# *Metallofluorescent nanoparticles for multi-modal applications*

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## 1. SI Methods. Table of synthesised particles

The following table (Table S1) lists the full set of particles prepared in this work, including the fluorescent and metallic modifications as well as additional modifications.

**Table S1.** Table of particles with different modifications.

| Particle | Size / nm | Dye | Metal | Other modifications |
|---|---|---|---|---|
| **$NH_2$-PEG-$NP_{500}$** | 500 | – | – | PEG spacer |
| **Cy5-PEG-$NP_{500}$** | 500 | Cy5 | – | PEG spacer |
| **Cy5-$PEG_2$-$NP_{500}$** | 500 | Cy5 | – | $(PEG)_2$ spacer |
| **Cy5-$PEG_3$-$NP_{500}$** | 500 | Cy5 | – | $(PEG)_3$ spacer |
| **Fmoc-Lys(Cy5)-$NP_{500}$** | 460 | Cy5 | – | Fmoc-protected lysine |
| **Fmoc-Lys(Cy5)-$NP_{900}$** | 900 | Cy5 | – | Fmoc-protected lysine |
| **Strp-Lys(Cy5)-$NP_{1200}$** | 1200 | Cy5 | – | Streptavidin |
| **A647-PEG-$NP_{500}$** | 500 | A647 | – | PEG spacer |
| **A647-$PEG_2$-$NP_{500}$** | 500 | A647 | – | $(PEG)_2$ spacer |
| **A647-$PEG_3$-$NP_{500}$** | 500 | A647 | – | $(PEG)_3$ spacer |
| **Fmoc-Lys(A647)-$NP_{900}$** | 900 | A647 | – | Fmoc-protected lysine |
| **Fmoc-Lys(A647)-$NP_{2500}$** | 2480 | A647 | – | Fmoc-protected lysine |
| **Fmoc-Lys(A647)-$NP_{5000}$** | 5000 | A647 | – | Fmoc-protected lysine |
| **Pd(II)-Cy5-PEG-$NP_{500}$** | 500 | Cy5 | Pd(II) | PEG spacer |
| **Ni(II)-Cy5-PEG-$NP_{500}$** | 500 | Cy5 | Ni(II) | PEG spacer |
| **Cu(I)-Cy5-PEG-$NP_{500}$** | 500 | Cy5 | Cu(I) | PEG spacer |
| **Pd(0)-Cy5-PEG-$NP_{500}$** | 500 | Cy5 | Pd(0) | PEG spacer |
| **$H_2N$-$NP_{500}$** | 500 | – | – | Glycine |
| **Ac-HN-$NP_{500}$** | 500 | – | – | Acetylated glycine |

## 2. SI Methods. Synthesis and loading of polystyrene nanoparticles

***Polystyrene NPs.*** The synthesis of the starting amino-methyl crosslinked polystyrene nanoparticles (NPs) was carried out following established protocols within the research group NanoChemBio group protocolos.[1-2] Figure S1 shows the dynamic light scattering (DLS) and scanning electron microscopy (SEM) characterization of the polystyrene NPs.

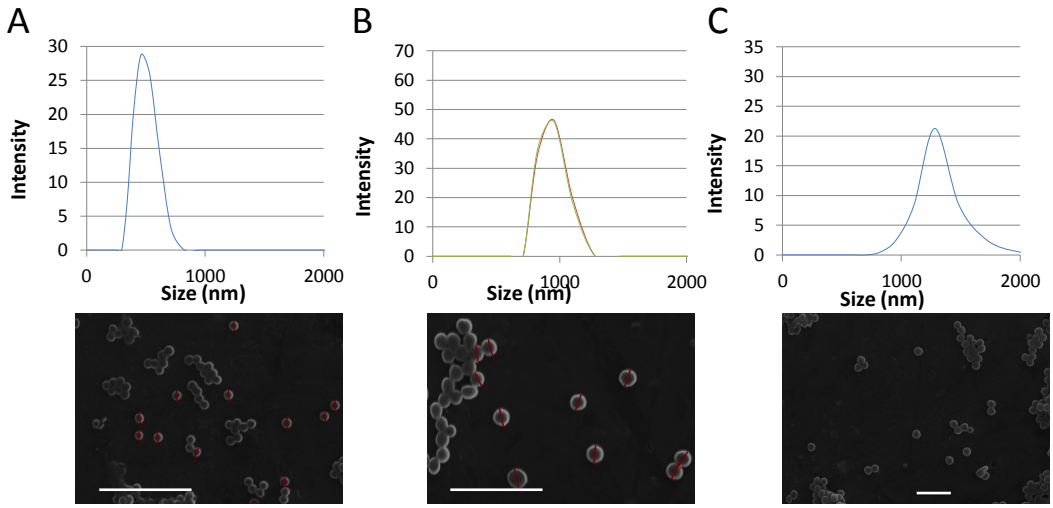
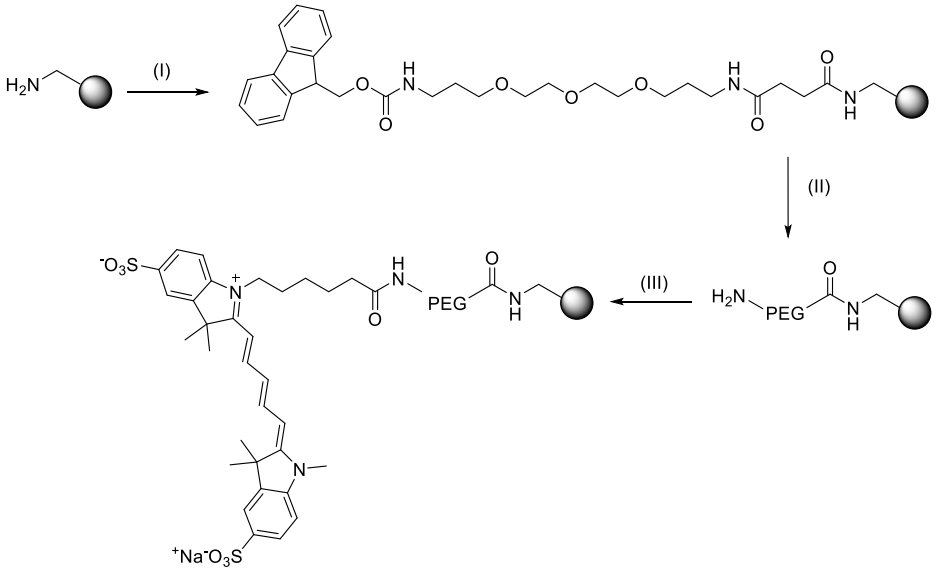



**Figure S1.** DLS (top) and SEM (bottom) characterization of 500-nm (A), 900-nm (B), and 1,200-nm (C) polystyrene NPs. The SEM analysis of, at least, 10 different particles resulted in the following average particle diameter: 490±70 nm (A); 910±110 nm (B); and 1,220±130 nm. Scale bars in SEM images represent 5 μm.

***Sulfo-Cy5-coupled NPs.*** To synthesise sulfo-cyanine 5 (sulfo-Cy5)-coupled NPs, 500-nm NPs (loading: 58 μmol/g, solid contents: 2%) were used. These nanoparticles were first conditioned by washing them three times with 1 mL of dimethylformamide (DMF) each time through suspension-centrifugation (13,400 rpm, 3 min) cycles. Next, to conjugate a polyethylene glycol (PEG) spacer to the NPs, fluorenylmethyloxycarbonyl-4,7,10-trioxa-1,13-tridecanediamine succinamic acid (Fmoc–PEG–OH) (75 equiv.) was dissolved in DMF (1 mL) with oxyma (75 equiv.) and N,N'-diisopropylcarbodiimide (DIC) (75 equiv.). The mixture was stirred at room temperature for 10 minutes. Then, the solution was added to the dry NPs, and the suspension was stirred at 60°C and 1,400 rpm for 2 h. Subsequently, the NPs were washed with three cycles of suspension-centrifugation (13,400 rpm, 3 min) to obtain Fmoc-PEGylated NPs. Fmoc was removed using a 20% piperidine/DMF solution (3 × 20 min) and sequential washing steps, as described above.

Four different sets of Sulfo-Cy5-coupled NPs were synthesised with decreasing concentrations of the fluorophore (Sulfo-Cy5 NHS ester) from a stock solution in DMF (1 mg/mL, 1.313 mM). The different sets were prepared with solutions of the dye at concentrations of 0.1 mg/mL (131.3 μM), 0.01 mg/mL (13.13 μM), 0.001 mg/mL (1.313 μM), and 0.0001 mg/mL (0.1313 μM). Then, 50 μL of each sulfo-Cy5 NHS ester solution were mixed separately with 2 μL of N,N-diisopropylethylamine (DIPEA), and the mixtures were added to the corresponding dry PEGylated NPs and suspended. The suspensions were stirred at room temperature at 1,000 rpm for 14 h in the dark. The following day, the NPs were washed with three cycles of suspension-centrifugation with DMF (13,400 rpm, 3 min), methanol (13,400 rpm, 5 min) and water (13,400 rpm, 8 min), before finally being suspended in water (50 μL). The complete reaction process is shown in Scheme S1.



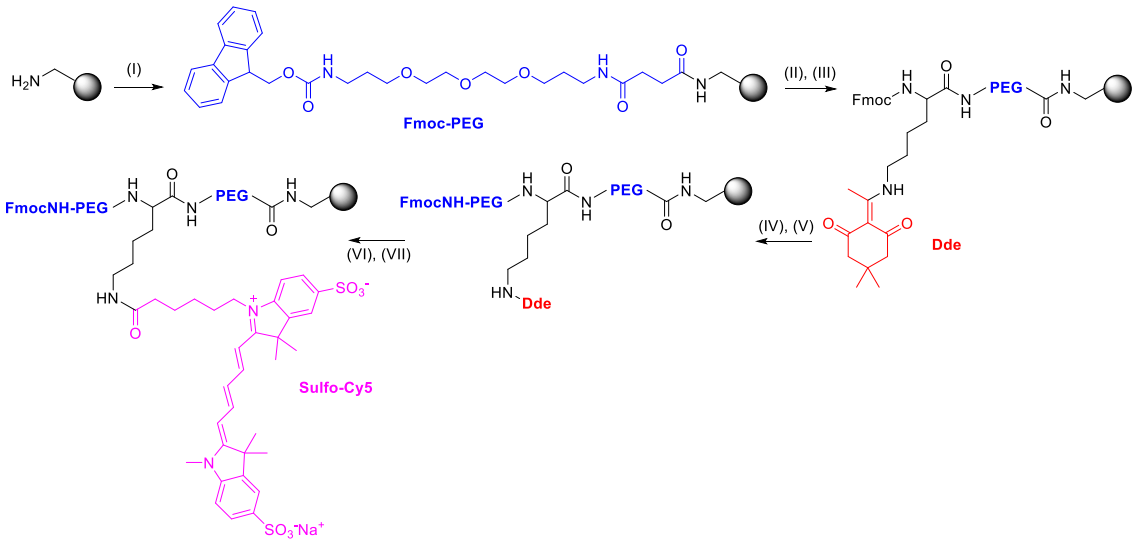

**Scheme S1. Synthesis of sulfo-Cy5-coupled NPs Cy5-PEG-NP$_{500}$.** (I) Fmoc-NH-PEG conjugation to naked aminomethyl polystyrene NPs (DIC/oxyma). (II) Fmoc deprotection (20% piperidine). (III) Sulfo-Cy5 coupling using NHS activated ester.

