2</td>
<td>3</td>
<td>0.07</td>
<td>16.2 ± 0.6</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>5/Pery</td>
<td>9.3</td>
<td>3</td>
<td>0.15</td>
<td>12.4 ± 0.8</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>6/Pery</td>
<td>18.7</td>
<td>3</td>
<td>0.14</td>
<td>6.6 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>
The $\Phi_{UC}$ of the BSA/SDS/1/DPA was 19.0 ± 0.7%, which is currently the record highest $\Phi_{UC}$ observed in a hydrogel system and it is among the highest $\Phi_{UC}$ values reported under aerobic conditions overall [92]. While 1 gave the highest $\Phi_{UC}$ among all of the sensitizers tested in the BSA/SDS hydrogel, most likely because of the high triplet yield brought on by Pd$^{2+}$ [68-71], the remaining sensitizers still achieved impressive $\Phi_{UC}$ values in their class. A $\Phi_{UC}$ of 10.7 ± 0.4% was measured in the BSA/SDS/2/Pery hydrogel, which was higher than the $\Phi_{UC}$ between 2 in Pery in deaerated toluene, hexane, heptane, and dimethyl sulfoxide [47]. These high $\Phi_{UC}$ values were testament to the level of oxygen protection provided by the BSA/SDS hydrogel. Furthermore, as shown in Figure 3.4A, phosphorescence at 665 nm was not observed from THF/1 solution because of triplet excited state quenching by molecular oxygen, whereas phosphorescence was observed from the BSA/SDS/1 hydrogel, and without fluorescence at 500-600 nm, which further affirmed the oxygen-protective capabilities of the BSA/SDS hydrogel. Also, SOCT-ISC sensitized TTA-UC occurred with $\Phi_{UC}$ values ranging from 1.0 ± 0.1% to 3.5 ± 0.2%; the only reported $\Phi_{UC}$ values for bodipy-based SOCT-ISC sensitizers in soft matter to date. The SOCT-ISC sensitized $\Phi_{UC}$ of 3.5 ± 0.2%, achieved by the BSA/SDS/4/Pery hydrogel, was relatively high when compared to some reports of TTA-UC in other soft matter systems [60,62-67], which remain ineffective even when using traditional, heavy metal and heavy atom-containing sensitizers.

3.6. Effects of BSA/SDS Hydrogel on Sensitizer Photophysics

When put in the BSA/SDS hydrogel, the photophysical behavior of the SOCT-ISC sensitizers was altered compared to their reported photophysics in acetonitrile (MeCN), as shown in Figure 3.12A. The $\Phi_{UC}$ of the BSA/SDS/3/Pery hydrogel was 2.5 ± 0.2%, which was surprising considering that the triplet yield of 3 was only 33% in acetonitrile (MeCN) [37]. The $\Phi_{UC}$ of the BSA/SDS/5/Pery hydrogel was much lower, 1.1 ± 0.2%, even though the triplet yield of 5 was
90% in MeCN [37]. Furthermore, TTA-UC was not observed at all in the BSA/SDS hydrogel between Pery and another bodipy derivative, 7 (Figure 3.12B), despite it having a triplet yield of 61% in MeCN [37]. Therefore, the $\Phi_{\text{UC}}$ was not higher for SOCT-ISC sensitizers that had a higher reported triplet yield in MeCN. These inconsistencies indicated that the inherent triplet yields of the SOCT-ISC sensitizers must have changed drastically within the BSA/SDS hydrogel. Therefore, SOCT-ISC may have been deactivated or activated for sensitizers 3-7, and to different extents.
**Figure 3.12.** (A) Table of the reported $\Phi_{PL}$ and triplet yields ($\Phi_T$) in the specified organic solvent.

Data retrieved from [37,47,94]. (B) Structure of 7 accompanied by photophysical results in the BSA/SDS hydrogel.
For example, the photophysics of 7 were most affected by the environment within the BSA/SDS hydrogel, as the \( \Phi_{PL} \) of 7 was 60 ± 1% in the BSA/SDS hydrogel while it was a mere 10% in MeCN [37]. Since no TTA-UC was observed from the BSA/SDS/7/Pery hydrogel at all, it was likely that the enhanced \( \Phi_{PL} \) of the BSA/SDS/7 hydrogel consisted of mostly charge-transfer emission and that the SOCT-ISC mechanism of 7 was deactivated by the environment within the BSA/SDS hydrogel.

The \( \Phi_{PL} \) values of 2-6 in the BSA/SDS hydrogel increased relative to those reported in MeCN [37], which hinted at two possibilities: 1) a larger fraction of triplets excited states underwent decay, or 2) a larger fraction of singlet excited states underwent radiative decay rather than SOCT-ISC. We believe that the latter possibility occurred for 2-5 in the BSA/SDS hydrogel because it is known that these SOCT-ISC sensitizers have lower triplet yields in nonpolar solvent [37], so the nonpolar environment within the SDS micelles could have been responsible for reducing their triplet yields. To test this hypothesis, we studied the photophysics of 6 as a negative control, which has a \( \Phi_{PL} \) of 1% and a triplet yield of 0.5% in MeCN, but a triplet yield of 20% in toluene, a nonpolar solvent [37]. Therefore, the polarity-dependent photophysics of 6 could be measured within the BSA/SDS hydrogel to probe the local environment in which the sensitizers reside and to provide contrast against the deactivation of SOCT-ISC observed for 2-5.

The \( \Phi_{UC} \) of the BSA/SDS/6/Pery hydrogel was 1.0 ± 0.1%, which was higher than the reported triplet of 6 in MeCN [37]. Therefore, the triplet yield of 6 must have increased in the BSA/SDS hydrogel relative to MeCN due to the nonpolar environment within SDS micelle. Not only did this increase confirm that the sensitizers were indeed situated in the SDS micelle, but it also showed that the environment within the BSA/SDS hydrogel may activate SOCT-ISC just as well, depending on the molecular design of the sensitizer.
The rule that sensitizers with low fluorescence yields and high triplet yields will perform better in TTA-UC holds true here [12], of course. Unfortunately, SOCT-ISC sensitizers may have difficulties following this rule because the environment of the soft matter system may negatively affect the photophysics of SOCT-ISC. These conclusions call for the design and synthesis of SOCT-ISC sensitizers that have: 1) fluorescence yields below 1%, 2) triplet yields above 90%, and 3) SOCT-ISC photophysics that are positively influenced by the polarity of the soft matter environment.

