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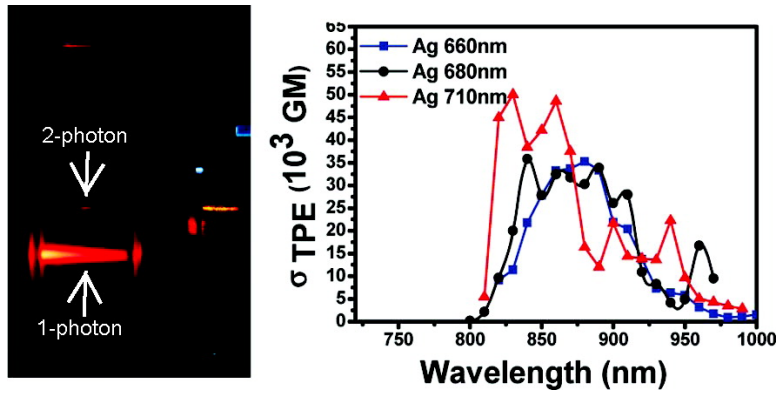

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Water-Soluble Ag Nanoclusters Exhibit Strong Two-Photon-Induced Fluorescence

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The constraints imposed by high sensitivity cellular and medical imaging, especially through tissue, have fueled efforts in developing red and near-infrared (NIR) fluorophores. Ideal for excitation and emission within the optical window from 630−1100 nm, two-photon excited fluorescence offers high spatial resolution owing to its inherent quadratic intensity dependence, while also providing spectral selectivity and improved sensitivity resulting from decreased NIR absorption, scattering, and fluorescence in tissue.[1,2] Many fluorophores have been created and characterized for multiphoton bioimaging.[3] Push−pull organic dyes have been created with high absorption cross sections,[4] but water-soluble organic dyes are plagued by low two-photon absorption (TPA) cross sections and rapid photobleaching.[5] Semiconductor quantum dots exhibit large two-photon excitation (TPE) cross sections[6] but pose problems due to their large physical size and toxicity concerns.[7,8] Recently, gold clusters and nanoparticles ($Au_{\geq 38}$) have been reported with high TPA cross sections, but their solubility in hexane and low total fluorescence currently limit application as biolabels.[9]

Here we present a new class of two-photon dyes with high TPE cross sections, providing bright, photostable emission with versatile tunability of excitation and emission wavelengths. We have previously reported sequence-dependent oligonucleotide encapsulation of silver clusters to selectively create emissive species in the blue, green, red, and near-IR,[10] that also have utility as intracellular fluorophores.[11,12] Further refining microarray experiments have enabled creation of three distinct red/NIR-emitting species (Figure 1). These nanoclusters all exhibit indistinguishable hydrodynamic radii and yield spectrally pure solutions emitting at 660, 680, or 710 nm, after synthesis according to a previously described procedure.[10] Two-photon excitation (150 fs, 80 MHz, 680−1040 nm, Coherent Mira) yields emission indistinguishable from one-photon excitation (OPE) (Figure 1).

A function of TPA cross section, quantum yield, concentration, and excitation intensity, two-photon excited fluorescence was recorded on a CCD camera (Newton, Andor) through a monochromator. Quadratic excitation intensity dependent regions were identified for each Ag nanocluster compared with excitation and TPE fluorescence from reference dyes[3,13,14] (Figure 2). Accounting for concentration and quantum yield differences, the ratios of emission intensities in quadratically dependent excitation intensity regions enabled the calculation of cross sections. As OPE and TPE emission energies and the fluorescence lifetimes are indistinguishable for each nanocluster emitter (Table 1), one- and two-photon-excited quantum yields were treated as being the same for each species. Each nanocluster cross section measurement was referenced to two separate reference dyes (rhodamine B,[13] rhodamine 6G,[3] fluorescein (pH = 11),[3] or Cy5,[14] Table 1) to provide a cross-reference for accuracy, and measured TPE brightnesses further validate OPE and TPE Φ_F's being the same. To avoid dark state residence in either the nanodots or the reference dyes, all cross section measurements were performed at 8 kHz, ensuring that all molecules have returned to the ground state before the arrival of the next excitation pulse.