***Fmoc-Lys(Cy5)-NP$_{500}$, Fmoc-Lys(Cy5)-NP$_{900}$ and Strp-Lys(Cy5)-NP$_{1200}$.*** To synthesise **Fmoc-Lys(Cy5)-NP$_{500}$**, **Fmoc-Lys(Cy5)-NP$_{900}$**, and **Strp-Lys(Cy5)-NP$_{1200}$**, 460-nm NPs (loading: 64 µmol/g, solid contents: 2.5%), 900-nm NPs (loading: 70 µmol/g, solid contents: 3.3%) and 1,200-nm NPs (loading: 35 µmol/g, solid contents: 4%) were used, respectively. These NPs were first conditioned by washing them three times with 1 mL of DMF through suspension-centrifugation (13,400 rpm, 3 min) cycles. Next, to conjugate the PEG spacer to the NPs, Fmoc–PEG–OH (50 equiv.) was dissolved in DMF (1 mL) with oxyma (50 equiv.) and DIC (50 equiv.). The mixture was stirred at room temperature for 10 min. Then, the solution was added to the dry NPs, and the suspension was left to stir at 60°C and 1,400 rpm for 2 h. Subsequently, the NPs were washed with three cycles of suspension-centrifugation (13,400 rpm, 3 min) to obtain Fmoc-PEGylated NPs. Fmoc was removed using a 20% piperidine/DMF solution (3 × 20 min) and sequential washing steps, as described previously.

In the next step, $NH_2$-PEGlylated NPs were added to a mixture of Fmoc-lysine-Dde(OH) (50 equiv.), oxyma (50 equiv.) and DIC (50 equiv.), previously stirred for 10 min at room temperature, and resuspended. The suspension was stirred at 60°C and 1,400 rpm for 2 h. Subsequently, the NPs were washed with three cycles of suspension-centrifugation (13,400 rpm, 3 min) to obtain Fmoc-lysine-Dde-PEGylated NPs. Fmoc was removed using a 20% piperidine/DMF solution (3 × 20 min) and sequential washing steps, as described previously. Then, a mixture of Fmoc–PEG–OH (50 equiv.), oxyma (50 equiv.) and DIC (50 equiv.), previously stirred for 10 min at room temperature, was added to the Fmoc-lysine-Dde-PEGylated NPs, and the suspension was stirred at 60°C and 1,400 rpm for 2 h. Afterwards, the NPs were washed by three cycles of suspension-centrifugation (13,400 rpm, 3 min) to obtain Fmoc-Lys-NPs.

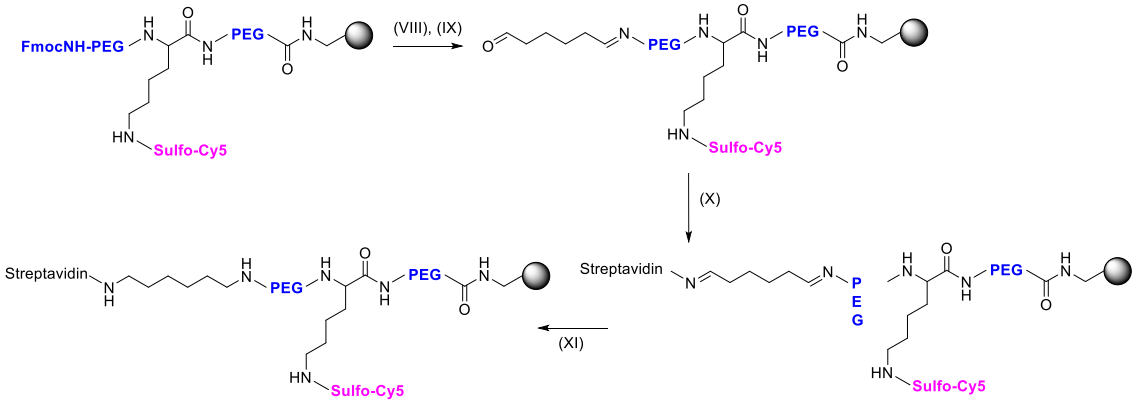
The Dde protecting group was then added by treating the NPs with hydroxylamine HCl (0.4 mmol) and imidazole (0.3 mmol) in a mixture N-methyl-2-pyrrolidone (NMP) and DMF (4:1). Subsequently, the NPs were washed with three cycles of suspension-centrifugation (13,400 rpm, 3 min). Then a sulfo-Cy5 NHS ester solution (0.1 mg/mL, 1.313 mM concentration) was mixed separately with 2 µL of DIPEA and added to the dry Fmoc-lysine-NPs. The particles were suspended and then stirred at room temperature at 1,400 rpm for 14 h in the dark. Finally, the **Fmoc-Lys(Cy5)-NP$_{500}$** and **Fmoc-Lys(Cy5)-NP$_{900}$** were washed with three cycles of suspension-centrifugation

with DMF (13,400 rpm, 3 min), methanol (13,400 rpm, 5 min) and water (13,400 rpm, 8 min), before finally being suspended in water. All the synthesis steps are depicted in Scheme S2.




**Scheme S2. Synthesis of Fmoc-Lys(Cy5)-NPs.** (I) Fmoc-NH-PEG conjugation to naked polystyrene nanoparticles (NPs). (II), (III) Fmoc deprotection and Fmoc-lysine-Dde(OH) coupling. (IV), (V) Fmoc deprotection and Fmoc-NH-PEG conjugation. (VI), (VII) Dde group selective removal and sulfo-Cy5 coupling.

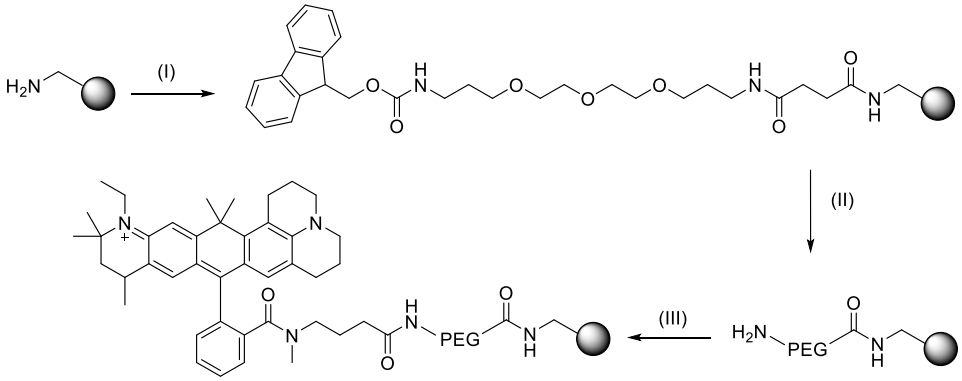
For the **Strp-Lys(Cy5)-NP$_{1200}$** particles, conjugation continued from the Fmoc deprotection step, which was accomplished by the treatment with a 20% piperidine/DMF solution (3 × 20 min) and sequential washing steps, as described previously. Next, the NPs were washed with sequential suspension-centrifugation cycles with phosphate buffered saline (PBS) (pH 7.4), NaOH (500 mM), and water, before the NPs were finally suspended in a 25% (v/v) glutaraldehyde solution in water. The suspension was shaken at 1,000 rpm at room temperature for 15 h. Subsequently, the NPs were washed with sequential suspension-centrifugation cycles with PBS (pH 7.4), and a 1 mg/mL streptavidin solution in PBS, before mixing at room temperature at 1,000 rpm for 15 h. The NPs were then washed with two cycles of suspension-centrifugation with PBS (pH 7.4). Next, the NPs were suspended in a 20 mM solution of $NaBH_3CN$ in PBS:EtOH (3:1), and the mixture was shaken at 1,000 rpm for 2 h at room temperature. The reaction was stopped by adding a quenching solution (40 mM ethanolamine in 1% BSA solution in PBS), after a washing suspension-centrifugation step with PBS (pH 7.4). The suspension was quickly centrifuged, and the NPs were washed with three cycles of suspension-centrifugation with PBS (pH 7.4), before being finally suspended in PBS (pH 7.4). These final coupling steps are shown in Scheme S3.




**Scheme S3. Final steps in the synthesis of Strp-Lys(Cy5)-NP$_{1200}$.** (VIII), (IX) Fmoc deprotection and glutaraldehyde coupling to **Fmoc-Lys(Cy5)-NPs**. (X) Streptavidin conjugation to glutaraldehyde. (XI) Reduction step using $NaBH_3CN$.

***Atto 647N-coupled NPs.*** To synthesise Atto 647N (A647)-coupled NPs, 500-nm NPs (loading: 58 µmol/g, solid contents: 2%) were used. These nanoparticles were first conditioned by washing them three times with 1 mL of DMF each time through suspension-centrifugation (13,400 rpm, 3 min) cycles. Next, to conjugate the PEG spacer to the NPs, Fmoc–PEG–OH (75 equiv.) was dissolved in DMF (1 mL) with oxyma (75 equiv.) and DIC (75 equiv.). The mixture was stirred at room temperature for 10 min. Then, the solution was added to the dry NPs, and the suspension was left to stir at 1,400 rpm at 60°C for 2 h. Subsequently, the NPs were washed with three successive suspension-centrifugation cycles (13,400 rpm, 3 min) to obtain Fmoc-PEGylated NPs. Fmoc was removed by treating the NPs with a 20% piperidine/DMF solution (3 × 20 min) and sequential washing steps, as described above.

To achieve A647-coupled NPs, 50 µL of an A647 NHS ester solution (0.1 mg/mL, 1.2 mM) was mixed separately with 2 µL of DIPEA and then added to the dry PEGylated NPs and suspended. The suspension was stirred at 1,000 rpm at room temperature for 14 h in the dark. The following day, the NPs were washed with three successive suspension-centrifugation cycles with DMF (13,400 rpm, 3 min), methanol (13,400 rpm, 5 min), and water (13,400 rpm, 8 min), before finally being suspended in water (50 µL). All the synthesis steps are shown in Scheme S4.

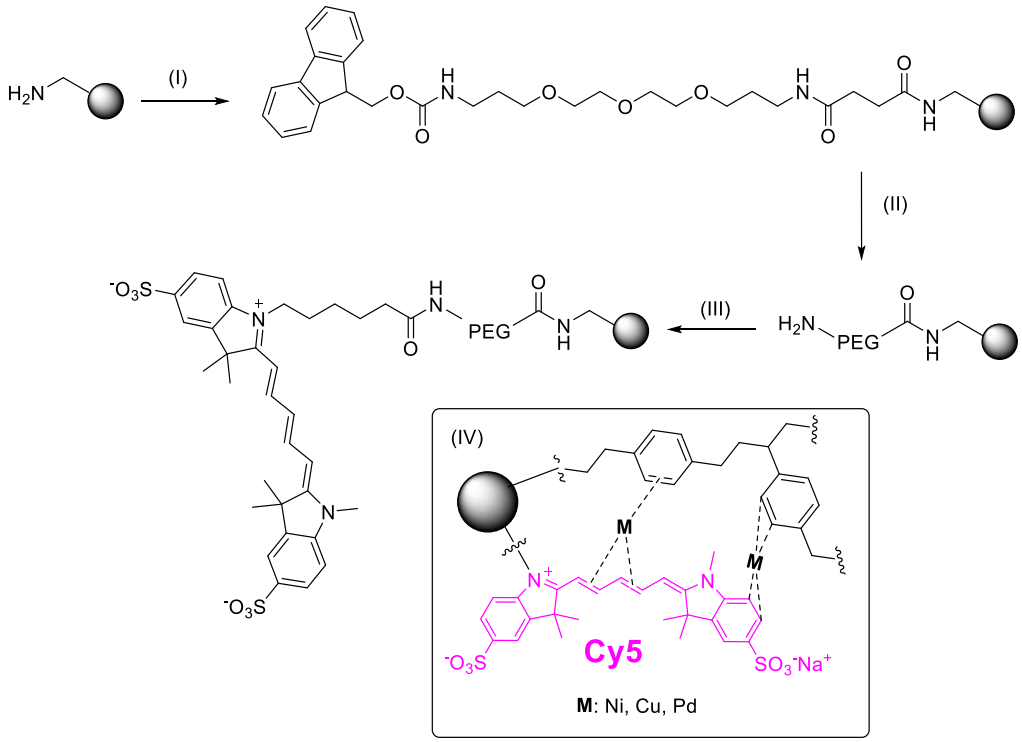



**Scheme S4.** (I) Fmoc-NH-PEG conjugation to naked polystyrene nanoparticles (NPs). (II) Fmoc deprotection. (III) A647 conjugation.