3.7. **Time-Resolved Studies**

The photophysics of the BSA/SDS/1-3 hydrogels, as well as analogous samples containing the respective annihilators were studied via transient absorption spectroscopy (Figure 3.13). The transient absorption spectra of the BSA/SDS/1 hydrogel depicted the microsecond-scale decay of a positive band around 430 nm that corresponded to the long-lived triplet excited state expected for 1. Long-lived, positive sensitizer triplet bands were also observed in the transient absorption spectra of the BSA/SDS/2 and BSA/SDS/3 hydrogels at 450 nm and 430 nm, respectively. In the transient absorption spectra of BSA/SDS hydrogels, containing both sensitizer and annihilator, different bands at 450 nm and 490 nm appeared 1 μs after the excitation pulse, which corresponded to triplet excited states of DPA and Pery, respectively. These bands were shorter-lived than those of the sensitizer, which indicated that TTA occurred relatively fast upon the formation of the annihilator triplet excited state.
Figure 3.13. Transient absorption spectra of the BSA/SDS/1-3 hydrogels (A-C), and analogous BSA/SDS hydrogels containing both, sensitizer and annihilator (D-F). The optimized sensitizer and annihilator concentrations were used for these measurements.
The triplet absorption decay for each of the BSA/SDS/1-3 hydrogels was then measured at their respective sensitizer triplet absorption maxima. The sensitizer triplet excited state lifetimes of 1-3 within the BSA/SDS hydrogel were 517 ± 16, 270 ± 20, and 670 ± 80 μs, respectively. Within the BSA/SDS hydrogel, there was a four-fold reduction in the lifetime of 1 [94] and a four-fold increase in the lifetime of 2 [47] compared to their reported triplet excited state lifetimes in deaerated octane and toluene, respectively. This four-fold increase in the triplet excited state lifetime of 2 may have resulted in the high $\Phi_{UC}$ value observed in the BSA/SDS/2/Pery hydrogel, although there are other factors involved. Also, the triplet excited states of 1-3 were quenched in the presence of annihilator, as represented by triplet absorption curves that decayed successively faster with increasing annihilator concentrations (Figure 3.14).
Figure 3.14. Sensitizer triplet absorption decay curves of 1 at 430 nm (A), 2 at 450 nm (B), and 3 at 430 nm (C) with increasing concentrations of their respective annihilator inside of the BSA/SDS hydrogel. The optimized sensitizer concentration was used.
Using the triplet excited state lifetimes of 1-3, and their lifetimes obtained in the presence of annihilator, the Stern-Volmer relationship was invoked to determine the quenching constant (k_q) (Figure 3.15). The value of k_q within the BSA/SDS hydrogel was on the order of $10^8$ M^{-1} s^{-1} for sensitizers 1-3, which was similar to reported k_q values of other dyes inside of SDS micelle environments [95-99], further supporting that the upconverters were located within micellar SDS environments of the BSA/SDS hydrogel. Specifically, the k_q values were $1.01 \times 10^8$, $1.23 \times 10^8$, and $1.30 \times 10^8$ for sensitizers 1, 2, and 3, respectively, so the bodipy-based sensitizers experienced about ~20-30% more bimolecular collisions per second than 1. A linear trend was also observed in all of the Stern-Volmer plots, which indicated that the mechanism of quenching was dynamic and that the upconverters were freely diffusing in a SDS micelle-like environment, rather than aggregating [96]. As calculated per equation 16, the $\Phi_{TET}$ values of the BSA/SDS/1/DPA, BSA/SDS/2/Pery, and BSA/SDS/3/Pery hydrogels were 99.7%, 99.0%, and 99.7%, respectively. Although the $\Phi_{UC}$ resulting from 3 was probably low because of its reportedly low triplet yield and further environment-dependent penalties toward SOCT-ISC, it is conclusive that 3 did sensitize TTA-UC just as efficiently as 1 and 2 because of its long triplet excited state lifetime and high mobility in the BSA/SDS hydrogel.
Figure 3.15. Stern-Volmer plots of the BSA/SDS/1/DPA (A), BSA/SDS/2/Pery (B), and BSA/SDS/3/Pery hydrogels.
The normalized time-resolved emission spectra of the BSA/SDS/1/DPA, BSA/SDS/2/Pery, and BSA/SDS/3/Pery hydrogels were also recorded (Figure 3.16). The time-resolved TTA-UC emission maxima at 434 and 476 nm matched the steady-state fluorescence peaks of DPA and Pery in the BSA/SDS hydrogel, respectively. Since the singlet excited states of DPA and Pery are known to decay within nanoseconds [100], the delayed blue emission from the BSA/SDS/upconverter hydrogels observed 10 μs following photoexcitation further supported that TTA-UC was taking place. Emission between 520 nm and 700 nm was observed from the BSA/SDS/2/Pery and BSA/SDS/3/Pery hydrogels 10 μs following photoexcitation, and this emission increased at longer timescales. Therefore, we believe that a small fraction of the TTA-UC emission from the BSA/SDS/2-6/Pery hydrogels could have been high enough in energy to re-sensitize TTA-UC, considering that the emission of Pery was close to the absorption of 2 and 3 in the BSA/SDS hydrogel. Although minor, this re-sensitization of TTA-UC may have negatively influenced the inherent \( \Phi_{UC} \), which could have been slightly higher than the observed steady-state \( \Phi_{UC} \) values.
Figure 3.16. The time-resolved emission spectra of the BSA/SDS/1/DPA, BSA/SDS/2/Pery, and BSA/SDS/3/Pery hydrogels 10 μs following the excitation pulse.
The TTA-UC emission from the BSA/SDS/1/DPA hydrogel at 434 nm and from the BSA/SDS/2/Pery and BSA/SDS/3/Pery hydrogels at 476 nm was also measured as a function of time (Figure 3.17). Time constants were extracted from both, the rise and the decay of TTA-UC. The TTA-UC rise times were 2.2, 3.0, and 15.8 μs in the BSA/SDS/1/DPA, BSA/SDS/2/Pery, and BSA/SDS/3/Pery hydrogels, respectively. Meanwhile, the TTA-UC decay lifetimes were 136, 128, and 250 μs in the BSA/SDS/1/DPA, BSA/SDS/2/Pery, and BSA/SDS/3/Pery hydrogels, respectively. The photophysical parameters obtained from all of the time-resolved studies are summarized in Table 3.2. The microsecond-scale decay indicated that TTA-UC occurred rapidly in the BSA/SDS hydrogel due to the fast diffusion of upconverters within the micellar SDS environment relative to other reported TTA-UC lifetimes in soft matter that decay over milliseconds [60,93]. The speed at which TTA-UC occurred in the BSA/SDS hydrogel made sense, considering the highly efficient $\Phi_{TET}$ values and high rates of upconverter diffusivity in the BSA/SDS hydrogel.
Figure 3.17. The TTA-UC lifetimes recorded at 434, 476, and 476 nm for the BSA/SDS/1/DPA (A), BSA/SDS/2/Pery (B), and BSA/SDS/3/Pery (C) hydrogels, respectively. The initial TTA-UC rise seen in A-C and fitted (D-F) to obtain the respective, combined lifetime of TET and TTA.
**Table 3.2.** Collection of photophysical parameters extracted from time-resolved studies of the BSA/SDS/1/DPA, BSA/SDS/2/Pery, and BSA/SDS/3/Pery hydrogels.

<table>
<thead>
<tr>
<th>BSA/SDS/... Gel</th>
<th>$\tau_{\text{TTR-UC em}}$ (µs)</th>
<th>$\tau_{\text{Rise}}$ (µs)</th>
<th>$\tau_{\text{Sensitizer}}$ (µs)</th>
<th>$\Phi_{\text{TET}}$ (%)</th>
<th>$k_q$ ($10^8$ M$^{-1}$ s$^{-1}$)</th>
<th>$K_{sv}$ ($10^3$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/DPA</td>
<td>136 ± 1</td>
<td>2.2</td>
<td>517 ± 16</td>
<td>99.7</td>
<td>1.01</td>
<td>5.22</td>
</tr>
<tr>
<td>2/Pery</td>
<td>128 ± 1</td>
<td>3.0</td>
<td>270 ± 20</td>
<td>99.0</td>
<td>1.23</td>
<td>3.32</td>
</tr>
<tr>
<td>3/Pery</td>
<td>250 ± 6</td>
<td>15.8</td>
<td>670 ± 80</td>
<td>99.7</td>
<td>1.30</td>
<td>8.71</td>
</tr>
</tbody>
</table>
3.8. TTA-UC Dependence on Temperature

The steady-state TTA-UC emission at 434 nm and 476 nm for the BSA/SDS/1/DPA and BSA/SDS/3/Pery hydrogels, respectively, was recorded at increasing temperatures (Figure 3.18). Over the tested temperature range, the TTA-UC emission from the BSA/SDS hydrogels exhibited an inverse temperature dependence. This was surprising because, according to other work done using lipid membranes, the TTA-UC emission should increase with temperature, as the lipid environment becomes less viscous and this increases the mobilities of upconverters, leading to enhanced TTA-UC [101-103]. However, the upconverter mobilities and consequent $\Phi_{\text{TET}}$ values were already saturated in the BSA/SDS hydrogel, as determined by time-resolved studies, so we believe that the observed inverse TTA-UC emission dependence on temperature was caused by thermal deactivation of the upconverter excited states. While some soft matter systems undergo a gel-sol transition at high temperatures, which enables oxygen to diffuse quickly and quench TTA-UC [93], the BSA/SDS hydrogel was characterized by an irreversible sol-gel transition with intact oxygen-protective capabilities, so thermal deactivation of upconverter excited states was the next most reasonable explanation for the observed inverse TTA-UC emission dependence on temperature. It is worth noting that the decrease in TTA-UC emission was linear for the BSA/SDS/upconverter hydrogels, whereas most reported systems have nonlinear TTA-UC temperature dependencies [101-103], so BSA/SDS/upconverter micro-hydrogels may find a secondary application as potentially useful sensors for biomedical purposes.
Figure 3.18. The relationship between TTA-UC emission and temperature for the BSA/SDS/1/DPA and BSA/SDS/3/Pery hydrogels under 7.4 W cm\(^{-2}\) laser excitation. The optimized upconverter concentrations were used in this study.
3.9. Conclusions

