Supporting Information

Non-Toxic Polymeric Dots with the Strong Protein-Driven Enhancement of One- and Two-Photon Excited Emission for Sensitive and Non-Destructive Albumins Sensing

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**PDs’ notations**

Two hydrophilic fractions of acetone-derived PDs are considered in this work:

- **C1Na** denotes PDs received from sodium hydroxide-mediated (NaOH) synthesis;
- **C1K** corresponds to PDs fabricated using potassium hydroxide (KOH) as a catalyst.

More details regarding the synthesis and purification processes along with fundamental characterizations of their structural and linear optical properties.\(^1\)
Absorption and fluorescence spectra of serum albumins

**Figure S1.** Normalized absorption (black) and fluorescence (blue) spectra of native albumins.
Molar mass from gel permeation chromatography

**Figure S2.** Chromatograms of standard proteins and pure PDs.

**Figure S3.** The calibration curve for the calculation of molar masses.

| Sample | M (kDa) |
|--------|--------|
| C1K    | 2.07   |
| C1Na   | 2.18   |
Two-photon excited fluorescence of free PDs

Figure S4. TPE FL spectra of pure PDs upon excitation in the wide wavelength range.

Figure S5. Excitation-dependent TPE fluorescence of pure PDs. Correlations between OPE and TPE wavelengths and emission maxima.

Figure S6. Log (I_{TPE FL}) vs. log (P_{laser}) for pure PDs with the slope values.
Figure S7. OPE (lower) and TPE (upper) excitation-emission maps of free PDs.
**Table S2.** Two-photon absorption cross-section values of fluorescein in alkalic solution as reference sample – results were reported by Makarov et al.²

| Excitation wavelength (nm) | Two-photon absorption cross-section (GM) |
|----------------------------|------------------------------------------|
| 720                        | 21                                       |
| 740                        | 30                                       |
| 760                        | 45                                       |
| 780                        | 45                                       |
| 800                        | 36                                       |
| 820                        | 28                                       |
| 840                        | 13                                       |
| 860                        | 12                                       |
| 880                        | 12                                       |
| 900                        | 12                                       |
| 920                        | 14                                       |
| 940                        | 15                                       |
| 960                        | 16                                       |
| 980                        | 16                                       |
Photobleaching resistance

**Figure S8.** Changes in the fluorescence intensity as a function of laser irradiation time.
Enhanced one-photon excited fluorescence of PDs

**Figure S9.** Evolution of BSA-induced enhanced OPE fluorescence of C1K-including systems in aqueous media (Mill-Q) as representative examples. Three excitation wavelengths are also indicated.
Figure S10. Evolution of peak positions in albumin-induced enhanced OPE fluorescence of C1K-including systems in different aqueous media: Milli-Q (red), cacodylate buffer (blue), and PBS (green). Each excitation wavelength is plotted separately.
Figure S11. Evolution of peak positions in albumin-induced enhanced OPE fluorescence of C1Na-including systems in different aqueous media: Milli-Q (red), cacodylate buffer (blue), and PBS (green). Each excitation wavelength is plotted separately.
Linear optical properties of free albumins

**Figure S12.** The comparison of OPE emission spectra for C1K, C1K-HSA, and free albumins at varying concentrations. The fluorescence intensity values were scaled with respect to the fluorescence signal of free PDs.

**Figure S13.** The linear correlation of absorbance values vs. concentrations of HSA.

**Table S3.** The normalized intensity values of C1K-HSA and free HSA.

|                   | Normalized Intensity | Fluorescence maxima (nm) |
|-------------------|----------------------|---------------------------|
| C1K+52.3 µM HSA   |                      |                           |
| 350 nm            | 1.65                 | 0.0766                    | 434 |
| 377 nm            | 1.77                 | 0.0545                    | 449 |
| 450 nm            | 3.01                 | 0.0350                    | 505 |
Enhanced two-photon excited fluorescence of PDs

Figure S14. Evolution of protein-induced enhanced TPE fluorescence of C1Na-including systems. Three excitation wavelengths are also indicated.
Figure S15. Evolution of protein-induced enhanced TPE fluorescence of C1K-including systems. Three excitation wavelengths are also indicated.
Figure S16. Log I vs. log P for protein-nanostructure assemblies.
The fluorescence enhancement coefficients

The fluorescence enhancement coefficients were estimated from OPE and TPE emission spectra, according to the following equation:

\[
FEC(\lambda_{exc}) \approx \frac{100\%}{I_{free PDS}} \cdot \frac{I_{complex} - I_{free PDS}}{I_{free PDS}}
\]

where \(FEC(\lambda_{exc})\) denotes the fluorescence enhancement coefficient (%), \(I_{complex}\) and \(I_{free PDS}\) represent the integrated fluorescence intensity for a protein-nanostructure complex and unbound PDs (a.u.), respectively.

