Electronic Supplementary Material (ESI) for RSC Advances.
This journal is © The Royal Society of Chemistry 2017

Electronic Supporting Information to

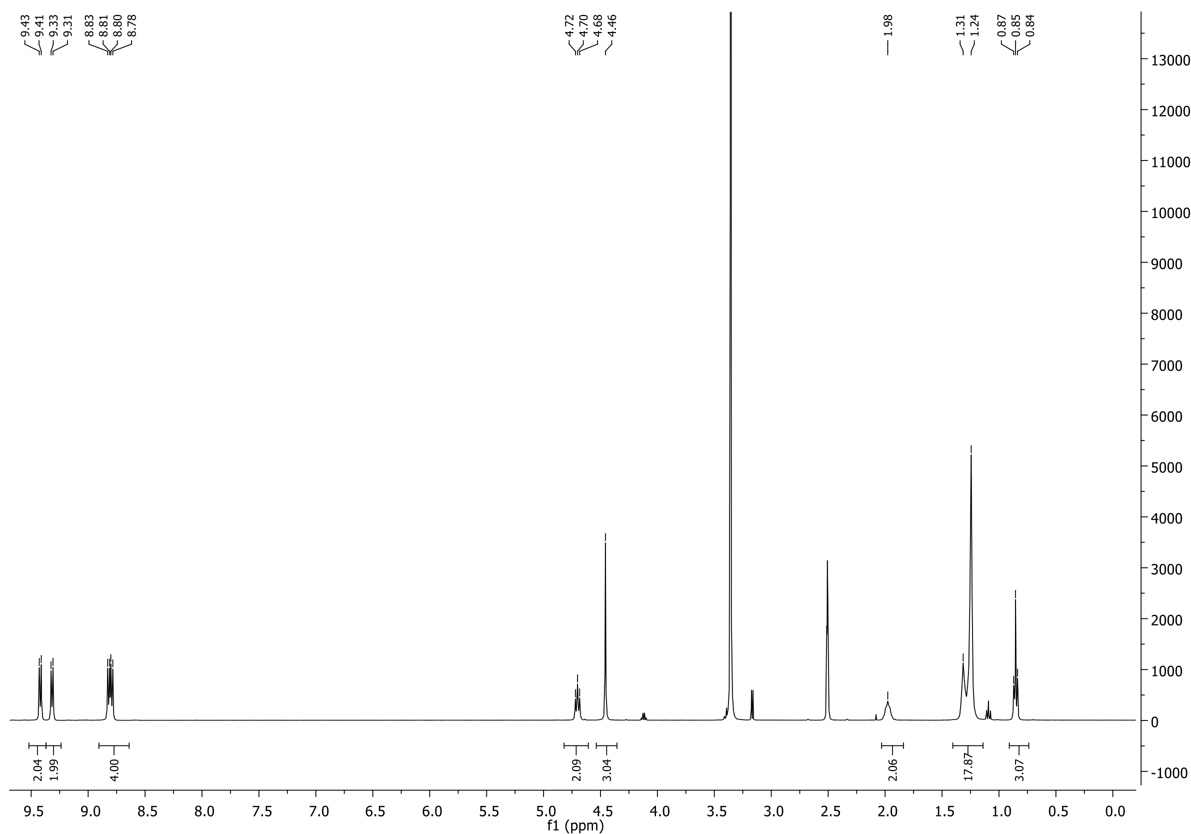
Targeting protein-loaded CB[8]-mediated supramolecular nanoparticles to cell

Emanuela Cavatorta[a], Jens Voskuhl[a], Dorothee Wasserberg[b], Jenny Brinkmann[a], Jurriaan Huskens[a] and Pascal Jonkheijm[a]