The environmentally conscious, low-cost, multiphasic BSA/SDS hydrogel proved to be an excellent medium for facilitating green-to-blue TTA-UC under aerobic conditions, with a Φ_{UC} of 19.0 ± 0.7%, only achievable in air-tolerant environments. Not only is this the record highest Φ_{UC} value achieved by a hydrogel, but it is among the highest reported in aerobic conditions overall. The Φ_{UC} between 2 and Pery was higher in the BSA/SDS hydrogel than in deaerated toluene and phosphorescence was predominant in the steady-state emission spectrum of the BSA/SDS/1 hydrogel, which further indicated a high degree of oxygen protection afforded by the dense BSA network. Completely organic SOCT-ISC sensitizers (3-6) gave Φ_{UC} values ranging up to 3.5 ± 0.2%, the second highest reported in soft matter thus far. However, these Φ_{UC} values were dependent on the polarity of the local environment within the BSA/SDS hydrogel, as the SOCT-ISC mechanism of 3-5 was deactivated while 6 was activated. If SOCT-ISC sensitized TTA-UC is ever to be as efficient as heavy metal or heavy atom aided TTA-UC, high triplet-yielding SOCT-ISC sensitizers must be designed with consideration to the polarity of the medium, or vice versa, although 3 did sensitize Pery with a competitive Φ_{TET} of 99.7%. The steady-state studies conducted herein supported that the densely-layered BSA network slowed oxygen diffusion while the time-resolved studies proved that the upconverters diffused quickly and collided efficiently within a SDS micelle-like environment, permitting more efficient TTA-UC to occur under aerobic conditions. The antagonistic relationship between oxygen protection and upconverter mobility was not previously addressed in the literature, but by studying the dynamics of a uniquely designed medium that accounted for both of these facets simultaneously, we showed that multiphasic soft matter resulted in generally higher Φ_{UC} values under aerobic conditions. Ultimately, we hope that this study will inform the design of other TTA-UC soft matter systems, so that they too can achieve...
higher $\Phi_{\text{UC}}$ values, and we also advocate for the use of completely organic, low-cost materials and modern SOCT-ISC sensitizers, which are likely to be the next challenges in the TTA-UC field.
CHAPTER 4: TTA-UC Protein Nanoparticle Size Control via Millifluidic Synthesis

4.1. Preface

So far, this thesis has been focused on TTA-UC, but chapter 4 strays from previous discussion in that it involves a completely different topic; that is, the large-scale, size-controlled production of BSA protein-based nanoparticles according to a theoretical model, explained later. While this topic seems unrelated to the subject of TTA-UC in hydrogels, a BSA hydrogel is just the bulk form of the BSA nanoparticle discussed in this chapter. In most recent work, TTA-UC may have been accomplished in the nanoparticles made by the method described here. For convenience, explanations of TTA-UC will not be repeated here. Rather than to improve solar cell efficiency, TTA-UC nanoparticles treat an entirely different range of applications in the biomedical industry. Since TTA-UC absorbs produces high-energy emission from the absorption of two low-energy photons, a nanoparticle that facilitates TTA-UC is desired from a biomedical imaging perspective because low-energy excitation sources have better penetrance into organic tissues than higher-energy excitation sources, which can also be damaging to nucleic acids [12]. Therefore, the use of TTA-UC nanoparticles in biomedical applications may allow enable bioimaging using safer light sources that can excite at a greater depth in tissues [12]. As mentioned in section 2.3., lanthanides-doped nanoparticles are currently being developed for biomedical imaging applications, but these can be toxic at certain dosages [12]. There is a vacuum in the upconversion imaging subfield that organic TTA-UC can fill; therefore, the synthesis of a completely organic nanoparticle that can perform TTA-UC without any heavy metals was an
attractive topic for our groups, which aim to replace inorganic technologies with organic alternatives.

### 4.2. Introduction

The popularity of nanoparticles (NPs) in biomedical applications can be attributed to their competency in imaging, drug delivery, and a variety of other properties that surpass the perks of current technologies [104-111]. In response to the demand for advanced tools to address biomedical problems, the push to implement NPs into existing clinical procedures and newly discovered solutions has become increasingly apparent. This realization has stimulated rapid growth in the bio-nano research fields [107,108]. As a result, many NPs have been approved for use in everyday practice, and an expanding inventory of NPs are currently under clinical study for potential future applications in the biomedical industry [111-113]. Already, NPs are being utilized for the targeted delivery of therapeutic drugs to diseased tissues, treatment of cancer via photodynamic therapy, imaging of cells and tissues, and sensing of biomolecules [111-113]. NPs can be effective in these applications because of their size-dependent optical behavior, magnetic properties, high surface area to mass ratio, and potential for surface modification [105-111]. However, the application of most NPs to biomedical problems has been limited by their high toxicity, poor biodegradability, and scarcity of functional groups for surface modification [109,114,115]. If these limitations are addressed successfully by modern research, there may be an immediate increase in the frequency of NPs being implemented in clinical settings.

Protein-based nanoparticles (PNPs) do not suffer from the aforementioned drawbacks [106]. For this reason, all future discussion will regard PNPs. A PNP, termed GlowDot, is the model system that will be the focus of this mechanistic study on the controlled, large-scale
synthesis of PNPs. GlowDots are made from bovine serum albumin (BSA) protein, a nontoxic, biodegradable, and affordable reagent, also approved by the United States Food and Drug Administration (FDA) for medical applications [116]. BSA’s biological function is to shuttle steroids, lipids, hormones, and other molecules capable of hydrophobically binding with it [117]. This hydrophobic binding can also be taken advantage of to form complexes with fluorescent probes or therapeutic drugs [118-122]. Abraxane, for example, is an albumin-bound paclitaxel complex that has been FDA approved for the treatment of early-stage lung cancer [123]. BSA has 99 carboxyl groups and 59 amine groups that are capable of reacting with other BSA molecules via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry to form GlowDots [124]. The number of noncovalent binding sites for biomedically relevant substrates in a single BSA molecule is therefore multiplied in GlowDot. The unreacted functional groups remaining on the surface of GlowDot can additionally be exploited for surface modification. As shown previously, labeling the surface of GlowDot with up to two-hundred fluorophores yielded a single PNP with advanced fluorescence properties [124]. The populations of binding sites or reactive functional groups required to support advanced functionality depend on the size of the PNP [107-110,118]. Control over the synthesis to afford PNPs of a desired size is therefore essential for manipulating their properties and dictating specific biomedical applications.

The ascribed particle size to property correlation necessitates the development of reliable synthetic methods to attain fine control over the size of PNPs. Precise and predictable control over Glowdot’s size was not established by the previously reported bulk mixing method [124], an outcome that is all too familiar for many synthetic approaches to making NPs [104-111,115,116,124,125]. A different approach is warranted for preselecting a desired particle diameter and then dependably producing it for a prescribed application. Previous successful
attempts to control the size of PNPs involve post-synthetic treatment, but this extra step typically requires the use of toxic solvents, high temperatures, or high-cost procedures [124,126-128] To circumvent these arduous steps, control over the PNP synthesis itself has been attempted using unreliable and impractical techniques. These techniques perform the synthesis in bulk reaction vessels, microfluidic reactors, or microemulsions under varying conditions, such as temperature, ionic strength, reagent concentration, and pH [128,129]. The preparation of calcium-loaded BSA PNPs at varying pH values by a modified desolvation method, for example, gave particle sizes with a standard deviation above 35% [129]. On the other hand, microfluidic reactors do offer precise particle size control, but they are impractical because their low throughput limits the production of PNPs, unless a series of reactions are run in parallel [130]. Microfluidic reactors are also easily clogged, further limiting the production to milligram scale quantities [130]. Since parameterized bulk methods merely provide general control with poor precision and microfluidic reactors are unable to produce large quantities of PNPs [123,127,130], a robust method for controlling the size of PNPs, as well as producing substantial amounts, is needed.

Largescale quantities of monodisperse PNPs are needed to assess their toxicity, biodistribution, physiological side effects, and effectiveness before they can be widely used for biomedical applications [131,132]. Despite the growing demand to implement PNPs in biomedical practice, few methods to reliably produce them on a scale greater than 50 mg have been developed [130]. The previous bulk mixing approach to prepare GlowDots succeeded at producing 150 mg, but had little reliability, standard deviations ranging up to 30%, and required post-synthetic annealing at high temperatures [124]. In addition, bulk NP syntheses are not easily scaled up without the loss of size control because the rates of thermal transport and reagent diffusion are significantly affected by changes to the reaction volume and reagent concentrations [133].
Methods have been reported that can produce a gram of particles, but not without sacrificing some ability to control their size. The implementation of most NPs into biomedical applications is therefore restricted by low-throughput, high-waste production, and imprecise synthetic strategies, as well as difficult purification methods, that are not amenable to scale up without challenges [130,134,135]. These restrictions can be avoided by switching from a bulk synthesis platform to a millifluidic reactor.