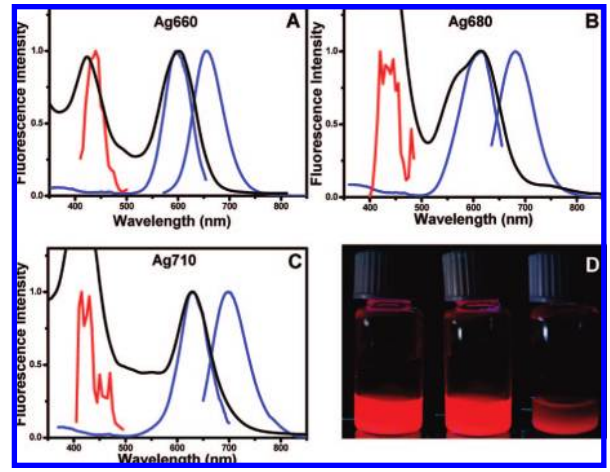

Figure 1. OPE and fluorescence spectra (blue), TPE spectra (red), and absorption spectra (black) of (A) 660 nm emitting species created in 5′-CCCATATTCCCC-3′; (B) 680 nm emitting species created in 5′-CCCTATAACCCC-3′; (C) 710 nm emitting species created in 5′-CCCTAACTCCCC-3′. (D) Picture showing from left to right the OPE emission of the 660, 680, and 710 nm species

As shown in Figure 2C, the cross section for the 660 nm emitter peaks at 35 000 Goppert−Mayer (GM) units (10^{-50} cm^4photon/s), while the 680 nm emitter peaks at 34 000 GM, and the 710 nm emitter reaches 50 000 GM. These cross sections are close to the value of water-soluble quantum dots (66 000 GM),[6] with brightnesses far exceeding that observed for the best water-soluble two-photon dyes.[5] Ag nanoclusters, however, not only are much smaller than quantum dots but also do not exhibit the heavy metal toxicity. Two-photon fluorescence correlation spectroscopy experiments confirm that the hydrodynamic radii of Ag660, Ag680, and Ag710 (all ∼2.3 nm) are smaller than that for Cy-5 labeled 12-mer DNA (2.7 nm).

For each of the species, an excitation spectrum was measured and calibrated for the cross section measurement in GM units. The excitation maxima are blue-shifted with respect to the OPE peaks (Figure 1), indicating that TPE accesses a higher excited electronic state than does OPE. The OPE excitation spectra of the three species all show, in addition to the primary electronic transitions at or beyond 600 nm, much less efficient excitation at higher energy that overlaps with the doubled TPE transition energy (Figure 1). Although OPE and TPE transitions typically exhibit different selection rules,[15] the absorption, OPE, and TPE spectra suggest that the selection rules for this transition are loosened, and the clusters can be directly excited to this high energy state.

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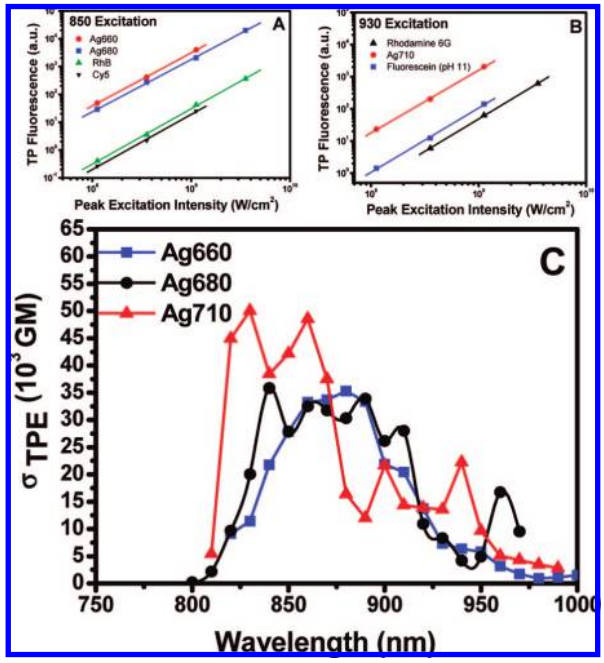

Figure 2. (A and B) Excitation intensity dependence taken at 8 kHz repetition rate, plotted on a log−log scale, along with references rhodamine B and Cy5 (for A), and rhodamine 6G and fluorescein (for B). (C) TPE cross sections as a function of wavelength for the three species. The slopes of the fitted lines are 1.9 ± 0.1 for Ag660, 1.9 ± 0.1 for Ag680, and 2.0 ± 0.1 for Ag710.