## 3. SI Methods. Metallofluorescent nanoparticle synthesis

To synthesise $M^+$-Sulfo-Cy5-coupled NPs using Ni(II), Cu(I), and Pd(II) as metal ions, 460-nm NPs (loading: 64 μmol/g, solid contents: 2.5%) were used. The nanoparticles were first conditioned by washing them three times with 1 mL of DMF each time through suspension-centrifugation (13,400 rpm, 3 min) cycles. Next, to conjugate the PEG spacer to the NPs, Fmoc–PEG–OH (75 equiv.) was dissolved in DMF (1 mL) with oxyma (75 equiv.) and DIC (75 equiv.). The mixture was stirred at room temperature for 10 min. Then, the solution was added to the dry NPs, and the suspension was left to stir at 1,400 rpm at 60°C for 2 h. Subsequently, the NPs were washed with three successive suspension-centrifugation cycles (13,400 rpm, 3 min) to obtain Fmoc-PEGylated NPs. Fmoc was removed using a 20% piperidine/DMF solution (3 × 20 min) and sequential washing steps, as described above.

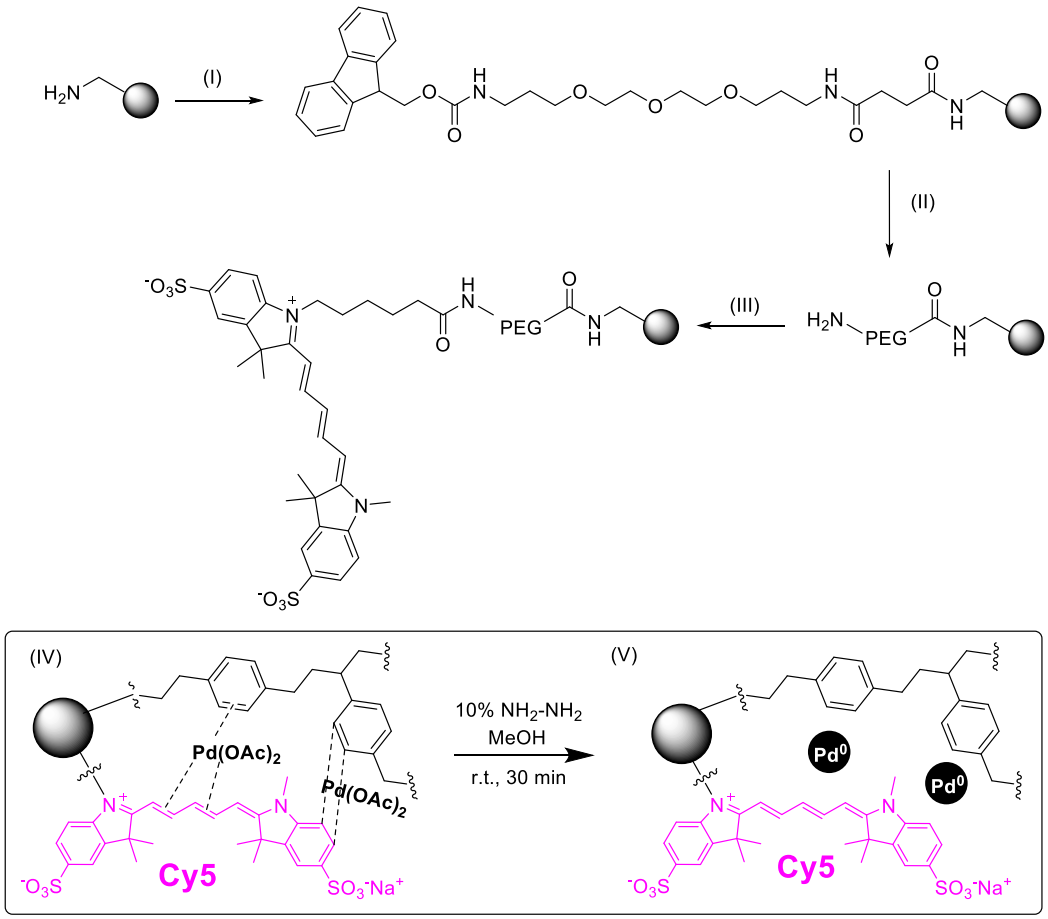
Next, 50 μL of a sulfo-Cy5 NHS ester solution (0.1 mg/mL, 1.313 mM) was mixed separately with 2 μL of DIPEA before being added to the dry PEGylated NPs, which were then suspended. The suspension was stirred at 1,000 rpm at room temperature for 14 h in the dark. Afterwards, the NPs were washed with three cycles of suspension-centrifugation with DMF (13,400 rpm, 3 min). Next, 100 μL of a 10 mM metal ion solution ($NiCl_2$, CuBr, or $Pd(OAc)_2$) in DMF was added to the dry NPs, and they were stirred at 1,000 rpm at room temperature for 14 h in the dark. After the reaction was complete, the NPs were washed with three successive suspension-centrifugation cycles with DMF (13,400 rpm, 3 min), methanol (13,400 rpm, 5 min) and water (13,400 rpm, 8 min), before finally being suspended in water (50 μL). The complete reaction process is shown in Scheme S5.




**Scheme S5. Synthesis of metallofluorescent NPs.** (I) Fmoc-NH-PEG conjugation to naked polystyrene nanoparticles (NPs). (II) Fmoc deprotection. (III) Sulfo-Cy5 conjugation. (IV) Metal (M) coordination *via* π-π stacking interaction.

To extend the type and nature of the metallofluorescent NPs, the **Pd(0)-Cy5-PEG-NP$_{500}$** particles were obtained by the *in situ* reduction of Pd(II) to Pd(0). The synthesis of these particles followed the same protocol for sulfo-Cy5 coupling and coordination with Pd(II) described above. Then, Pd(II) was reduced to Pd(0) by adding 150 μL of

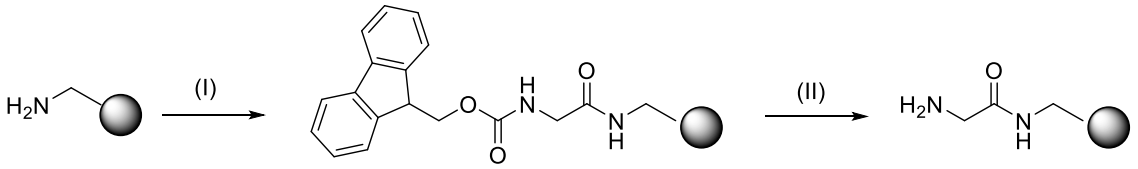
10% hydrazine in methanol. The reaction mixture was left to stir at room temperature for 30 min. The NPs were then washed with three suspension-centrifugation cycles with methanol (13,400 rpm, 5 min) and water (13,400 rpm, 8 min), before finally being suspended in water. This synthesis is shown in Scheme S6 and Scheme 1 (in main text).




**Scheme S6. Synthesis of Pd(0)-Cy5-PEG-NP$_{500}$.** (I) Fmoc-NH-PEG conjugation to naked polystyrene nanoparticles (NPs). (II) Fmoc deprotection. (III) Sulfo-Cy5 conjugation. (IV) Pd(II) coordination. (V) Pd(II) reduction to Pd(0) (particle)

## 4. SI Methods. Glycine and capped glycine nanoparticle synthesis and reaction with Pd(II)

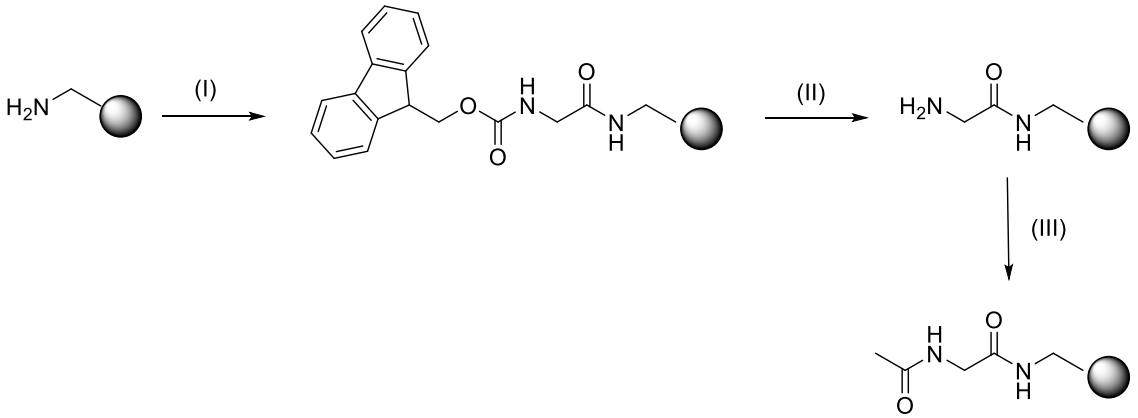
***Glycine NPs.*** To synthesise **$H_2N$-$NP_{500}$**, 500-nm NPs (loading: 58 μmol/g, solid contents: 2%) were used. These nanoparticles were first conditioned by washing them three times with 1 mL of DMF each time through suspension-centrifugation (13,400 rpm, 3 min) cycles. Next, to conjugate a glycine spacer to the NPs, fluorenylmethyloxycarbonyl-Glycine (Fmoc–Gly–OH) (75 equiv.) was dissolved in DMF (1 mL) with oxyma (75 equiv.) and DIC (75 equiv.). The mixture was stirred at room temperature for 10 min. Then, the solution was added to the dry NPs, and the suspension was stirred at 60°C and 1,400 rpm for 2 h. Subsequently, the NPs were washed with three cycles of suspension-centrifugation (13,400 rpm, 3 min) to obtain Fmoc-Gly NPs. Fmoc was removed using a 20% piperidine/DMF solution (3 × 20 min) and sequential washing steps, as described above. The process is shown in Scheme S7.




**Scheme S7. Synthesis of $H_2N$-$NP_{500}$.** (I) Fmoc-NH-Glycine conjugation to naked polystyrene NPs. (II) Fmoc deprotection.

***Capped Glycine NPs***. To synthesise **Ac-HN-$NP_{500}$**, 500-nm NPs (loading: 58 μmol/g, solid contents: 2%) were used. These nanoparticles were first conditioned by washing them three times with 1 mL of DMF each time through suspension-centrifugation (13,400 rpm, 3 min) cycles. Next, to conjugate a glycine spacer to the NPs, Fmoc–Gly–OH (75 equiv.) was dissolved in DMF (1 mL) with oxyma (75 equiv.) and DIC (75 equiv.). The mixture was stirred at room temperature for 10 min. Then, the solution was added to the dry NPs, and the suspension was stirred at 60°C and 1,400 rpm for 2 h. Subsequently, the NPs were washed with three cycles of suspension-centrifugation (13,400 rpm, 3 min) to obtain Fmoc-Gly NPs. Fmoc was removed using a 20% piperidine/DMF solution (3 × 20 min) and sequential washing steps, as described above.

In order to perform capping to the NPs (amide bond formation), these were suspended in a solution of acetic anhydride and DIPEA (50 equiv.) in DMF. The reaction mixture was left to stir at room temperature for 30 min. After that, the NPs were washed with three cycles of suspension-centrifugation with DMF (13,400 rpm, 3 min). The process is shown is Scheme S8.




**Scheme S8. Synthesis of Ac-HN-NP$_{500}$.** (I) Fmoc-NH-Glycine conjugation to naked polystyrene NPs. (II) Fmoc deprotection. (III) Amine groups capping (amide bond) using acetic anhydride and DIPEA.

***Reaction with Pd.*** The NPs were initially washed with three cycles of suspension-centrifugation with DMF (13,400 rpm, 3 min). Next, 100 µL of a 10 mM metal ion solution ($Pd(OAc)_2$) in DMF was added to the dry NPs, and they were stirred at 1,000 rpm at room temperature for 14 h in the dark. After the reaction was complete, the NPs were washed with three successive suspension-centrifugation cycles with DMF (13,400 rpm, 3 min) and methanol (13,400 rpm, 5 min). Subsequently, Pd(II) was reduced to Pd(0) by adding 150 µL of 10% hydrazine in methanol. The reaction mixture was left to stir at room temperature for 30 min. The NPs were then washed with three suspension-centrifugation cycles with methanol (13,400 rpm, 5 min) and water (13,400 rpm, 8 min), before finally being suspended in water.