A millifluidic reactor may allow for the continuous, high-throughput production of monodisperse, functionalizable NPs, usually in gram-scale quantities [130,133]. The production capacity of millifluidic reactors can be scaled up to kilogram levels through their high-speed, high-throughput continuous operation or by running multiple reactions in parallel, thus circumventing the limited production capacity consistent with the bulk and microfluidic approaches [136-138]. The millifluidic reactor also improves the monodispersity of NPs, as its narrow reaction channels facilitate precise laminar mixing, uniform heat distribution, and mass transport due to an increased surface area to volume ratio [135]. In addition, NP syntheses are made facile due to the ease with which millifluidic reactors can be operated. The millifluidic approach has been previously used to prepare largescale quantities of metal NPs and nanocomplexes, for which size control was demonstrated by varying a number of reaction parameters, such as pH, flow rate, channel diameter, reagent concentration and residence time [128,129,138,139]. The relationships between the reaction parameters and particle sizes deduced from these studies, however, are nonlinear, and provide a low degree of size control and reproducibility. Millifluidic reactors have not yet been implemented to produce large-scale quantities of PNPs with predictable, linear size control.

The millifluidic approach reported herein is the first of its kind to synthesize PNPs, and it allows for the production of GlowDot in up to kilogram quantities, while maintaining linear,
nanometer-resolution size control over particle diameter. In the development of this millifluidic method, we drew inspiration from astrophysics to hypothesize that PNP growth might follow a natural phenomenon known as the planetary accretion model [140,141]. That is, a planet grows when a disk of radially distributed particles begins to condense around a relatively massive core due to its gravitational pull. Such accretion is also responsible for the formation of fullerenes in space [142], among other carbon-based nanomaterials [142-144]. The originality of our approach lies in its ability to emulate the phenomenon of accretion on a scale below 100 nm to produce definite, controlled sizes of PNPs. The planetary accretion model suggests that the size of the planet formed is related to the amount of protoplanetary dust confined to the accretion disk [144]. Similarly, the size of the PNP might be related to the density of reagents in the accretion disk. This accretion density was manipulated to exert kinetic control over the nucleation and growth phases of PNP formation.

The EDC-mediated crosslinking was controlled by varying the diameter of the reaction channel and the concentration of BSA and EDC, hence the accretion density. This approach did not boast vague, nonlinear, size control, only capable of producing polydisperse PNPs with an average size far off the expected mark. By varying the parameters described above, the density of the accretion disk was systematically perturbed to obtain linear trends in the resulting GlowDot diameter. Our robust millifluidic method provides one with the ability to predictably synthesize large amounts of a desired PNP size, namely GlowDot, according to a linear fit, with a standard deviation less than 3.0 nm. The high precision of this method in controlling PNP size can then translate into fine control over its properties if bound or functionalized with biomedically relevant substrates. Therefore, this high-throughput, millifluidic approach would be indispensable for supplying biomedical researchers with the large quantities of size-specific testing materials needed.
to help bring PNPs into everyday applications, thus making bionanomedicine much more accessible.

4.2. Experimental

**Materials.** Bovine serum albumin (> 98%) (fatty acid) was purchased from Equitech Bio. (Kerrville, TX). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (> 98%) was purchased from TCI America (Portland, OR). Agarose (type I, low EEO), sodium phosphate dibasic (> 99%), and Perylene (Pery) (> 99%) was purchased from Sigma Aldrich. Tris base (> 99.8%) was purchased from Fisher Scientific. Sodium chloride was purchased from Avantor Performance Materials (> 99%).

**Millifluidic Reactor Assembly.** An NE-1800 8 channel syringe pump from New Era Pump Systems Inc. was used as the millifluidic reactor for all syntheses. 10 mL syringes from BD were used to eject the reagents. Ferrules, fittings, and junctions from Fisher Scientific were used to connect the reactor channels. The reactor channels (0.034" ID X 0.066" OD, 1/16" ID X 1/8" OD, 3/32" ID X 5/32" OD, 1/8" ID 3/16" OD, 5/32" ID X 1/4" OD) made of PTFE, were purchased from Fluorotherm. Amicon centrifugal filters were purchased from EMD Millipore (Billerica, MA).

**Synthesis of GlowDot in the Millifluidic Reactors.** BSA (varying concentrations) was dissolved in deionized water. EDC (varying concentrations) was dissolved in deionized water. The reagents were added into their own syringes and fastened to the millifluidic reactor. The syringes were pumped at a constant rate of 0.5 mL min⁻¹, and the reagent streams met each other at the same time across a Y-junction. The Y-junction flowed into the reaction channel (varying dimensions). The pumping was stopped at the desired volume for the reaction. The reaction was allowed to
reach completion (5 h), after which the contents of the reaction channel were drained, and then diluted with two times the volume of 10 mM phosphate buffer, pH 9.

**Dynamic Light Scattering.** GlowDot was diluted to 2.0 mg mL\(^{-1}\) with 100 mM phosphate buffer, pH 7. All samples were filtered with a 0.22 μM filter (PVDF, 13 mm, Restek) and measured in 1 cm x 1 cm plastic cuvettes. The hydrodynamic diameter of GlowDot was measured by photon correlation spectroscopy with Precision Detectors (Varian Inc., part of Agilent Technologies), and CoolBatch+ dynamic light scattering apparatus, with a 658 nm excitation laser source and a 90° geometry. Each of the measurements was carried out at room temperature for 1 s, 10 repetitions with 100 accumulations each. Precision Elucidate v 1.1.0.9 was used to collect the data and Precision Deconvolve v 5.5 was used to analyze the data.

**Purification.** GlowDot was diluted to 2.0 mg mL\(^{-1}\) with deionized water and then centrifuged against a 10 kDa Amicon centrifugal filter at 13,300 rpm for 15 min. The purified GlowDot was diluted back to its initial volume and concentration with deionized water.

**Transmission Electron Microscopy.** Purified GlowDot samples were diluted with deionized water to 35 µg mL\(^{-1}\) and drop-cast onto a TEM grid (TED PELLA, Inc., CA: Ultrathin carbon film supported by a lacey carbon film on a 400 mesh copper grid. Product No. 01824). The sample was then stained with 1% uranyl acetate (filtered through 0.22 μm filter before use) and excess stain was removed by dabbing with Whatman 1 filter paper. The sample was dried at room temperature before being taken for imaging. An FEI Tecnai T12 S/TEM was used for imaging with an accelerating voltage of 100 kV.

**Agarose Gel Electrophoresis.** Agarose gels were prepared by dissolving agarose (0.5% w/v) in heated 40 mM Tris-acetate buffer, pH 7. The agarose solution was poured into an
electrophoresis apparatus (Gibco model 200, Life Technologies Inc., MD) and 40 mM Tris-acetate buffer, pH 7, was used as the running buffer. Purified GlowDot samples (0.5 mg mL\(^{-1}\)) were loaded into the wells at the center of the gel in 50% (v/v) loading buffer (50% v/v glycerol, 0.01 m/m bromophenol blue. Electrophoresis was carried out for 30 min at 100 mV. The gel was stained (0.02 m/m Coomassie Blue, 10% v/v acetic acid) overnight and then destained (10% v/v acetic acid) overnight.

**Zeta Potential.** A Brookhaven Zeta Plus instrument capable of laser doppler velocimetry was to measure the zeta potentials of purified GlowDot samples (1.5 mg mL\(^{-1}\) GlowDot, 1.6 mL, 0.2 mM phosphate buffer, pH 9, 12.3 μM KCl) in a 1 cm x 1 cm plastic cuvette. The zeta potential values were obtained through the Smoluchowski fit by software supplied by the manufacturer.

**Preparation of TTA-UC GlowDot.** 1 mL 300 mg mL\(^{-1}\) solution of purified GlowDot was placed in a microfuge tube and stirred overnight with 3 mg of solid Pery and 0.1 g of solid diiodo-bodipy (2, defined previously). The resulting mixture was purified 200 μL at a time by centrifuging against a 10 kDa Amicon centrifugal filter at 13,300 rpm for 15 min. 60 μL of purified GlowDot/2/Pery solution was collected, and diluted up to a 3 mL volume with dH2O.

**Absorption Spectroscopy.** Absorbance spectra were recorded by a HP 8450 diode array spectrophotometer (Varian Inc.). For each absorption measurement, the baseline between 700 nm and 900 nm was averaged and then subtracted over the range. All samples were measured under aerobic conditions in a 1 cm x 1 cm glass cuvette at room temperature.

**Steady-state Spectrophotometry.** Absorbance spectra were recorded by a HP 8450 diode array spectrophotometer (Varian Inc.). For each absorption measurement, the baseline between 700 nm and 900 nm was averaged and then subtracted over the range. Fluorescence spectra were
recorded by a FLS1000 fluorescence spectrophotometer (Edinburgh Instruments) under lamp excitation. All samples were measured under aerobic conditions in a 1 cm x 1 cm glass cuvette at room temperature.