Table 1. OPE and TPE Photophysical Values of Ag Nanoclusters

	TPE ex. max (nm)	σ (GM)	Φ	τ_{OPE} (ns)	τ_{TPE} (ns)	R_{hd} (nm)
Ag660	880	35 300	0.18	3.0 ± 0.02	3.1 ± 0.02	2.3
Ag680	890	33 900	0.37	3.0 ± 0.02	3.0 ± 0.02	2.3
Ag710	830	50 000	0.31	3.5 ± 0.02	3.4 ± 0.02	2.3
RhB	840	210	0.31	1.8		0.7
Cy5-DNA	785	400	0.27	1.0		2.7
Fluor	930	24	0.95	4.0		
R6G	700	150	0.95	4.1		

Though the Ti-sapphire range does not permit us to directly excite at half the transition energy of the primary OPE transition for the emitters reported here (i.e., ≥1150 nm excitation), we excited another emitter (540 nm excitation, 620 nm emission,[10] Figure 3), much closer to one-half the OPE excitation energy. No preresonance by TPE was observed upon approaching one-half the OPE transition energy. Although high energy overlap in OPE and TPE spectra exists, direct TPE into the primary OPE peak yields no observable fluorescence, suggesting that transitions may be doubly enhanced by both intermediate and final state resonances.

According to theoretical sum rules,[16] the fundamental limit of the TPA cross section can be estimated as a function of the number of participating electrons, surrounding refractive index, and the energy of the OPE and TPE transitions. For a system resonant only with the final two-photon state, the limit is ~210 000 GM for a spherical, five-electron system with $n = 1.5$ (considering the higher index of the surrounding DNA[17]). Though the number of participating electrons N is not precisely known, previously reported results suggest that $N \leq 5$,[18,19] which would lead to $\sigma^{TPE}/\sigma^{TPE}_{max} \geq 0.24$. Push−pull organic dyes typically exhibit values of <0.01.[16] The free electrons of Ag clusters, however, exhibit large polarizabilities

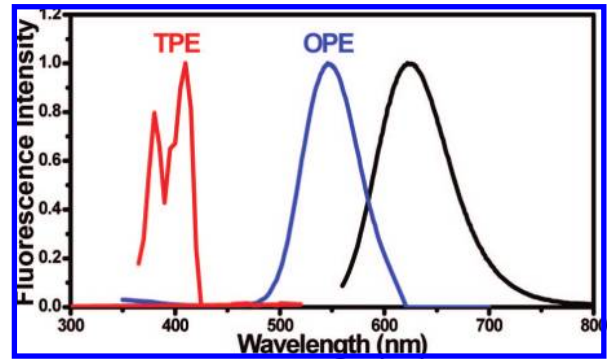

Figure 3. OPE and TPE (doubled in energy) spectra are shown along with the emission spectrum (black) for 620 nm emitting Ag nanoclusters.[10] Only a small portion of the OPE overlaps with the TPE spectrum (out as far as 1040 nm), but no overlap with the principal OPE peak at 540 nm is observed.

through free movement of electrons within the cluster,[20,21] possibly accounting for more efficient two-photon absorption per electron.

These Ag nanoclusters exhibit TPE cross sections in buffer far surpassing all known water-soluble fluorophores and are comparable to much larger quantum dot cross sections. With some of the largest water-soluble TPE action cross sections known, the single point of attachment, small size, and excellent one- and two-photon brightness, metal clusters hold great promise as high sensitivity biolabels.

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Supporting Information Available: Full author lists for ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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