## 5. SI Methods. Fluorescence lifetime imaging microscopy (FLIM) and spectroscopy

***FLIM and spectroscopy instrumentation.*** Fluorescence lifetime images were obtained using a MicroTime 200 microscope system (PicoQuant GmbH). To focus on the unexpected green fluorescence detected in the Cy5-labelled particles, the excitation source was a 470-nm pulsed laser (LDH-P-C-470, PicoQuant) controlled by a "Sepia" driver (PicoQuant) to adjust the power and repetition rate to 20 MHz. The excitation pulses were directed into the specimen through a 1.4 NA, 100× oil immersion objective of an IX-71 inverted confocal microscope (Olympus), after being reflected on a 510 DCXR dichroic mirror (Chroma). The fluorescence emission was filtered by a 500LP (AHF/Chroma) cut-off filter and focused onto a 75-μm pinhole. Then, a 600 DCXR dichroic mirror (Chroma) separated the fluorescence emission into two detection channels: green ($I_G$, with a 520/35-nm bandpass filter, Omega Filters) and red ($I_R$, with a 685/70-nm bandpass filter, Omega Filters). Two single-photon avalanche diode SPCM-AQR 14 modules (Perkin Elmer) detected individual photons and sent the corresponding signal to a TimeHarp 200 single-photon timing (SPT) module (PicoQuant).

To collect the spectra, the laser was focused onto a single particle after the area was pre-scanned. The fluorescence was collected past the pinhole, diverted to a fibre coupler and sent to an F-matcher attached to an Andor Shamrock 303i-A spectrograph equipped with a 1,200-line/mm grating. The fluorescence photons were spectrally separated and directed to an ultrasensitive Andor Newton electron multiplying CCD camera (EM-CCD) to simultaneously collect the entire fluorescence emission spectra. Every spectrum was collected as 20 accumulations of 0.2-s readouts, from 300 to 854 nm, with a wavelength resolution of 0.351 nm. A 15-point Gaussian smoothing was performed on the spectra to reduce random noise.

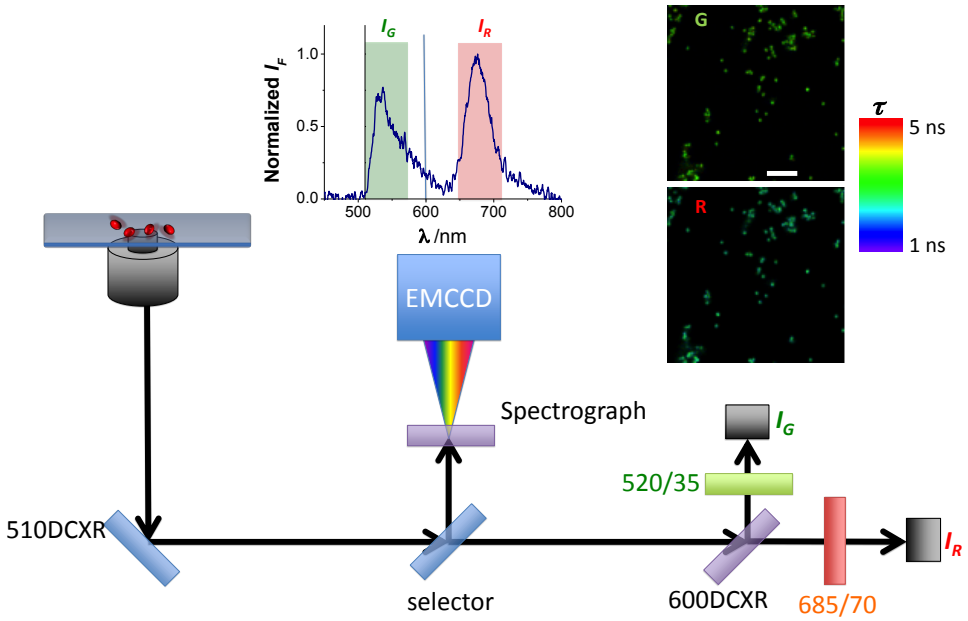



**Figure S2. Scheme of the instrumentation for FLIM and fluorescence spectroscopy employed in this work.** A representative spectrum of a particle containing dual-band emission is shown to highlight the bandpass filters used to define the green ($I_G$) and red ($I_R$) channels. The scale bar in the image represents 5 μm.

***FLIM images and spectra collection and analysis*.** The particle samples were sonicated for 3 min before performing a 10-fold dilution with a phosphate buffer (10 mM) at pH 6. The diluted particle sample was then placed on the microscope slide. To locate a set of fluorescent particles, an initial pre-scan of an 80 × 80 $\mu m^2$ area was performed at a very low laser power to avoid photobleaching effects. Then, a small region containing several particles was selected and FLIM imaged with a 512 × 512 pixel resolution. To collect the spectra of the particles, a different set of particles was selected to avoid potential photobleaching effects occurred during the FLIM acquisition via a low-power pre-scan. The laser was then focused onto different particles, and the spectra were collected from at least 5 different particles.

The FLIM image data were analysed using SymphoTime 32 software (PicoQuant) by fitting the fluorescence decay trace within each pixel to a single exponential decay function and depicting the obtained lifetime in an adequate pseudo-colour scale. $I_G/I_R$ ratio images were obtained by dividing the intensity images collected in each channel, and the ratio represented by a pseudo-colour scale, with the image intensity modulated by the total $I_G+I_R$ intensity, via a home-coded script in MathCad 15.0 (PTC).

## 6. SI Results. Additional results of Cy5-carrying NPs

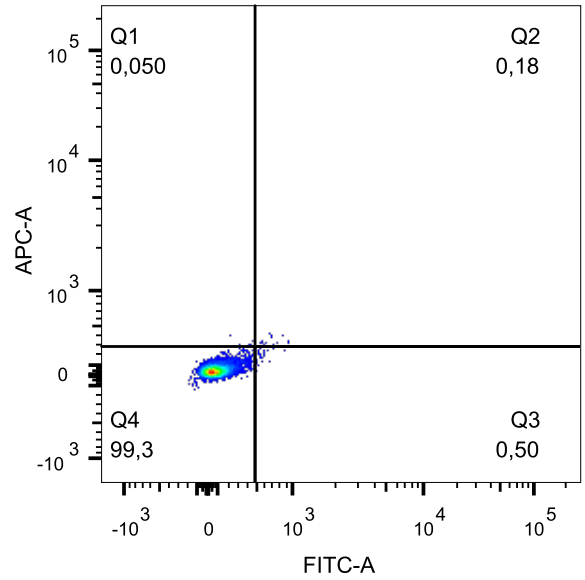



**Figure S3.** FACS result of a blank particle: **$NH_2$-PEG-$NP_{500}$**. This control shows that the blank particles show negligible fluorescence in both channels and that the unexpected green fluorescence does not arise from any contamination of the reagents during the synthetic process.

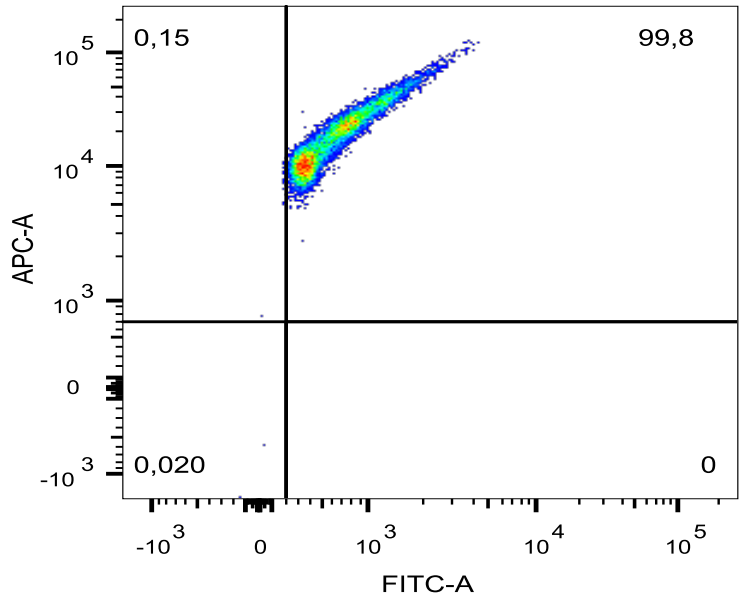



**Figure S4.** A) Dual-colour FLIM image of **Strp-Lys(Cy5)-$NP_{1200}$** particles in the red emission channel. The pseudo-colour scale indicates the average fluorescence lifetime of the emission in each pixel. The scale bar represents 2.5 μm. B) Corresponding $I_G/I_R$ ratio image, with the pseudo-colour scale indicating the $I_G/I_R$ value in each pixel. C) Fluorescence emission spectra of different particles in the image. d) FACS correlogram of detected fluorescence in the red (APC-A) and green (FITC-A) channels of **Strp-Lys(Cy5)-$NP_{1200}$** particles.

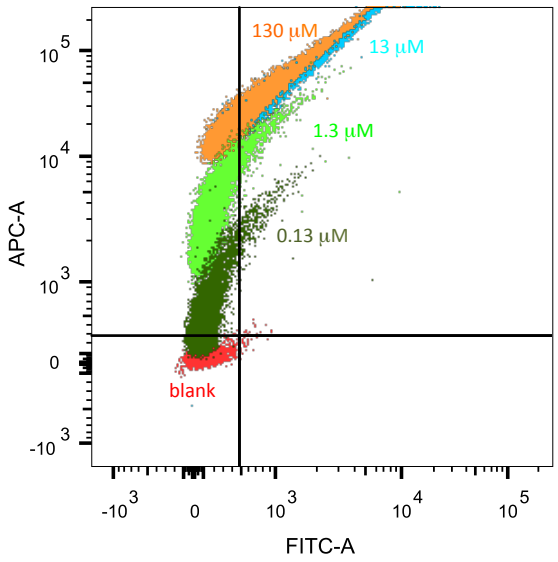



**Figure S5.** FACS results of **Cy5-PEG-NP$_{500}$** particles carrying different loadings of Cy5 in the conjugation reaction from 0.13 to 130 μM.