**Transient-Absorption Spectroscopy.** The transient absorption setup was based on Edinburgh Instruments LP920. The excitation pulse at 532 nm was provided by a Nd:YAG-pumped laser (Continuum Surelite I) with a 10 Hz repetition rate and 5-7 ns pulse width. The energy of the pump beam was adjusted between 1 mJ and 25 mJ depending on the samples. The probe beam was supplied by a Xe Model 920, 450 W xenon arc lamp. A monochromator, with a P928 photomultiplier detector (Hamamatsu), was used for decay measurements. An iCCD camera (Andor Technology) was used for spectral measurements. All samples were measured under aerobic conditions in a 1 cm x 1 cm glass cuvette at room temperature.

**Statistical Analysis.** All particle sizes measured via DLS are reported as the average of triplicate syntheses, and the standard deviation (error bars) describes the reproducibility of obtaining a particular particle diameter. The particle diameter determined via TEM is the average of the diameters across X sample size, and the standard deviation describes the variety of diameters present in the sample. The average of three zeta potential measurements on a given sample is reported with the standard deviation of the measurements.

**Continuous Flow Dynamics Simulations.** The Y-junction was built in COMSOL, with varying reaction channel diameters (0.86, 1.68, 2.16, 2.38, 3.30, 4.00, 10.0). Time-independent laminar flow, transport of concentrated species, and reacting flow studies physics were added to the study. The fluid properties were that of water. The flow velocity at each inlet was 0.5 mL min⁻¹. The transport properties were kept at default values and mixture-averaged convection was taken
into account for. The reacting flow physics were kept at default, except for the fluid flow setting, which was switched to laminar.

4.3. Design of the Millifluidic Reactor

An affordable, commercially available, programmable syringe-pump flow reactor was used in this study to facilitate the high-throughput, precise mixing of two reagents, BSA and EDC, into a millifluidic channel (Figure 4.1.). The result of this approach was the large-scale synthesis of monodisperse GlowDots having a desired size. The driving force of the millifluidic reactor originated from the mechanical motion of a motor operating about a threaded iron rod. The motor could traverse the iron rod, either backwards or forwards, to withdraw or dispense liquid from syringes, respectively. Flow rates between 0.1 mL min\(^{-1}\) and 19.4 mL\(^{-1}\) were possible. The millifluidic reactor was assembled from commercially available syringes, polyethylene joints, junctions, ferrules, and polytetrafluoroethylene (PTFE) tubing with diameters on the millimeter scale. The material of the tubing had a significant effect on the outcome of the GlowDot synthesis (Figure 4.2.). TYGON polyvinyl tubing was inadequate for use in this millifluidic system because protein adsorption onto the polymer might have disrupted the mechanism of size control, proposed later. The synthesis of GlowDot in the millifluidic reactor was initiated by the mixing of two solutions, a BSA solution and an EDC solution that mediated the crosslinking of BSA. The reaction was set up such that BSA and EDC solutions, initially located in separate syringes, were ejected by the flow reactor through a Y-junction and into the millifluidic reaction channel (Figure 4.1.).
Figure 4.1. The millifluidic reactor used to mass-produce size-controlled, monodisperse GlowDot.  
(A) A full picture of the millifluidic reactor for GlowDot synthesis. The components of the reactor in which reagent flow took place are labeled. (B) Close-up pictures of the millifluidic reactor setup from a bird’s-eye view. The length of the reaction channel could be varied depending on the desired volume of the synthesis. (C) Diagram of the millifluidic reactor that illustrates the flow of reagents throughout the reactor while under operation. The synthesis could be operated continuously over 6 hours at a flow rate of 0.5 mL min$^{-1}$ or the flow could be stopped when the reagents were depleted.
Figure 4.2. Relationship between GlowDot particle size and reaction channel size, when channels made from TYGON were used. The TYGON reaction channels had inner diameters of 0.79, 1.58, 3.18, 4.76, 6.35, and 9.53 mm. For all syntheses here, the injected BSA concentration was 300 mg mL\(^{-1}\), while the injected EDC concentrations was 83 mM, pH 7. The GlowDots were made and measured in triplicate. GlowDot diameters were measured via DLS in 100 mM PBS, pH 7.0, 500 mM NaCl.
The two reagent streams converged at a Y-junction, but turbulent mixing did not occur. Rather, a characteristically laminar flow was established, in which reagent streams met without lateral mixing. For a flow rate of 0.5 mL min\(^{-1}\) and for reaction channels with a diameter of 4.0 mm or below, the flow between both reagents was found to be in the laminar regime according to COMSOL Multiphysics continuous flow dynamics simulations (Figure 4.3.). Reactions run under these specifications also had calculated Reynolds numbers well within in the laminar regime, below 2,100 [145,146]. The mixing between the two parallel reagent streams occurred primarily by diffusion due to the flow being in the laminar regime. During typical experiments, the flow reactor was allowed to operate for 1 minute, ejecting 0.5 mL of each reagent into a single reaction channel at a flow rate of 0.5 mL min\(^{-1}\). The small volumes of reagents that we used can, of course, be scaled up to a full syringe of any desired volume, but small volumes of reagents were used in this study as it required over 300 millifluidic GlowDot samples for thorough analysis. After both reagent streams were fused at the Y-junction, the reaction mixture was typically allowed to sit in the reaction channel for 6 hours. To proceed with reactions conveniently, the reaction channel was disconnected from the millifluidic reactor and laid on the laboratory bench.
Figure 4.3. COMSOL Multiphysics continuous flow dynamics simulations of reagents mixing at the Y-junction of the millifluidic reactor. A flow rate of 0.5 mL min$^{-1}$ was simulated across the range of reaction channel diameters tested in this study, (A) – (F): 0.86 mm – 4.00 mm, labeled within each simulation. (G) A simulation for reagent mixing in a bulk reaction vessel is also depicted, in which laminar flow was not observed.
During the time that the reaction channel was disconnected, the reactor could be equipped with another PTFE tube to perform consecutive reactions. Multiple reactions could be run in parallel by using the reaming syringe slots of the flow reactor. All opportunities to scale up were considered in our design rationale, so a facile millifluidic synthesis could be easily integrated into industrial settings with the purpose of mass-producing GlowDots, as well as other PNPs, without complications. This millifluidic approach can be accomplished in an industrial setting, alternatively, by letting the reactor operate for 6 hours in reaction channels long enough to flow kilogram quantities of reagent. Throughout this study, we demonstrated the ability of our millifluidic reactor to produce large quantities of monodisperse PNPs without the loss of size control, while showcasing its convenience in application to both, laboratory and industrial settings.

4.4. Size-Controlled Millifluidic Synthesis of GlowDot

Herein, we demonstrate that our millifluidic approach can be used to precisely control the size of GlowDot, and that it may also be suitable for application to a number of different PNP syntheses. The covalent crosslinking between multiple BSA molecules that occurs in the presence of EDC (Scheme 4.1.) to form GlowDot is a commonly used reaction among many syntheses of PNPs [124,147,149-153]. The investigation of this EDC-mediated crosslinking reaction in our millifluidic reactor provides guidelines to attain size control in the synthesis of other PNPs. At a flow rate of 0.5 mL min$^{-1}$, the timescale of reagent mixing was fast enough (1 minute) for the EDC to be injected into the reaction channel before significant hydrolysis. The iso-urea by-product produced by this reaction was so small in size compared to the resulting GlowDot that it could be easily removed from the product mixture by ultracentrifugation against a 10 kDa filter or even by industrial purification techniques. EDC-mediated reactions with proteins in solution are therefore
non-problematic on an industrial level, widely applicable to a variety of PNP syntheses, and avoid the use of organic solvents.
Scheme 4.1. Synthesis of GlowDot by the EDC-Mediated Crosslinking of Carboxyl and Amine Functionalities on the Protein Surface

The GlowDot synthesis (A) – (C) is carried out in a millifluidic environment (D).

\(^{a}\)The GlowDot synthesis (A) – (C) is carried out in a millifluidic environment (D).
First, to establish the size control of GlowDot using our method, we had to arrive at trends describing the product particle diameter as a function of the reaction parameters. Before deciding on which reaction parameters to vary, the widely accepted nanoparticle nucleation and growth mechanism was considered, and a variant of this mechanism was theorized based on the accretion model. The parameters that might effect meaningful changes in the product particle size were decided by analyzing the consequences of the accretion model when applied to PNP synthesis in our millifluidic reactor. These parameters were: 1) the diameter of the reaction channel and 2) the concentration of reagents. These ‘accretion parameters’ were chosen because they were directly responsible for forming the so-called accretion disk. By applying the accretion model to our millifluidic approach, a new understanding of PNP formation in millifluidic reactors was arrived at, which is discussed in the following section alongside experimental results. This accretion growth hypothesis was tested by measuring the GlowDot diameter from syntheses in which the accretion parameters were varied. Dynamic light scattering (DLS) was used as the primary method to measure the diameter of GlowDot.