Table S4. The FEC parameters (%), calculated from OPE fluorescence spectra for samples in Mili-Q, cacodylate buffer, and PBS.

| Sample   | 350 nm       | 377 nm       | 450 nm       |
|----------|--------------|--------------|--------------|
| C1K-BSA  | 29.8±1.3     | 34.4±1.4     | 91.2±3.2     |
|          | (36.2±1.4)   | (36.6±1.5)   | (83.2±2.9)   |
| C1K-HSA  | 66.9±2.7     | 73.8±3.0     | 110.2±3.9    |
|          | (58.5±2.3)   | (61.1±2.4)   | (110.6±3.9)  |
| C1Na-BSA | 63.4±2.5     | 64.0±2.6     | 152.6±5.0    |
|          | (51.5±2.1)   | (56.2±2.2)   | (147.3±4.9)  |
| C1Na-HSA | 119.2±6.1    | 131.5±6.0    | 276.3±10.8   |
|          | (93.1±4.7)   | (112.7±5.2)  | (179.5±7.0)  |
|          | (100.5±5.1)  |              | (201.6±7.9)  |

Table S5. The FEC parameters (%), calculated from TPE fluorescence spectra.

| Sample   | 750 nm       | 800 nm       | 850 nm       |
|----------|--------------|--------------|--------------|
| C1K-BSA  | 44.6±2.2     | 66.6±3.3     | 84.2±4.2     |
| C1K-HSA  | 60.6±3.0     | 100.5±5.0    | 136.8±6.8    |
| C1Na-HSA | 68.0±3.4     | 96.4±4.8     | 175.3±8.8    |
| C1Na-BSA | 55.5±3.0     | 88.4±4.6     | 93.8±4.9     |
**Absolute fluorescence quantum yields**

To quantify how albumins improve fluorescence properties of PDs, the absolute fluorescence quantum yields (FQYs) were estimated, as shown below:\(^{3-4}\)

\[
FQY = \frac{S_2 - S_3}{S_0 - S_1}
\]

where $FQY$ is the calculated fluorescence quantum yield (%), $S_2$ denotes the integrated fluorescence intensity of sample (a.u.), $S_0$ and $S_1$ are the integrated intensity of an excitation peak in the absence and presence of a sample (a.u.), respectively, and $S_3$ denotes the background in the emission region (a.u.);

**Table S6.** The FQYs of free and protein-treated PDs.

| FQYs (%)  | C1K  | C1K-BSA | C1K-HSA | C1Na  | C1Na-HSA | C1Na-BSA |
|-----------|------|---------|---------|-------|----------|----------|
|           | 7.20 | 9.58    | 12.4    | 5.70  | 10.5     | 8.55     |
Two-photon absorption cross-section and brightness

**Figure S17.** Molar-weight scaled TPA cross-section spectra of free and protein-including C1Na systems and their corresponding two-photon brightness spectra.
Fluorescence decays

All fluorescence decays were fitted with commercially available analysis software (SPCImage, Becker&Hickl), using the tri-exponential equation as follows:

\[ I(t) = I_0 + A_1 e^{\frac{t - t_0}{\tau_1}} + A_2 e^{\frac{t - t_0}{\tau_2}} + A_3 e^{\frac{t - t_0}{\tau_3}} \]

where \( I(t) \) is the fluorescence intensity (a.u.), \( t \) represents time (ns), \( \tau_1, \tau_2, \tau_3 \) denote lifetime components, and \( A_1, A_2, \) and \( A_3 \) are the relative decay amplitudes.