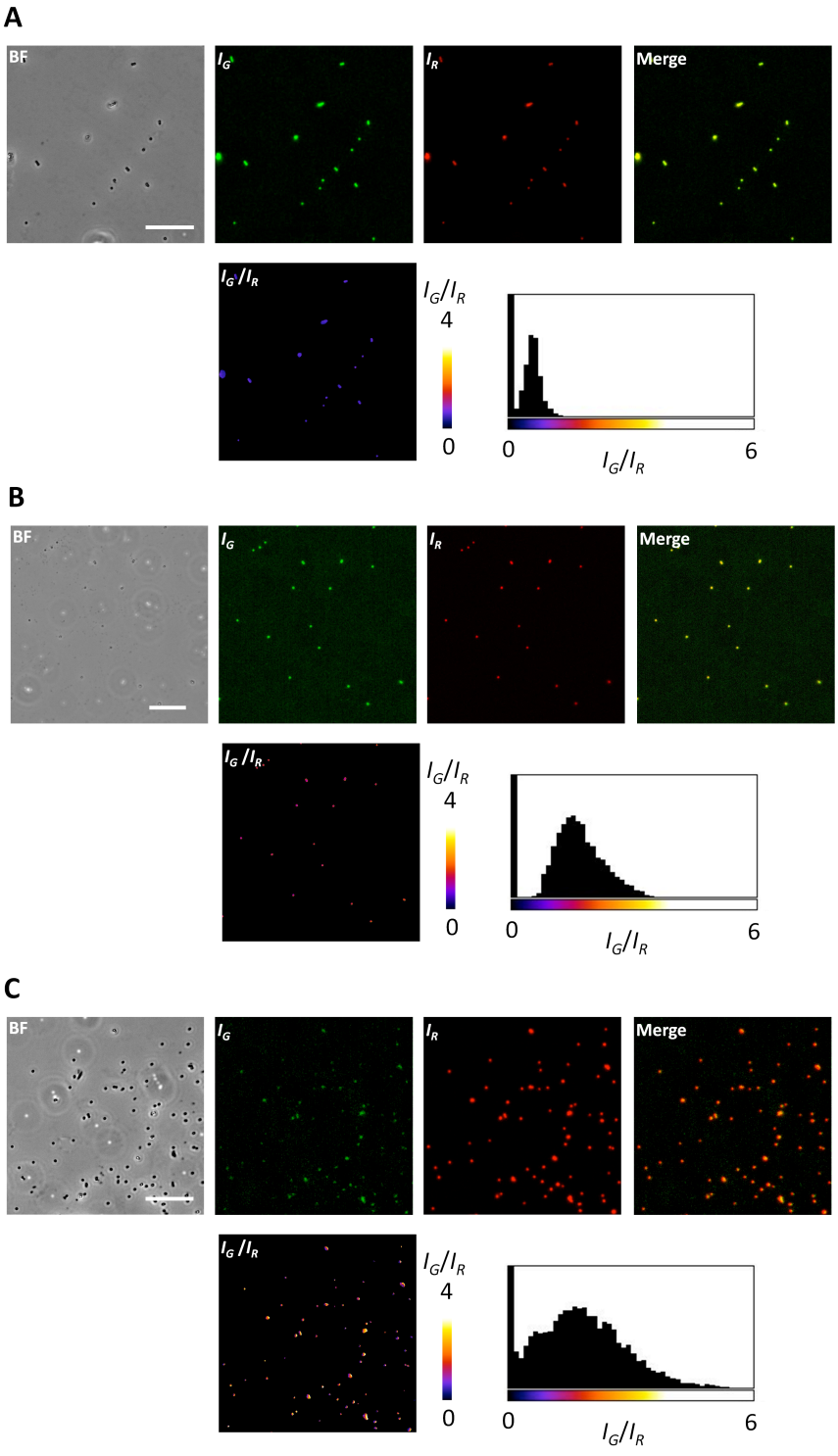



**Figure S6.** Dual-colour fluorescence microscopy of **Cy5-PEG-NP$_{500}$** particles carrying different loadings of Cy5 in the conjugation reaction: 130 (A), 13 (B), or 1.3 μM (C). The figure shows representative images of NPs in the bright-field (BF), green-channel ($I_G$), red-channel ($I_R$), merged $I_G$ and $I_R$ channels, and the $I_G/I_R$ ratio image. The $I_G/I_R$ histogram of just the particles surpassing an automatic set threshold is also shown. Scale bars represent 10 μm.

## 7. Scheme of π-π stacking interactions causing green fluorescence and FRET

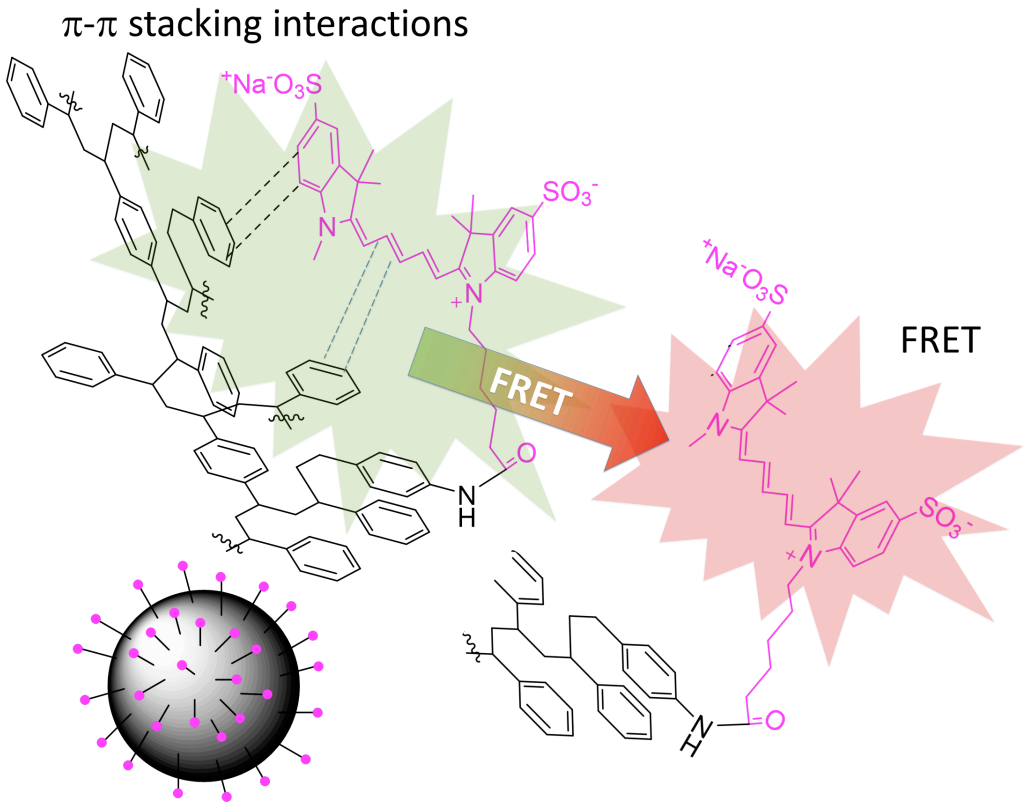



**Figure S7.** The π-π stacking interactions between the Cy5 polymethine chain and aromatic side groups with the aromatic groups in the polystyrene core cause the green fluorescence emission. The excitation energy on the interacting moieties can be transferred to non-interacting Cy5 dyes via FRET.

## 8. SI Results. Additional results of Atto 647N-carrying NPs

An important control is evaluating whether the unexpected green fluorescence emission is a specific behaviour of the Cy5 dye. For this control, we prepared a series of particles, following exactly the same protocols, but carrying another red fluorophore A647. We chose this dye because it is spectrally equivalent to Cy5 (Fig. S8), but the dye's core moiety is not a cyanine, but a carbopyronin.[3] Figure S9 shows that the green fluorescence is negligible in the A647-labelled **A647-PEG-NP$_{500}$** particles, in all the dual-channel FLIM microscopy experiments, the associated spectral profile of the particles, and the FACS measurements. Similar results were obtained with **A647-PEG$_2$-NP$_{500}$** and **A647-PEG$_3$-NP$_{500}$**. We also repeated the syntheses of **A647-PEG-NP$_{500}$** particles with different dye loadings using different concentrations during the coupling reaction in the same range of concentrations as that described for Cy5. The FACS measurements showed that the number of events exhibiting green fluorescence was always less than 10% (Fig. S11). Large particle conjugates carrying a polypeptide chain and the A647 dye were also synthesised (see Table S1). In all these particles, the green fluorescence emission was negligible (Fig. S10), as evidenced in the $I_G/I_R$ images, with $I_G/I_R$ values generally below 0.1. Hence, the green fluorescence emission is confirmed to be a specific effect of Cy5 and not related to any factor in the synthesis of the conjugates.

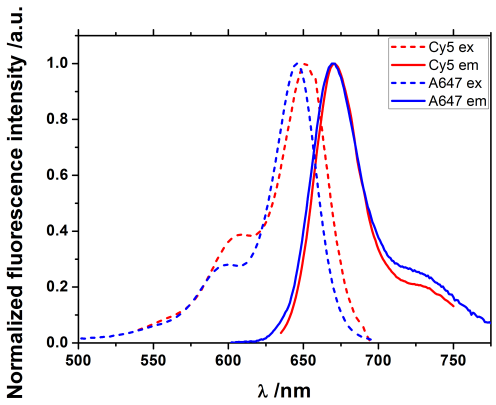



**Figure S8.** Comparison of the Cy5 (red) and A647 (blue) excitation (dashed lines) and emission (solid lines) spectra.

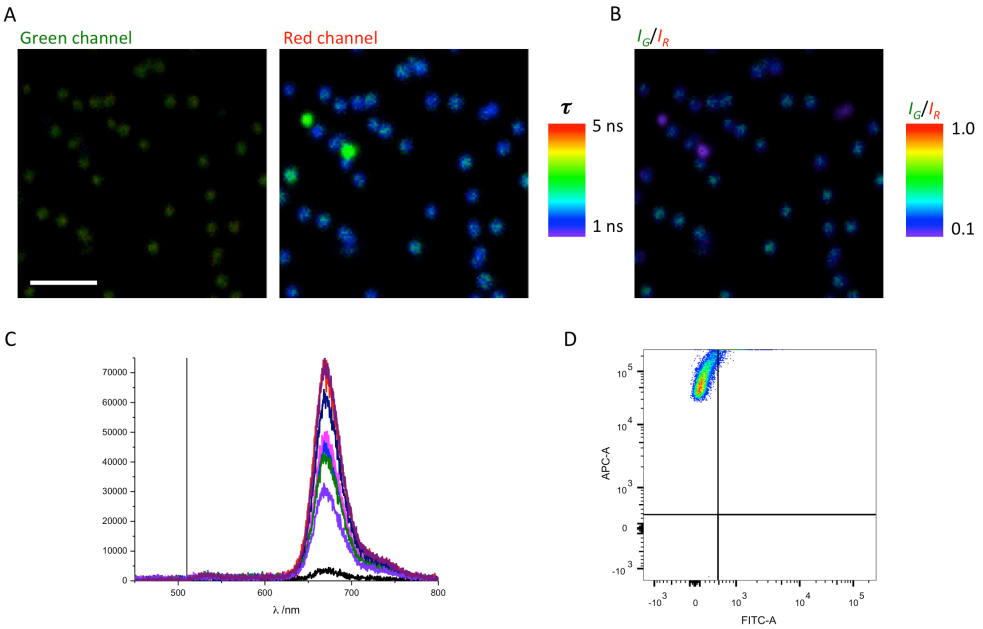



**Figure S9.** A) Dual-colour FLIM images of **A647-PEG-NP$_{500}$** particles in the green and the red emission channels. The pseudo-colour scale indicates the average fluorescence lifetime of the emission in each pixel. The scale bar represents 2.5 μm. B) $I_G/I_R$ ratio image of the images shown in A). The pseudo-colour scale indicates the $I_G/I_R$ value in each pixel. C) Fluorescence emission spectra of different particles in the images, exclusively showing emission in the red channel and negligible emission on the green channel. d) FACS correlogram of detected fluorescence in the red (APC-A) and green (FITC-A) channels of **A647-PEG-NP$_{500}$** particles.

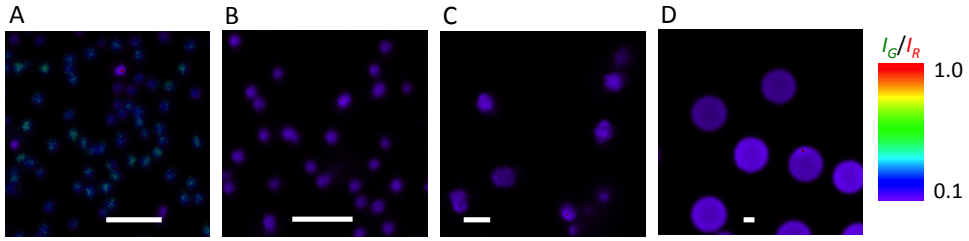



**Figure S10.** $I_G/I_R$ ratio images of A647-loaded particles. The pseudo-colour scale indicates the $I_G/I_R$ value in each pixel. The scale bars represent 2.5 μm. A) **A647-PEG-NP$_{500}$**; B) **Fmoc-Lys(A647)-NP$_{900}$**; C) **Fmoc-Lys(A647)-NP$_{2500}$**; and D) **Fmoc-Lys(A647)-NP$_{5000}$**.