4.5. Mechanism of Accretion PNP Growth

We propose that GlowDot nucleation occurs at the laminar interface between reagent streams, and then uniform growth might proceed (supported by data) by the accretion of BSA molecules, affording nearly monodisperse GlowDots (Figure 4.4.). The accretion growth can be manipulated to linearly control the size of GlowDot. Our accretion model is as follows: First, a GlowDot nucleus with a discrete size initially formed through the crosslinking of BSA molecules at the laminar interface between reagents. After some time, laminar flow broke down, and the reaction became dominated by the diffusion of BSA molecules around the GlowDot nucleus. The diffusion could have been influenced by protein-protein, hydrophobic, and ionic interactions to
form a radial distribution of BSA about the relatively large GlowDot nucleus, termed an accretion disk. Over the 6 h reaction period, rapid radial growth of the GlowDot nucleus then occurred by the EDC-mediated condensation of BSA molecules. Such growth is defined here as accretion growth, a phenomenon that has been artificially facilitated in our millifluidic reactor using proteins and the noncovalent interactions that exist between them. In most cases, the EDC crosslinking and particle growth finished within 5 hours (Figure 4.5.). The reaction time of 6 hours was chosen to allow for particle growth until completion of the reaction. This observation also implied that, while in the laminar regime, the GlowDot size was independent of the flowrate because EDC crosslinking occurred over a longer timescale than the flow needed to combine the two reagent streams. As illustrated in Figure 4.5, the formation of a discrete GlowDot nucleus at the laminar interface was a necessary step in ensuring precise control over the eventual size of the GlowDot product in the millifluidic reactor. If uniform nuclei did not form at the core of the reaction channel, enabled by laminar flow, then a uniform accretion disk would not have been able to form about the scattered nuclei, resulting in the formation of polydisperse GlowDots.
Figure 4.4. Illustration of the proposed reaction mechanism for the formation of GlowDot in the millifluidic reactor. The reaction occurred in two steps, nucleation and accretion growth. In nucleation, GlowDot nuclei are formed at the laminar interface between reagent streams (red = BSA, blue = EDC). An accretion disk formed about the GlowDot nucleus and accretion growth proceeded radially over a 6 h period. This growth was aided by noncovalent interactions between proteins and the growing particle.
Figure 4.5. GlowDot diameter as a function of time. The GlowDots in the 2.16 mm reaction channel were synthesized by injecting 110 mM EDC and 300 mg mL$^{-1}$ BSA. The GlowDots in the 0.86 mm reaction channel were synthesized by injecting 83 mM EDC and 300 mg mL$^{-1}$ BSA. Depending on the EDC concentration, the reactions were complete in 5 h or earlier. 10 mM phosphate buffer, pH 9 was used to inhibit further EDC reaction [124].
Experimental results agreed with this negative consequence. When the reaction was run outside of the laminar flow regime, the resulting GlowDot diameter resembled that of GlowDot produced by bulk mixing with a polydisperse (20-100 ± 10 nm) mixture [124]. Deviations from laminar flow probably caused non-accretion growth to proceed about the nuclei, and GlowDots of all different sizes were produced by the reaction. Therefore, normal, non-accretion NP growth and accretion growth can be distinguished from each other. The mechanism of accretion-controlled PNP synthesis is supported by the fact that the disruption of laminar flow resulted in polydisperse GlowDots, while the occurrence of laminar flow yielded monodisperse GlowDots. It was therefore a requirement for the flow of the two reagent streams to be in the laminar regime for accretion growth to occur, otherwise reliable particle size control could not be established. The negative result obtained from performing the millifluidic reaction outside of the laminar regime, in accordance with the accretion model, indicated that the diameter and monodispersity of GlowDot might depend on the density and uniformity of reagents in the accretion disk. The accretion model was then probed by varying the density of reagents in the accretion disk, which could be accomplished by varying the accretion parameters, channel diameter and reagent concentration.

4.6. Diameter Dependence of GlowDot on Reaction Parameters

To assess the validity of the accretion model, we measured the diameter of GlowDot via DLS resulting from the millifluidic synthesis at a variety of reaction channel diameters. This relationship is shown at a variety of EDC concentrations (Figure 4.6.), using a constant BSA concentration of 300 mg mL⁻¹. The notably high concentrations of BSA compatible with this method are beneficial for easily mass-producing large amounts GlowDot, achievable both in laboratory and industrial settings. At smaller channel diameters, the growth of the GlowDot was described by a sigmoidal curve, which connotes a slow nucleation phase followed by a rapid
growth phase [152]. As described by the Finke-Watsky model, such sigmoidal growth curves represented a two-step cooperative phenomenon, mathematically modeled by the equations below:

\[
\begin{align*}
\text{eqn 21:} & \quad nA + \text{EDC} & \rightarrow & \nB \\
\text{eqn 22:} & \quad A + nB + \text{EDC} & \rightarrow & B_{n+1}'
\end{align*}
\]

where \(A\) is the unreacted BSA molecule, \(n\) is the number of species, \(B\) is the covalently crosslinked nucleus, and \(B'_{(n+1)}\) is the growing nucleus. The GlowDot diameter as a function of EDC concentration at varying channel diameters (Figure 4.6. A), using constant BSA concentration, also clearly depicted the nucleation and growth steps in sigmoidal curves. The first step (equation 21) represented an induction period in which the nucleus slowly formed due to the relatively low diffusion coefficient of BSA and the long diffusion timescale required for BSA to cross the laminar interface. During this period, there was little change in the size of the particle. A sudden increase in slope, relating the diameter of GlowDot to the diameter of the reaction channel, was observed (Figure 4.6.) at the beginning of the second step (equation 22). This increase might have been due to the role of noncovalent interactions in forming the accretion disk. The growing PNP then underwent iterations of BSA accretion, but with progressively lower rate constants because the large size of the growing particle could have sterically inhibited further reaction. This decrease in the rate constant was represented by the plateau region of the growth curves (Figure 4.6.). The steric hindrance was observed particularly in Figure 4.6. A as the GlowDot product failed to surpass a 60 nm limit, even at EDC concentrations higher than an 83 mM, which was when the limit was first reached.
Figure 4.6. GlowDot particle size curves shown at varying reaction channel diameters and EDC concentrations. (A) Relationship between GlowDot diameter and channel diameter (0.86 – 4.00 mm) at varying [EDC] (0 – 110 mM) injected. (B) Relationship between GlowDot diameter and [EDC] (0 – 110 mM) injected at varying channel diameters (0.86 – 4.00 mm). A constant BSA concentration of 300 mg mL\(^{-1}\) was used for this study. GlowDots were made in triplicate and measured via DLS in 100 mM PBS pH 7.0, 500 mM NaCl.
Interestingly, a reflection occurred in the sigmoidal growth trend at combinations of high EDC concentration and small channel diameter (Figure 4.6A). This reflection could be explained by the occurrence of longitudinal reactions. That is, adjacent nuclei might have crosslinked to form large particles from the start, thus quenching the slow nucleation step. A particle that spans the length of the reaction channel formed at high EDC concentrations and low channel diameters due to extensive longitudinal reactions. This extensive particle was actually a solid BSA hydrogel, which could not be measured. These BSA hydrogels form readily in microfluidic reactors, even at lower EDC and BSA concentrations, and clog the microfluidic reaction channels. Thus, the high reagent concentrations used in this high-throughput millifluidic approach further demonstrated our reactor’s tolerance against clogging and its advantage over microfluidic reactors. The most remarkable aspect of this millifluidic approach, however, was that a linear relationship between the GlowDot diameter and the channel diameter existed at a particular EDC concentration (83 mM) that might have allowed for a blend of nucleation, accretion, and longitudinal reaction events to occur simultaneously. The result is that, by taking advantage of this linear relationship, GlowDots of a predicted diameter between 20 and 60 nm can be prepared with a precision of less than ± 3 nm.