**Figure S18.** Normalized fluorescence decays of free and protein-including PDs.

**Figure S19.** Evolution of fluorescence decays of PDs as a function of protein concentration. Red and green areas correspond to the error bars.
Figure S20. Stoichiometry determination derived from the OPE fluorescence titrations.
Circular dichroism

UV circular dichroism (CD) spectra of albumins and their corresponding bioconjugates at different luminophore/protein ratios were recorded, keeping constant concentration of albumins. To stimulate the inert environment, the sample chamber was deoxygenated with dry nitrogen, these conditions were held during the experiment. Spectroscopic measurements were performed, following the guidance provided by Norma J. Greenfield. Additionally, pure PDs’ dispersions were also measured to verify their chiroptical activity. Each CD spectrum were averaged after five accumulations. Afterward, as-obtained CD spectra were analysed with the K2D3 software to compute the secondary protein structure of native and bound albumins, as described by Caroline Louis-Jeune et al.
Figure S21. Changes in CD spectra of free proteins upon the titration with PDs dispersions. Evolution of the secondary protein structure as a function of PDs concentration.
Fourier-transform infrared spectra

The attenuated total-reflectance Fourier-transform infrared spectra (ATR-FTIR) of native proteins, pure PDs, and their conjugates were recorded in two different media, such as: Milli-Q and heavy water. All spectroscopic measurements and post-data treatment (incl. blank sample and nanostructure corrections) were performed, as reported in Huayan Yang et al.\textsuperscript{7}

\textbf{Figure S22.} Normalized Amide I and II bands of native proteins and conjugates. The ATR-FTIR spectra in the wavenumber region.
Figure S23. The Gaussian deconvolution of Amide I bands of native proteins and their PDs-including conjugates.
Table S7. Band assignments of peak components and their contribution in native proteins and conjugates.

| Peaks | Sample     | Peak position (cm⁻¹) | Band assignments | Content (%) |
|-------|------------|----------------------|------------------|-------------|
| 1     | BSA        | 1623.5               | β-sheet          | 7.8%        |
| 2     |            | 1636                 | Random coil      | 9.5%        |
| 3     |            | 1654                 | α-helix          | 60.1%       |
| 4     |            | 1674                 | β-turn           | 13.8%       |
| 5     |            | 1688                 | β-antiparallel   | 8.9%        |
| 1     | BSA-C1Na   | 1611                 | Intermolecular β-sheet | 3.5% |
| 2     |            | 1626                 | β-sheet          | 9.6%        |
| 3     |            | 1638                 | Random coil      | 8.4%        |
| 4     |            | 1654.5               | α-helix          | 61.1%       |
| 5     |            | 1679                 | β-turn           | 13.4%       |
| 6     |            | 1694                 | β-antiparallel   | 3.9%        |
| 1     | BSA-C1K    | 1615                 | Intermolecular β-sheet | 2.2% |
| 2     |            | 1623                 | β-sheet          | 5.0%        |
| 3     |            | 1634.5               | Random coil      | 11.4%       |
| 4     |            | 1654                 | α-helix          | 59.4%       |
| 5     |            | 1676                 | β-turn           | 12.0%       |
| 6     |            | 1690                 | β-antiparallel   | 7.5%        |
| 1     | HSA        | 1621                 | β-sheet          | 8.9%        |
| 2     |            | 1637                 | Random coil      | 11.4%       |
| 3     |            | 1653.5               | α-helix          | 64.9%       |
| 4     |            | 1679                 | β-turn           | 9.7%        |
| 5     |            | 1691                 | β-antiparallel   | 5.1%        |
| 1     | HSA-C1K    | 1615                 | Intermolecular β-sheet | 5.1% |
| 2     |            | 1623                 | β-sheet          | 12.6%       |
| 3     |            | 1639                 | Random coil      | 14.5%       |
| 4     |            | 1653                 | α-helix          | 59.9%       |
| 5     |            | 1674                 | β-turn           | 14.9%       |
| 6     |            | 1690                 | β-antiparallel   | 9.0%        |
| 1     | HSA-C1Na   | 1615                 | Intermolecular β-sheet | 4%  |
| 2     |            | 1629.5               | β-sheet          | 12.5%       |
| 3     |            | 1639                 | Random coil      | 9.5%        |
| 4     |            | 1652.5               | α-helix          | 59%         |
| 5     |            | 1675                 | β-turn           | 12%         |
| 6     |            | 1689                 | β-antiparallel   | 8%          |
Figure S24. The GPC chromatograms of albumin and albumin-PDs samples as a function of elution time. Comparison of GPC signals for free PDs and albumin-including composites.