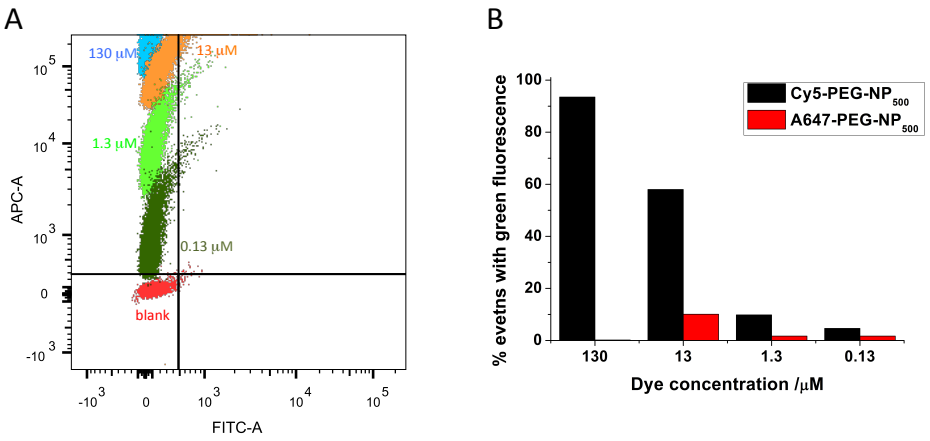
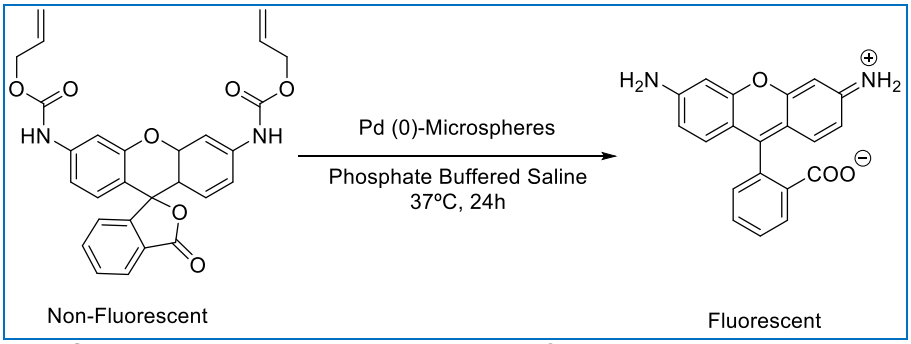



**Figure S11.** A) FACS results of **A647-PEG-NP$_{500}$** particles carrying different loadings of A647 in the conjugation reaction, from 0.13 to 130 μM. B) Percentage of events showing green events (FITC-A channel) in FACS experiments of **Cy5-PEG-NP$_{500}$** and **A647-PEG-NP$_{500}$** particles with different loadings of the dye in the conjugation reaction.

## 9. Fluorogenic reaction catalysed by Pd(0)

The effective reduction of Pd(II) to Pd(0) within the NPs was performed by probing the catalytic activity of these particles, using a reaction catalysed by Pd(0), i.e., removing allyloxycarbonyl protecting groups:[4-5] the non-fluorescent bis-allyloxycarbonyl rhodamine is transformed into fluorescent rhodamine dye once the protecting groups are removed (Scheme S9).




**Scheme S9.** Fluorogenic deprotection reaction of bis-allyloxycarbonyl rhodamine catalysed by Pd(0) microspheres.

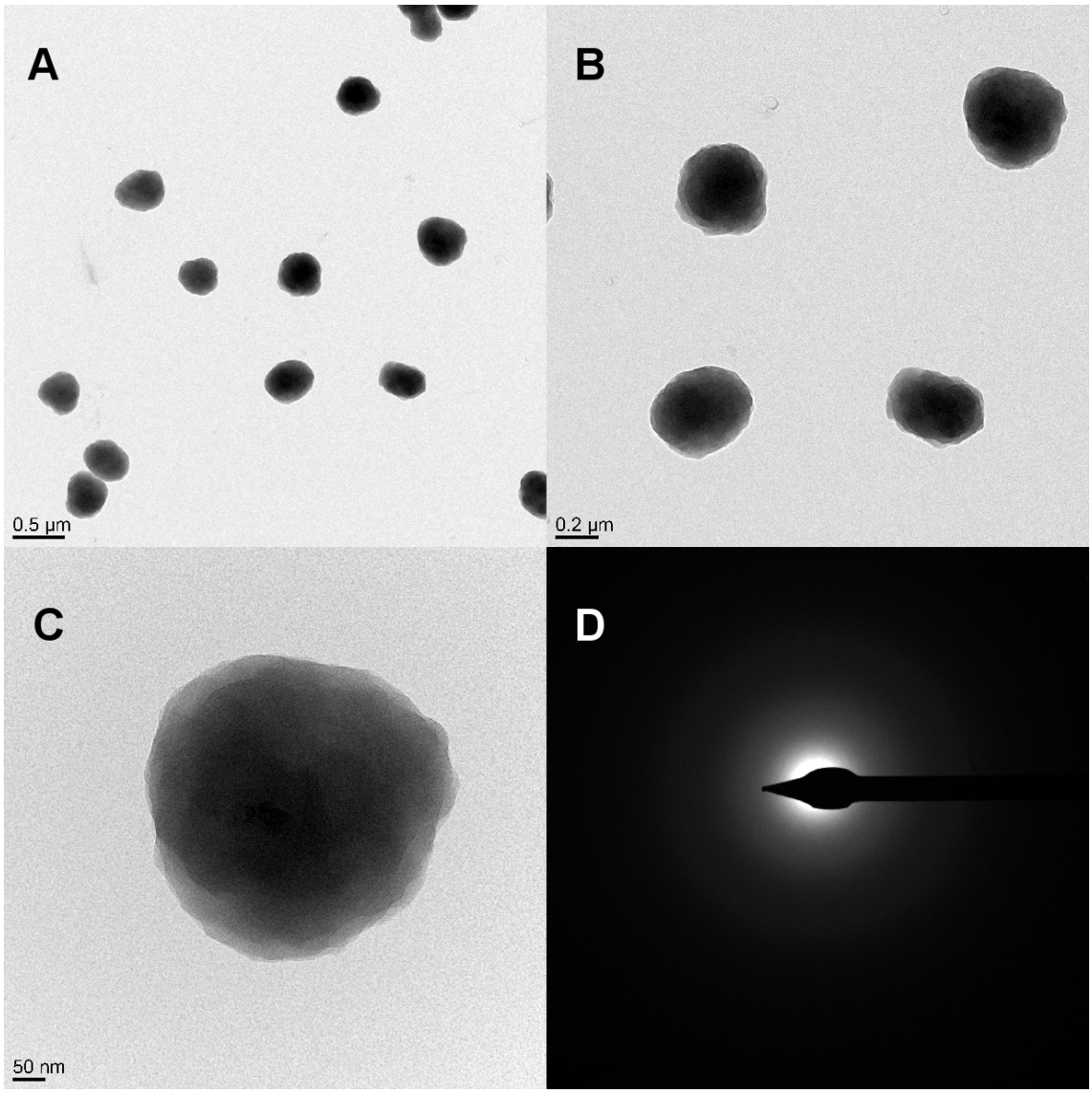



**Figure S12. A-C)** High resolution transmission electron microscopy (HRTEM) of **$NH_2$-PEG-$NP_{500}$** (NPs control). Scale bars represent 0.5 µm in **A**, 0.2 µm in **B** and 50 nm in **C. D)** X-ray diffraction (XRD) of **$NH_2$-PEG-$NP_{500}$**, showing that it is an amorphous structure.

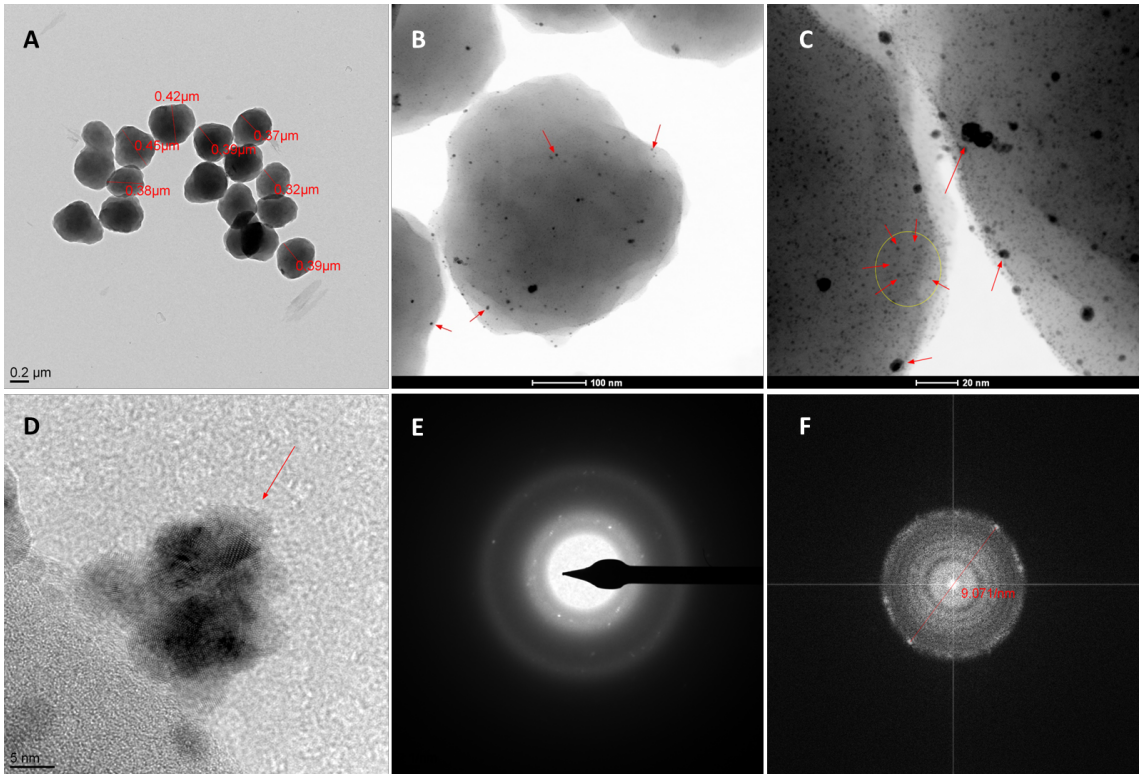



**Figure S13. A-D**) HRTEM of **Pd(II)-Cy5-PEG-NP$_{500}$**. Red arrows point to Pd particles. Panel **D** shows a Pd particle zoomed in. Scale bars represent 200 nm in **A**, 100 nm in **B**, 20 nm in **C**, and 5 nm in **D**. **E**) XRD of the Pd particle presented in **D**. **F**) Fast Fourier Transform (FFT) of the Pd particle presented in **D**.