The diameter of GlowDot was also measured as a function of BSA concentration at a variety of channel diameters (Figure 4.7.), using a constant EDC concentration of 83 mM. The growth of GlowDot was described by a sigmoidal curve at larger channel diameters, which further supported our nucleation and accretion growth hypothesis. A sudden increase in the GlowDot diameter indicated accretion growth at a critical BSA concentration of 150 mg mL⁻¹. High concentrations of BSA caused rapid accretion growth to occur until plateau was arrived at. This plateau was due to the GlowDot reaching a critical size at which the rate constant for the addition
of BSA was too low for the reaction to continue. However, the trend became less sigmoidal with decreasing channel diameter. A high degree of linearity was achieved at the smallest channel diameter of 0.86 mm, which provided fine control over the diameter of GlowDot from 10 to 27 nm with a precision of ± 2 nm.
Figure 4.7. The relationship between GlowDot diameter and the injected BSA concentration. The BSA concentration was between 50 mg mL\(^{-1}\) and 350 mg mL\(^{-1}\) at varying channel diameters (0.86 – 4.00 mm). The injected [EDC] was kept constant at 83 mM. GlowDots were made in triplicate trials and measured via DLS in 100 mM PBS pH 7.0, 500 mM NaCl.
This progression toward linearity could have been due to the formation of smaller accretion disks under the spatial constraints imposed by smaller reaction channels. It can be reasoned that a smaller channel diameter will contain smaller accretion disks, and smaller accretion disks facilitate less growth of GlowDot. If accretion growth, the second step of the two-step sigmoidal kinetic process, can be limited by decreasing the size of the accretion disk, hence the size of the reaction channel, there may be a greater likelihood for longitudinal reactions to occur as opposed to radial growth. Again, the occurrence of longitudinal reactions will quench slow nucleation and result in predictable, linear NP growth trends as opposed to sigmoidal trends. Microfluidic reactors also have small reaction channels that might be able to accomplish linear size control by the inhibition of accretion, but only over a narrow range. Gram-scale production is severely limited for these devices [147], so they are not optimal for applications that require mass-production. The millifluidic approach is better suited for maintaining size-control while being able to produce large quantities of product. Overall, the linear size control of GlowDot from 10 to 60 nm has been established through our millifluidic approach with a high precision of ±3 nm. Diameter control in the range of 10 to 27 nm was possible by varying the BSA concentration, while control over a large diameter range from 20 to 60 nm was possible by choosing the diameter of the reactor channel. Provided that the diameter of the reaction channel supported flow in the laminar regime, monodisperse PNPs of desired size, within a given range, could be produced by our millifluidic reactor in a variety of commercially available PTFE reaction channels.

4.8. Assessment of GlowDot Dispersity

The degree of GlowDot dispersity was related to the concentration of EDC and BSA that was used for the reaction. A bimodal distribution of GlowDot and unreacted BSA was observed (Figure 4.8.) when the EDC concentration was insufficient for a complete reaction. However, a
unimodal distribution of monodisperse GlowDot was observed when the EDC concentration was high enough for a complete reaction to occur. Thus, the data in Figure 4.7. were obtained using the particle diameters that were present at the highest mass percentage in the reaction mixture. When the BSA concentration was in excess, a bimodal distribution was observed (Figure 4.9.) Thus, the data in Figure 4.7. was obtained using the largest particle diameter present in the reaction mixture. In either case, the GlowDot product could be purified by ultracentrifugation against a filter to remove the unreacted BSA, as well as isourea by-product. The GlowDot product can also be dialyzed against a suitable cutoff membrane to remove isourea by-product, alone. DLS measurements of the purified GlowDot versus the unpurified GlowDot showed no difference in particle’s diameter, within experimental error (Figure 4.10). Although the accuracy of a DLS measurement can be influenced by the presence of impurities [153], this comparison validated that the measurement of unpurified GlowDots, rather than purified GlowDots, was accurate for this extensive study nonetheless.
Figure 4.8. Bimodal and unimodal GlowDot diameter distribution at constant BSA concentration. (A) DLS scan of unpurified GlowDot when made using 300 mg mL\(^{-1}\) BSA and 60 mM EDC in a 4.00 mm diameter reaction channel. (B) DLS scan of unpurified GlowDot when made using 300 mg mL\(^{-1}\) BSA and 110 mM EDC in a 4.00 mm diameter reaction channel. DLS scans of GlowDot were measured in 100 mM PBS, pH 7.0, 500 mM NaCl.
Figure 4.9. Unimodal and bimodal distribution of GlowDot diameter at constant EDC concentration. (A) DLS scan of unpurified GlowDot when made using 250 mg mL$^{-1}$ BSA and 83 mM EDC in a 2.16 mm diameter reaction channel. (B) DLS scan of unpurified GlowDot when made using 400 mg mL$^{-1}$ BSA and 83 mM EDC in a 2.16 mm diameter reaction channel. DLS scans of GlowDot were measured in 100 mM PBS, pH 7.0, 500 mM NaCl.
Figure 4.10. Comparison of unpurified GlowDot to purified GlowDot. (A) DLS scan of unpurified GlowDot (10 accumulations). (B) DLS scan of purified GlowDot (10 accumulations). DLS scans of GlowDot were measured in 100 mM PBS, pH 7.0, 500 mM NaCl.
The degree of precision achieved by the millifluidic method is showcased in Figure 4.11, which are DLS scans for two different syntheses of GlowDot. Both DLS scans showed a unimodal distribution, characteristic of a complete reaction between BSA and EDC, as there was no peak present for the diameter of unreacted BSA, which was 9.8 ± 0.2 nm. The reported diameter of GlowDot obtained from the bulk mixing method was 45 ± 3 nm following heat treatment of the product mixture for 5 min at 85 °C [124]. Within experimental error, the millifluidic approach was proven capable of producing the same-sized GlowDot without post-synthetic heat treatment. There was no need for post-synthetic treatment using this millifluidic approach because the size control and monodispersity were achieved by exerting kinetic control over the growing particle’s ability to accrete BSA. Furthermore, no loss in the monodispersity of the product was noted upon increasing the volume of the reaction, so there is a positive outlook for scaling the production level up to kilogram quantities without impairing the monodispersity of the product. Transmission electron microscopy (TEM) images to accompany these representative DLS scans of differently sized GlowDots are also shown (Figure 4.11.). The TEM images corroborated the accuracy of the DLS measurements, as the images roughly match the average size and the particle distribution shown in the corresponding DLS scans. The shape of the GlowDots depicted in the TEM images was a sphere. 97.6% of GlowDots were spherically shaped, with only 9 GlowDots from a sample size of 373 having a nonspherical or deformed shape. It was possible that the millifluidic method could have exerted spherical shape control over PNP synthesis, as accretion growth is theorized to occur in a radially symmetric fashion. Therefore, the millifluidic approach was effective in controlling the size and the shape of GlowDot up to high levels of monodispersity without additional, inconvenient post-synthetic steps, while at the same time, permitting the production of large quantities of GlowDot.
Figure 4.11. Assessment of GlowDot dispersity by DLS and TEM data. (A) DLS scan of 41 ± 2 nm GlowDot in 100 mM PBS pH 7.0, 500 mM NaCl. Synthesis: 2.38 mm channel, 300 mg mL⁻¹ BSA, 83 mM EDC, pH 7. (B) DLS scan of purified 61 ± 2 nm GlowDot in 100 mM PBS, 500 mM NaCl, pH 7. Synthesis: 4.00 mm channel, 300 mg mL⁻¹ BSA, 83 mM EDC, pH 7. (C) TEM of GlowDot from A. (D) TEM of purified GlowDot from B. Inset (C & D): histogram of particle diameter distribution corresponding to the GlowDots in the TEM images.
4.9. **Characterization of GlowDot**