Table S8. The hydrodynamic diameter values of BSA for different compositions, where $D_{\text{hydr.}}$ is the hydrodynamic diameter (nm) and SD denotes the standard deviation (nm).

| Sample      | BSA     | C1Na-BSA | C1K-BSA  |
|-------------|---------|----------|----------|
| $D_{\text{hydr.}} \pm \text{SD (nm)}$ | 8.3±2.4 | 8.6±2.7  | 8.5±2.7  |
Figure S25. The UV-Vis extinction spectra of C1K and C1Na at varying temperature values (top). The normalized temperature-dependent extinction map of C1K (bottom left). The evolution of absorbance values at 240 and 310 nm as a function of temperature (bottom right).
Temperature-dependent one-photon excited emission

Figure S26. The OPE fluorescence spectra of both PDs ($\lambda_{\text{exc.}} = 377$ nm) at varying temperature values as representative examples.
Figure S27. The evolution of the integrated OPE fluorescence intensity of C1Na and C1K-albumins samples as a function of temperature. As-calculated values were scaled by the integrated intensity arising from the emission spectrum at 20°C. The most crucial region for biological applications is highlighted as a yellow area.
**pH effect on the fluorescence activity**

**Figure S28.** Changes in the integrated OPE fluorescence intensity vs. pH value for C1K-including examples (top) and C1Na (bottom).
Molecular interferents vs. OPE fluorescence

**Figure S29.** Variations in the FEC values showing the interferents’ effect.
Selectivity towards proteins

Figure S30. Selectivity of C1K towards various proteins based on the changing FEC values.
NMR characterization of PDs

Raw NMR data were interpreted based on the peaks’ positions in the NMR spectroscopy handbooks, identifying the most crucial sub-units.

$^{13}$C NMR (151 MHz, D$_2$O) $\delta$: 215.4, 181.5, 180.7, 171.0, 69.9, 62.5, 57.4, 49.4, 30.2, 28.0, 27.7, 26.7, 23.2, 16.8

Figure S31. The $^{13}$C NMR spectrum of C1Na. The major sub-groups of PDs are indicated with their peak positions above.
Figure S32. The HSQC NMR spectrum of C1Na. Two areas with the most common sub-groups are indicated with blue and black frames. The heavy water signal is marked with a red cross.
\[^{13}\text{C} \text{NMR (151 MHz, D}_2\text{O)} \ \delta: 181.5, 180.7, 171.0, 69.9, 68.5, 51.2, 49.4, 28.0, 23.2, 20.0, 16.8.\]

**Figure S33.** The \[^{13}\text{C} \text{NMR spectrum of C1K. The three major sub-groups of PDs are indicated with their peak positions above.}**
Figure S34. The HSQC NMR spectrum of C1K. Two areas with the most common sub-groups are indicated with blue and black frames. The heavy water signal is marked with a red cross.
**Albumins’ sensing**

Table S8. LOD values of BSA and HSA.

|            | C1K-BSA | C1K-HSA | C1Na-HSA | C1Na-BSA |
|------------|---------|---------|----------|----------|
| **LOD (µM)** |         |         |          |          |
| 350 nm     | 4.3     | 4.7     | 3.2      | 4.6      |
| 377 nm     | 5.8     | 5.5     | 4.6      | 5.2      |
| 450 nm     | 6.8     | 6.8     | 5.4      | 4.4      |

Figure S35. Representative calibration curves for determination of LODs of HSA using C1K as a probe.
Table S9. Regression curve characteristics, where $a$ denotes the slope of the linear relation between the integrated fluorescence intensity of PDs and the concentration of albumins, $\sigma$ is the standard error of the regression line. Both parameters were rounded.

|       | C1K-BSA | C1K-HSA | C1Na-HSA | C1Na-BSA |
|-------|---------|---------|----------|----------|
|       | $a \times 10^5$ | $a \times 10^5$ | $a \times 10^5$ | $a \times 10^5$ |
|       | $\sigma \times 10^2$ | $\sigma \times 10^2$ | $\sigma \times 10^2$ | $\sigma \times 10^2$ |
| 350 nm | 4.3970  | 5.4857  | 6.7957   | 3.8207   |
|       | 6.3     | 8.6     | 7.3      | 5.8      |
| 377 nm | 4.0056  | 5.6856  | 6.695    | 3.3964   |
|       | 7.8     | 10      | 10       | 6.3      |
| 450 nm | 3.1793  | 2.8880  | 1.6583   | 1.7581   |
|       | 7.2     | 6.5     | 3.0      | 2.6      |
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