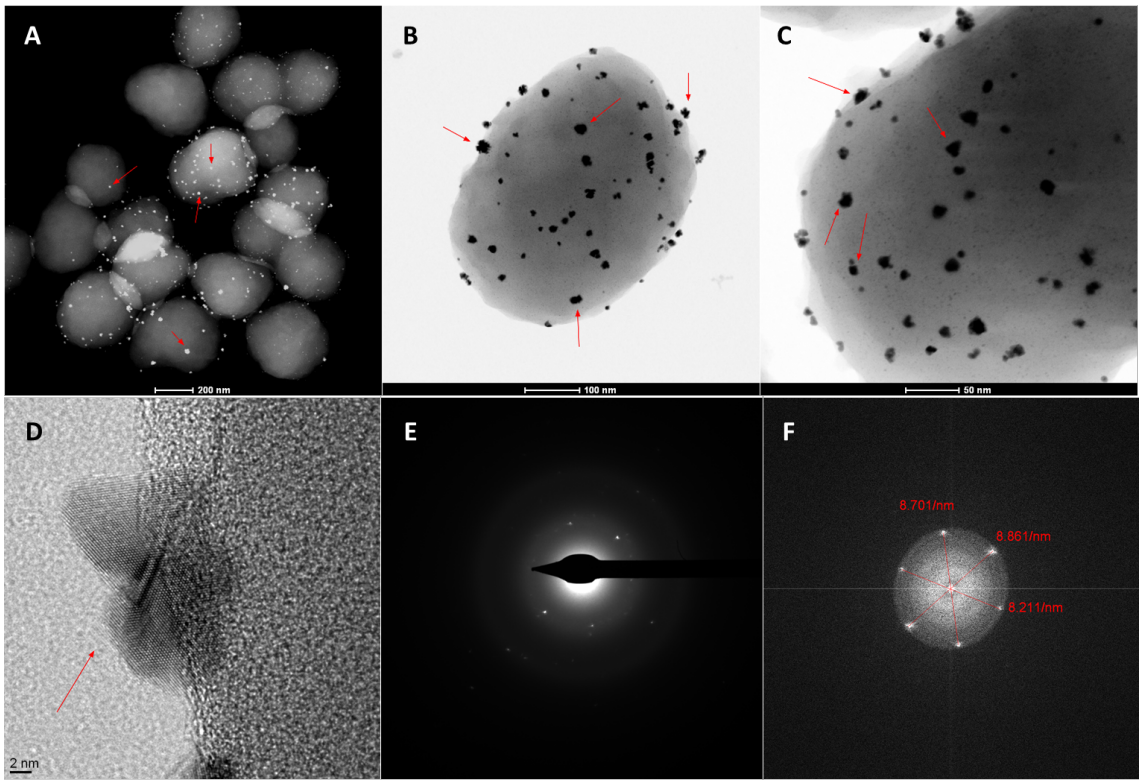



**Figure S14. A-D**) HRTEM of **Pd(0)-Cy5-PEG-NP$_{500}$**. Red arrows point to Pd particles. Panel **D** shows a Pd particle zoomed in. Scale bars represent 200 nm in **A**, 100 nm in **B**, 50 nm in **C**, and 2 nm in **D**. **E**) XRD of the Pd particle presented in **D**. **F**) Fast Fourier Transform (FFT) of the Pd particle presented in **D**.

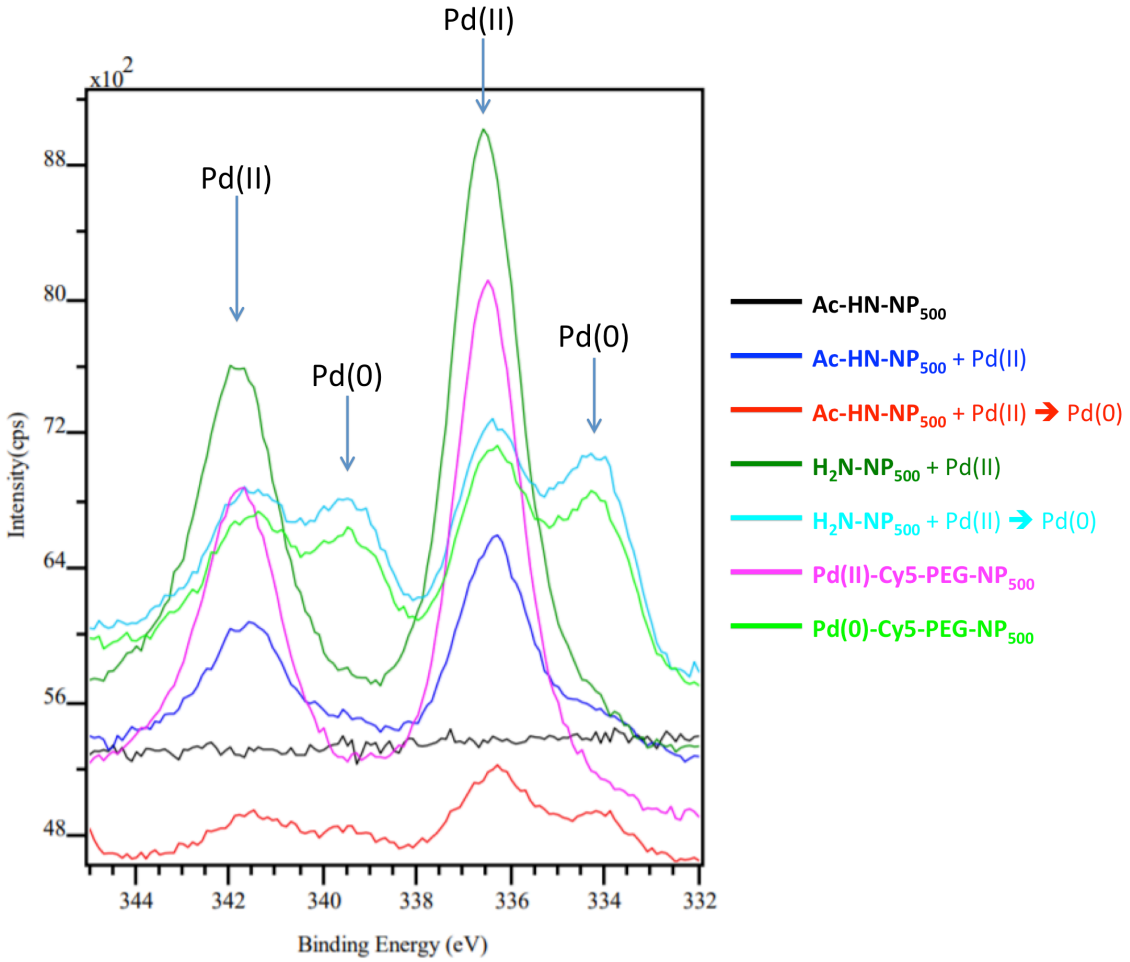



**Figure S15.** XPS spectra of different particles treated with Pd(II), and subsequent reduction to Pd(0). The particles **Ac-HN-NP$_{500}$**, with the amine groups capped with a glycine, showed very little binding of Pd(II) (blue line), and almost complete disappearance of Pd(II) and Pd(0) after the reduction (red line). In contrast, the **H$_2$N-NP$_{500}$** particles exhibited large binding of Pd(II) (olive line) and of Pd(0) after reduction (cyan line). The binding of Pd to the **H$_2$N-NP$_{500}$** particles is comparable to that of the **Cy5-PEG-NP$_{500}$** particles, giving rise to the **Pd(II)-Cy5-PEG-NP$_{500}$** (magenta line) and **Pd(0)-Cy5-PEG-NP$_{500}$** (light green line).

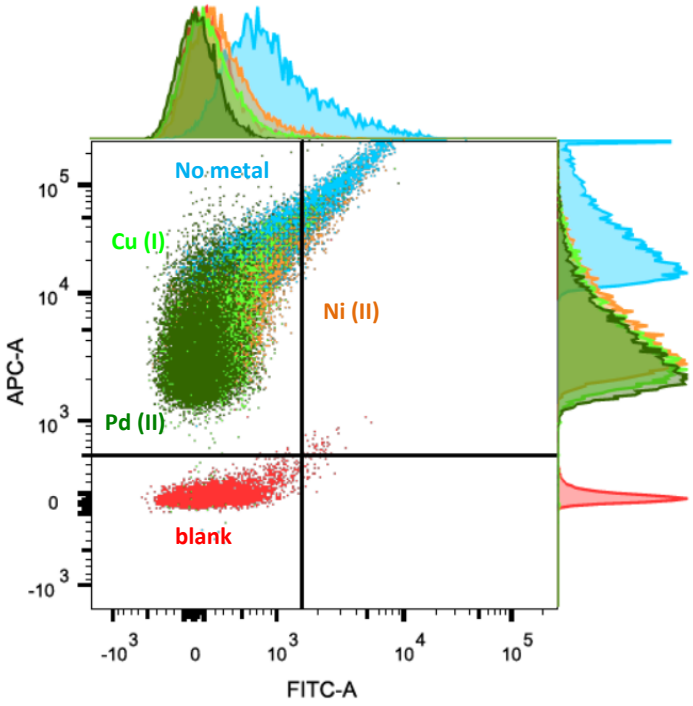



**Figure S16.** FACS correlogram of metallofluorescent particles in the red (APC-A) and green (FITC-A) emission channels. The correlogram shows results from **$NH_2$-PEG-$NP_{500}$** (blank, red), **Cy5-PEG-$NP_{500}$** (no metal, cyan), **Pd(II)-Cy5-PEG-$NP_{500}$** (dark green), **Cu(I)-Cy5-PEG-$NP_{500}$** (light green), and **Ni(II)-Cy5-PEG-$NP_{500}$** (orange). The fluorescence of Cy5 in all the metal-carrying particles was partially quenched. Moreover, the presence of Cu(I), Ni(II), and Pd(II) ions in the particles caused the disappearance of the green emission, suggesting that coordination of the metal ions prevents the interaction between the cyanine moieties and the aromatic groups on the particle surface.

## 11. SI Results. Au(III) ions catalyse the breakage of Cy5 conjugation

To test the catalytic effect of Au(III) ions on the breakage of the polymethine chain of Cy5, we added $HAuCl_4 \cdot 3H_2O$ to an aqueous solution containing sulfo-Cy5 ($1.5 \times 10^{-5}$ M). The absorption spectra of the dye before and after treatment are shown in Figure S17. The loss of conjugation is evidenced by the solution turning immediately colourless to the naked eye. This is corroborated by the loss of the typical absorption of Cy5 in solution. In contrast, when Au(III) ions were added to a Cy5 solution in DMF as solvent did not cause any colour loss.

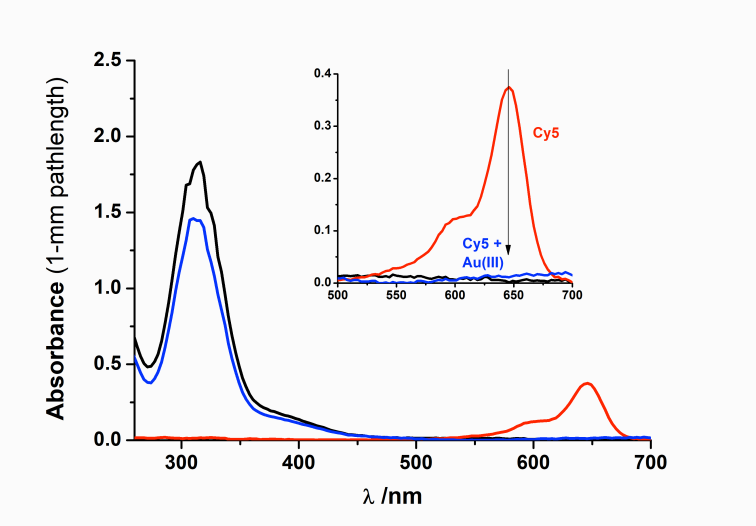



**Figure S17.** Absorption spectra of Cy5 in aqueous solution (red line) and after treatment with $HAuCl_4 \cdot 3H_2O$ (blue line). The absorption spectra of $HAuCl_4 \cdot 3H_2O$ in solution is also shown as a control (black line).

## 12. SI Methods and Results. Live cell metallofluorescent particle uptake

MDA-MB-231 cell line was maintained in Dulbecco's modified Eagle's medium supplemented with L-alanyl-L-glutamine, penicillin/streptomycin, pyruvate and 10% (v/v), foetal bovine serum in a standard incubator (95% humidity, 5% $CO_2$, 37°C) and subcultured twice per week.

For viability assays, 2,000 cells/well were seeded in 96-well plates and left overnight to stabilise. The stabilised cells were then nanofected with 20,000, 10,000, 5,000, 2,500, 1,250, 625, and 0 NP/cell with different metallofluorescent NPs, control fluorescent NPs or non-fluorescent NPs, in serum-free DMEM to avoid the formation of a protein corona. After 8 h, the serum concentration was restored to normal levels, and the cells were allowed to grow for 6 days. Viability/proliferation was measured by means of an MTT assay (550 nm with reference at 650 nm).