The electrophoretic mobility of GlowDots as a function of size was demonstrated by agarose gel electrophoresis (Figure 4.12.). The presence of protein in the individual bands was observed after the agarose gel was stained with Coomassie Blue. Similar to the GlowDots produced via bulk mixing [124], the GlowDots produced by the millifluidic reactor migrated slower than BSA in the agarose gel. This analysis was required to ensure that the GlowDots produced via the millifluidic approach were not just similar to the original particle in size and shape, but also in surface properties. It can be concluded that the GlowDots produced by the millifluidic method are of the same identity as those produced by the bulk mixing method. Confirmation of this fact meant that the millifluidic method described here might be applied to other existing PNPs currently produced by bulk mixing methods, without changing the particle identity and properties. The migration of particles in an agarose gel is controlled by their size and charge [154]. The increased size of GlowDot can explain its slow migration compared to that of BSA. Progressively slower migration in the agarose gel was also observed for GlowDots in the order of increasing diameter.
Figure 4.12. Agarose gel (40 mM Tris acetate, pH 7.0) electrophoresis image of BSA and GlowDots followed by staining with Coomassie Blue. The mobility of 9.78 nm BSA (lane 1) is compared to that of the GlowDots, each having varying diameters (lanes 2-5). The GlowDot diameters, labelled within the image, are 60.2, 48.7, 27.3, and 13.8 nm. GlowDots were purified via ultracentrifugation against a 10 kDa filter.
The slow migration of GlowDot could have also been caused by differences in its charge compared to that of BSA. The EDC-mediated crosslinking reaction was expected to not affect the net charge of GlowDot, as the consumption of a positively charged amine group and a negatively charged carboxyl group would yield the same effect on charge balance as if no reaction were to occur at all. However, the isoionic point of the GlowDot could have shifted as its residues became buried within environments that stabilize or destabilize them. These shifts in the isoionic point might be responsible for the charge difference between the GlowDots and BSA. This charge difference, along with the varying sizes between the GlowDots and BSA, provided an explanation for differential migration. The zeta potential of BSA and GlowDots of various sizes were also measured (Table 4.1.) to elucidate their differential mobility as a consequence of charge. The zeta potential of BSA was \(-26.4 \pm 0.8\) mV, while those of all the measured GlowDot sizes were about \(-8\) mV less, which explained the overall slower migration of GlowDots during electrophoresis, in addition to the larger sizes of the GlowDots. However, the zeta potential was roughly constant among the Glowdots of different sizes, within experimental error. The accretion of BSA added charge to the surface of GlowDot, but only at the expense of burying charges that were already on the surface. Therefore, the mechanism of accretion may be similar for the range of GlowDot sizes produced herein, although the extent of accretion can be controlled to afford custom GlowDot sizes. If this millifluidic method is applied to other PNPs, since the concept of accretion growth was similar for a variety of GlowDot sizes, the effects of channel diameter and reagent concentration on controlling the size of NPs might be replicable for similar systems.
Table 4.1. Zeta potentials of GlowDots

<table>
<thead>
<tr>
<th>Diameter (nm)</th>
<th>Zeta Potential (mV)</th>
<th>± Error (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.78</td>
<td>-26.4</td>
<td>0.8</td>
</tr>
<tr>
<td>60.2</td>
<td>-18.2</td>
<td>0.8</td>
</tr>
<tr>
<td>48.7</td>
<td>-19.4</td>
<td>0.9</td>
</tr>
<tr>
<td>27.3</td>
<td>-18.6</td>
<td>0.9</td>
</tr>
<tr>
<td>13.8</td>
<td>-19.2</td>
<td>0.5</td>
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</tbody>
</table>
4.10. TTA-UC GlowDot

While blue emission could not be observed from deaerated BSA/upconverter complexes upon 532 nm, 5 mW laser photoexcitation, blue emission could be observed from deaerated GlowDot/2/Pery complexes. Since GlowDot consists of hundreds of BSA molecules, most of which retain their binding capabilities according to previous studies [124], we believe that hundreds of upconverters may have bound to and saturated the hydrophobic binding sites of GlowDot. While these upconverters are not mobile in the binding pocket, nor completely protected from oxygen, TET may still occur in the GlowDot, permitting weak, long-range TTA-UC to occur. Excitation dependence, $\Phi_{uc}$ efficiency, and lifetime studies have yet to be performed for GlowDot/2/Pery, but suspected blue emission, suspected to be TTA-UC emission, was observed in the steady-state emission spectrum upon photoexcitation at 532 nm (Figure 4.13.). There was an intense TTA-UC emission peak at 390 nm in GlowDot/2Pery, which was odd because the maximum Pery emission typically occurred at longer wavelengths ( $> 476$ nm) in the BSA/SDS hydrogels. It seemed that the BSA environment had a significant effect in polarizing the orbitals of Pery, such that the absorption and emission features were altered and observed at shorter wavelengths than usual.
Figure 4.13. The emission and absorption spectra of diiodo-bodipy (2) and Pery in different environments.
While high enough signal in the transient absorption spectra of GlowDot/2/Pery (Figure 4.14.) could not be obtained to resolve the annihilator triplet peak or determine whether it had changed morphology relative to the BSA/SDS hydrogel, a significant reduction in the intensity of the sensitizer triplet excited state peak was observed, which indicated that the sensitizer was consumed when 2 and Pery were bound to GlowDot together. Therefore, TTA-UC is suspected in GlowDot/2/Pery based on this observed decrease in the sensitizer triplet excited state and the appearance of steady-state blue emission under 532 nm photoexcitation. If the TTA-UC is characterized further with excitation dependence studies, this could be the first instance of TTA-UC observed in a purely protein-based medium and the protein may be a good strategy for performing green-to-ultraviolet TTA-UC for applications in bioimaging and photodynamic cell therapy.
Figure 4.14. Transient absorption spectra of GlowDot/2 (left) and GlowDot/2/Pery.
4.11. Conclusions

In this study, we have reported a robust, millifluidic method capable of producing PNPs in gram to kilogram quantities, namely GlowDot, which also provided control over the size and the monodispersity of the particle. The mechanism for PNP growth in a millifluidic reactor was investigated to elucidate how the size of the reaction channels and concentrations of reagents might enable control over the size of PNPs. We believe that the growth mechanism might follow the accretion model. This accretion model, in relation to PNP synthesis in our millifluidic reactor, was examined by changing the diameter of the reaction channel and the concentration of the reagents across many millifluidic syntheses. However, uniform accretion growth could only proceed if the nuclei of the growing particles were located in the center of the reaction channel. Centralized nucleation was achieved by designing the reactor to facilitate laminar flow. The diameter of the GlowDot produced through the millifluidic method by varying the diameter of the reaction channel and the reagent concentrations followed a sigmoidal growth trend indicative of slow nucleation, proceeded by accretion growth. The sigmoidal trend could also be made linear under particular reaction conditions, which can be taken advantage of to reliably and predictably produce GlowDots that are 10 to 60 nm in diameter with a precision below ± 3 nm. The monodispersity achieved by this millifluidic PNP synthesis method was demonstrated in the DLS scans and TEM images of GlowDot. Furthermore, the GlowDots obtained from the millifluidic synthesis were characterized by agarose gel electrophoresis and zeta potential to show that the GlowDots obtained from the millifluidic method and the GlowDots prepared by the bulk mixing method are indistinguishable. The properties of the original GlowDot were preserved in the GlowDot product obtained from the millifluidic method. Therefore, our millifluidic method might serve as a capable tool for performing the largescale, size-controlled synthesis of other biomolecule-based NPs, while
maintaining their properties. The broad scope of the study presented here, which employed commonly used reagents and the widely-used EDC-mediated crosslinking to produce a model PNP, can easily encompass a variety of NPs already reported in the field, and those that are yet to be imagined. Our millifluidic method was also designed from the perspective of industrial usage and is capable of producing gram to kilogram scale quantities of GlowDots to supply researchers with large amounts of testable NP product. In previous studies, GlowDots and other PNPs have been used for the imaging of cells or the delivery of therapeutic drugs [124,154-158]. For now, our findings aim to support the mass-production of monodisperse PNPs and to provide rational guidelines for tuning PNP sizes, so their desired biomedical applications may be attained.
CHAPTER 5: Future Work

While green-to-blue TTA-UC does not promise a significant increase in solar cell efficiency relative to red-to-blue TTA-UC, red and near-infrared absorbing sensitizers, such as metallo- benzoporphyrins and phthalocyanines, can be incorporated into the BSA/SDS hydrogel in the same way that the sensitizers used in this study to offer high increases in solar cell efficiency. Nonetheless, efficient green-to-blue TTA-UC may still be helpful if used to improve the efficiency of solar cells that operate in greenhouses; since plants absorb a large range of the solar spectrum except for green light, TTA-UC enhanced solar cells that have improved capabilities of absorbing this excess green light and converting it into blue light for absorption by solar cells are extremely desired at the intersection between solar and agrilcultural industries.

Furthermore, for TTA-UC technologies to be practical in solar applications, strong annihilation behavior must be achieved at low excitation intensities, which the BSA/SDS hydrogel did not achieve. While our system did attain high TTA-UC efficiencies due to the dynamics in the multiphasic system, it did not achieve these efficiencies at the same power density as the sun. To further reduce excitation intensity needed for efficient TTA-UC to occur, much work is currently being done [19,25], but it is not yet known how to make a multiphasic system perform TTA-UC with high efficiency at low-power densities under aerobic conditions; the combination of which would make a TTA-UC material highly viable in industrial applications.

Personally, I separately been interested in the following potential ideas: a single molecule TTA-UC rotaxane system that can achieve 100% TTA and TET efficiencies; the use of antioxidants and catalytic oxygen removal cycles in soft matter TTA-UC systems; and the
modification of GlowDot for further advanced functional applications, such as lipid oxidation, aggregation-induced emission, and singlet fission.
REFERENCES