For flow cytometry assays, $5 \times 10^5$ MDA-MB-231 cells were seeded in 24-well plates and stabilised in an incubator for 18 h. Subsequently 2,000, 1,000, 500, 250, 125, or 0 metallofluorescent NPs/cell, **Cy5-PEG-NP$_{500}$**/cell or **NH$_2$-PEG-NP$_{500}$**/cell were incubated for 8 h in serum-free DMEM to evaluate cellular uptake. After washing with PBS, living cells were trypsinised for 5 min, washed twice with PBS, fixed with paraformaldehyde (2% v/v), washed again with PBS, and collected in a cytometer tube. Cells were analysed by means of flow cytometry with the instrumentation described above. The data were analysed according to previous reports of this group[6] using FACSDiva software for data acquisition and FlowJo® (Flowjo, LLC, OR, USA) for data analysis.

For dual mass- and flow-cytometry assays, $10^6$ MDA-MB-231 cells were seeded in 6-well plates and stabilised for 18 h in an incubator. Subsequently, 1,000, 500, 250, 100, 50 or 0 **Pd(0)-Cy5-PEG-NP$_{500}$**/cell were incubated for 8 h in serum-free DMEM. After washing with PBS, living cells were trypsinised for 5 min, washed twice with PBS (5 min, 450 g), and collected in a cytometer tube. Subsequently, cells were incubated with 330 µL of Cell-ID™ Intercalator-Ir (125 µM, 1:1000) into MaxPar® Fix and Perm Buffer at 4°C overnight to identify nucleated cells by CyToF® (Fluidigm Co., CA, USA) analysis. After two washes with 660 µL MaxPar® Cell Staining Buffer, cells were washed twice with MaxPar® Water. The cell concentration was adjusted to $5 \times 10^5$ cells/mL with MaxPar® Water immediately prior to CyToF® data acquisition and filtered twice into cell strainer cap tubes. The data were acquired on CyToF® for a minimum time of 20 min. Results were analysed using the Cytobank Community online software (Cytobank Inc., CA, USA) and compared to the results of FlowJo® analysis software, which yielded identical results (Fig. S18). Thresholds were set according to negative controls (0 NP/cell) to obtain percentages of palladium-particle containing cells. After each mass cytometry experiment, cells were analysed by means of flow cytometry as described above. The percentage of nanofected cells, as measured by flow cytometry and CyToF®, were compared by two-way ANOVA followed by the Bonferroni *post hoc* test.

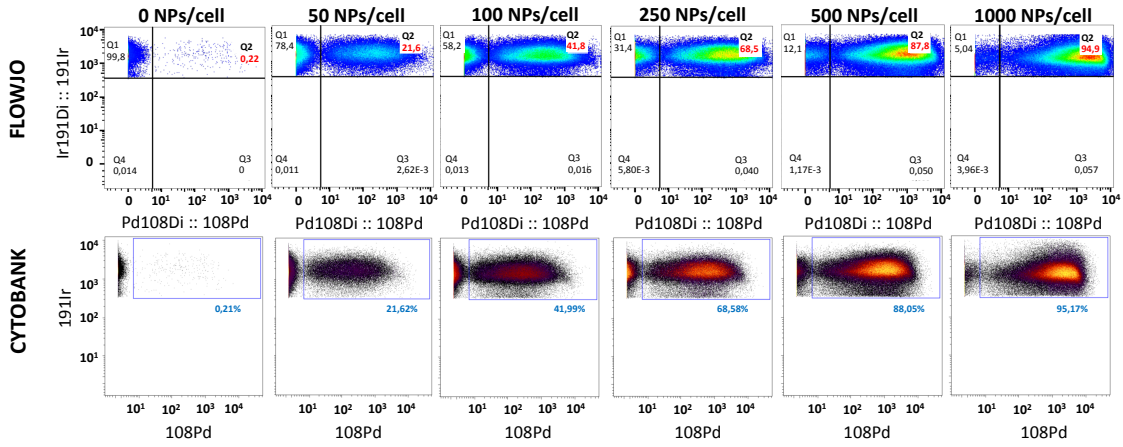



**Figure S18.** Comparative cellular uptake of **Pd(0)-Cy5-PEG-NP$_{500}$** analysed using two software programs: FlowJo (top row) and Community.Cytobank (bottom row). Both methods of analysis show practically indistinguishable cell uptake with increasing NPs/cell loading.

For confocal microscopy experiments, 1.5 × $10^3$ cells were seeded on poly-L-lysine (Sigma-Aldrich) pre-treated coverslips, stabilised overnight, and nanofected with 3,000 **Pd(0)-Cy5-PEG-NP$_{500}$**/cell or 3,000 **Cy5-PEG-NP$_{500}$**/cell in duplicate. After 8 h, the coverslips were collected, washed twice in PBS, and mounted with Mowiol® mounting medium (Sigma-Aldrich) on a slide. The nanoparticles in the fixed MDA-MB-231 cells were localised using a scanning confocal inverted microscope LSM 710 Axio Observer (Carl Zeiss, Jena, Germany). The images were acquired with a Plan-Apochromat 63×/1.4 OIL DIC M27 immersion objective and software ZEN 2010 (Carl Zeiss, Jena, Germany). Later, the images were processed with Software ZEN 2010 Black Edition (Carl Zeiss, Jena, Germany). Confocal images of MDA-MB-231 cells nanofected with either **Cy5-PEG-NP$_{500}$** or **Pd(0)-Cy5-PEG-NP$_{500}$** nanoparticles were obtained using the following settings: 1) a 488-nm Argon excitation laser (25 mW) at 2% power, an emission bandpass of 493-599 nm, and a pinhole of 1.0 Airy Unit (0.8 µm optical section); and 2) a 633-nm HeNe excitation laser (5.0 mW) at 18% power, an emission wavelength range of 635-759 nm, and a pinhole of 1.0 Airy Unit (0.8 µm optical section). Figure S19 shows representative images of cells containing **Cy5-PEG-NP$_{500}$** or **Pd(0)-Cy5-PEG-NP$_{500}$** nanoparticles. The exact number of NPs/cell was counted to determine the distribution of nanoparticles among the cell population. At least 70 cells were imaged to obtain the number of incorporated NPs. Figure S20 shows the number distribution of **Pd(0)-Cy5-PEG-NP$_{500}$** particles per cell after 3 h of incubation, with increasing loadings of nanoparticles under the MNF50.

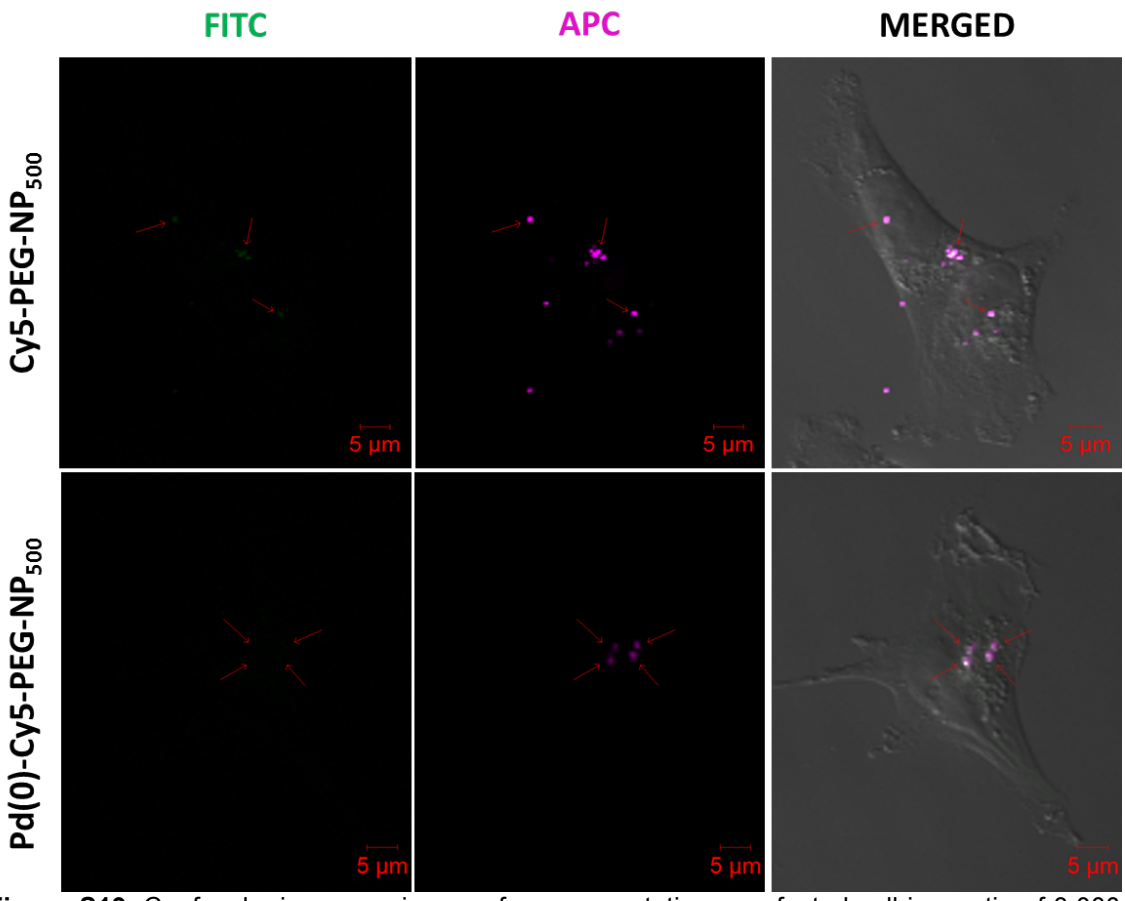



**Figure S19.** Confocal microscopy image of a representative nanofected cell in a ratio of 3,000 **Cy5-PEG-NP$_{500}$**/cell (upper row) and 3,000 **Pd(0)-Cy5-PEG-NP$_{500}$**/cell (lower row) through the different channels (FITC, APC and merged with the transmission image). Arrows point to single nanoparticles in the cell cytoplasm.

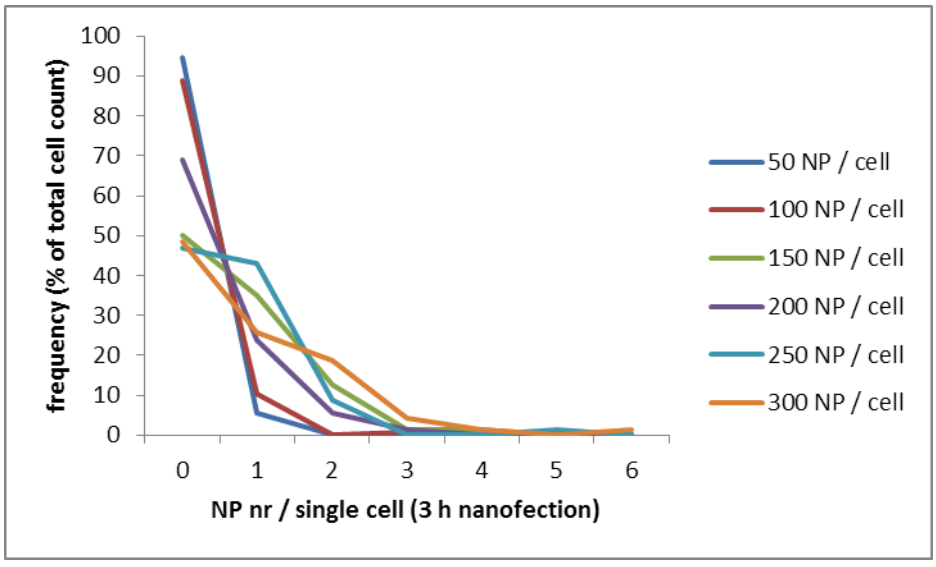



**Figure S20.** Exact number of **Pd(0)-Cy5-PEG-NP$_{500}$** per cell after 3 h of nanofection with increasing NP/cell ratios in a representative experiment, expressed as a percentage of the total number of cells counted by confocal microscopy. A minimum number of 70 cells per treatment were assessed.

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