Synthetic procedures

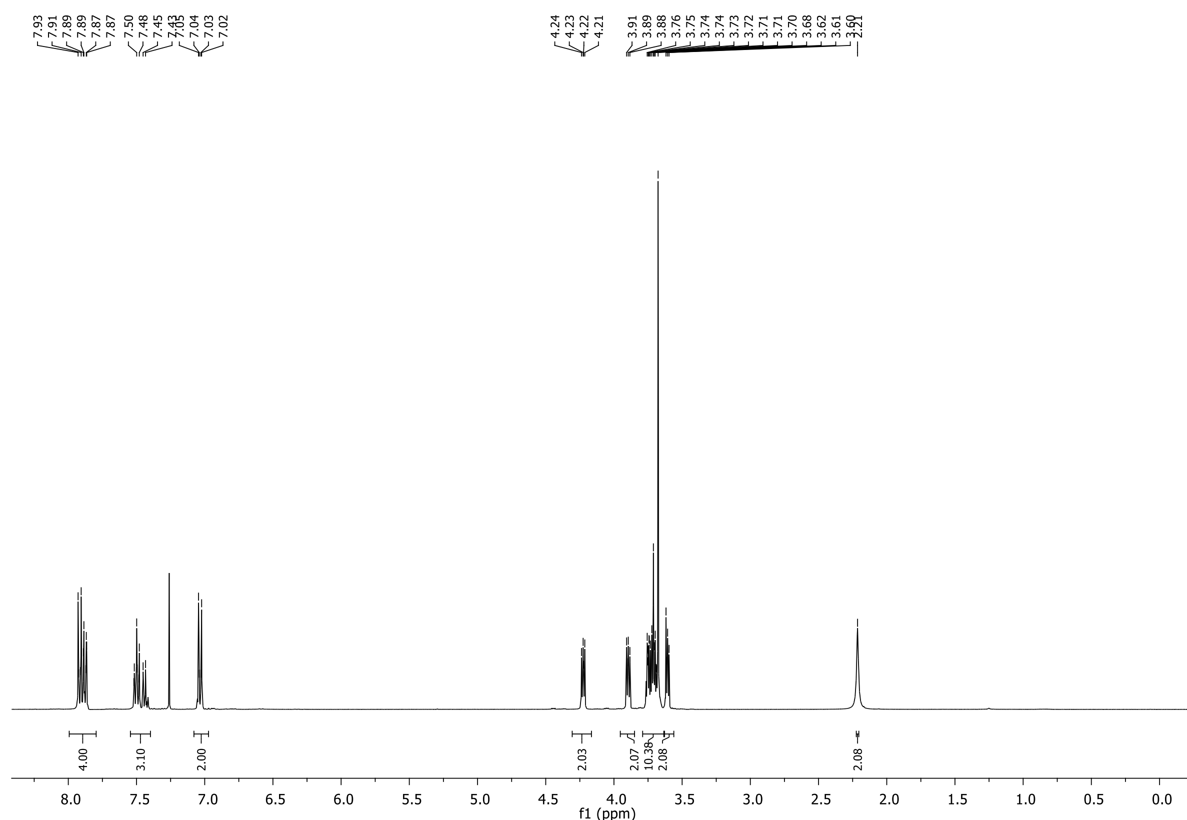
Synthesis of MV

Following a literature procedure[S1] 0.35 g (1.3 mmol) bromododecane was added to a stirred solution of 0.20 g (0.67 mmol) methylviologen in 25 mL of dry acetonitrile. The resulting solution was stirred at 70°C for 48 h followed by cooling to room temperature. The resulting precipitate was filtered and carefully washed with cold acetonitrile. The product (4, 4'-bipyridinium, 1-dodecyl-1'-methyl-, bromide iodide, MV) was obtained as orange solid. The yield, ^{1}H (see below) and ^{13}C-NMR data match the reported values in literature.



Synthesis of Azo

The synthesis of 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate and Azo (2-(2-(2-(2-(4-(phenyldiazenyl)phenoxy)ethoxy)ethoxy)ethoxy)ethanol) was carried out as described in literature.[S2] To a stirred solution of 10.0 g (51.0 mmol) of tetraethyleneglycol, 25 mg (cat.) of DMAP and 1.9 mL (13.3 mmol) of Et_3N in 100 mL of DCM, 2.4 g (13.0 mmol) of p-toluenesulfonylchloride was added keeping the temperature below 5°C. Stirring was continued for 1 h at this temperature and the solution was further stirred at room temperature for 24 h. The solution was then extracted with 1 M HCl (2 × 50 mL), water (2 × 50 mL) and brine (2 × 50 mL). The organic phase was dried over MgSO4 and the solvent was removed in vacuo. The residue was purified by silica gel column chromatography to yield 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate. The product was obtained as colorless oil. The yield, ^{1}H and ^{13}C-NMR data match the reported values in literature. Next, 1.1 g (5.3 mmol) of 4-phenylazophenol in 15 mL of acetonitrile was added to a stirred solution of 1.7 g (55.0 mmol) of 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate, 3.1 g (25.0 mmol) of K_2CO_3 and a catalytic amount of LiBr in 0.55 L of dry acetonitrile. The reaction mixture was refluxed for 2 days under argon. It was then allowed to cool to room temperature and the solvent was removed in vacuo. The residue was dissolved in DCM (30 mL), washed with water (30 mL) and brine (3 x 30 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel column chromatography to yield Azo. The product was obtained as orange oil. The yield, ^{1}H (see below) and ^{13}C-NMR data match the reported values in literature.



Synthesis of AzoRGD

First a carboxylic acid-functionalized azobenzene (ethyl 2-(4-(phenyldiazenyl)phenoxy)acetic acid) was synthesized.[S3] To a solution of 0.37 g (1.87 mmol) 4-phenylazophenol in 20 mL of dry acetone, 0.67 mL (6 mmol) of ethyl bromoacetate and 0.93 g (6.76 mmol) of K_2CO_3 were added. The reaction mixture was refluxed for 15 h and then allowed to cool to room temperature. After removal of the solvent under vacuum, the mixture was taken up in EtOAc and washed with water (3 x 50 mL). The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was purified by silica gel chromatography to afford ethyl 2-(4-(phenyldiazenyl)phenoxy)acetate as an orange solid. The ^{1}H and ^{13}C-NMR data are in agreement with reported values in literature. Next, to a solution of this product (1.55 mmol) in 20 mL of methanol, 4 mL of sodium hydroxide (1 N) was added and stirred for 2 h at room temperature. Then, methanol was removed under reduced pressure and 15 mL of EtOAc was added to the remaining solution. The aqueous layer was acidified to a pH of 2 with 1 M HCl. The aqueous phase was separated and the organic layer was further washed with brine (3 x 50 mL), dried over $MgSO_4$ and concentrated in vacuo to yield the desired product 2-(4-(phenyldiazenyl)phenoxy)acetic acid. The product was obtained as orange solid. The yield, ^{1}H and ^{13}C-NMR data match the reported values in literature. Peptide Azo-GSGGRGDSG (AzoRGD) was synthesized using a Multisyntech automatic solid phase peptide synthetic robot (Syro II) following standard Fmoc protocols.[S4] The carboxylic acid-functionalized azobenzene was added to the N-terminal of the peptide on the resin. Purification of the peptide was done by reversed phase HPLC in a gradient of H_2O/acetonitrile (10/90% to 0/100% in 70 min) and characterized by analytical HPLC and mass spectrometry (Fig. S1). $[M+H]^+$ = 985.88 (calc. 986.39); $[M+2H]^{++}$ = 493.49 (calc. 493.69).

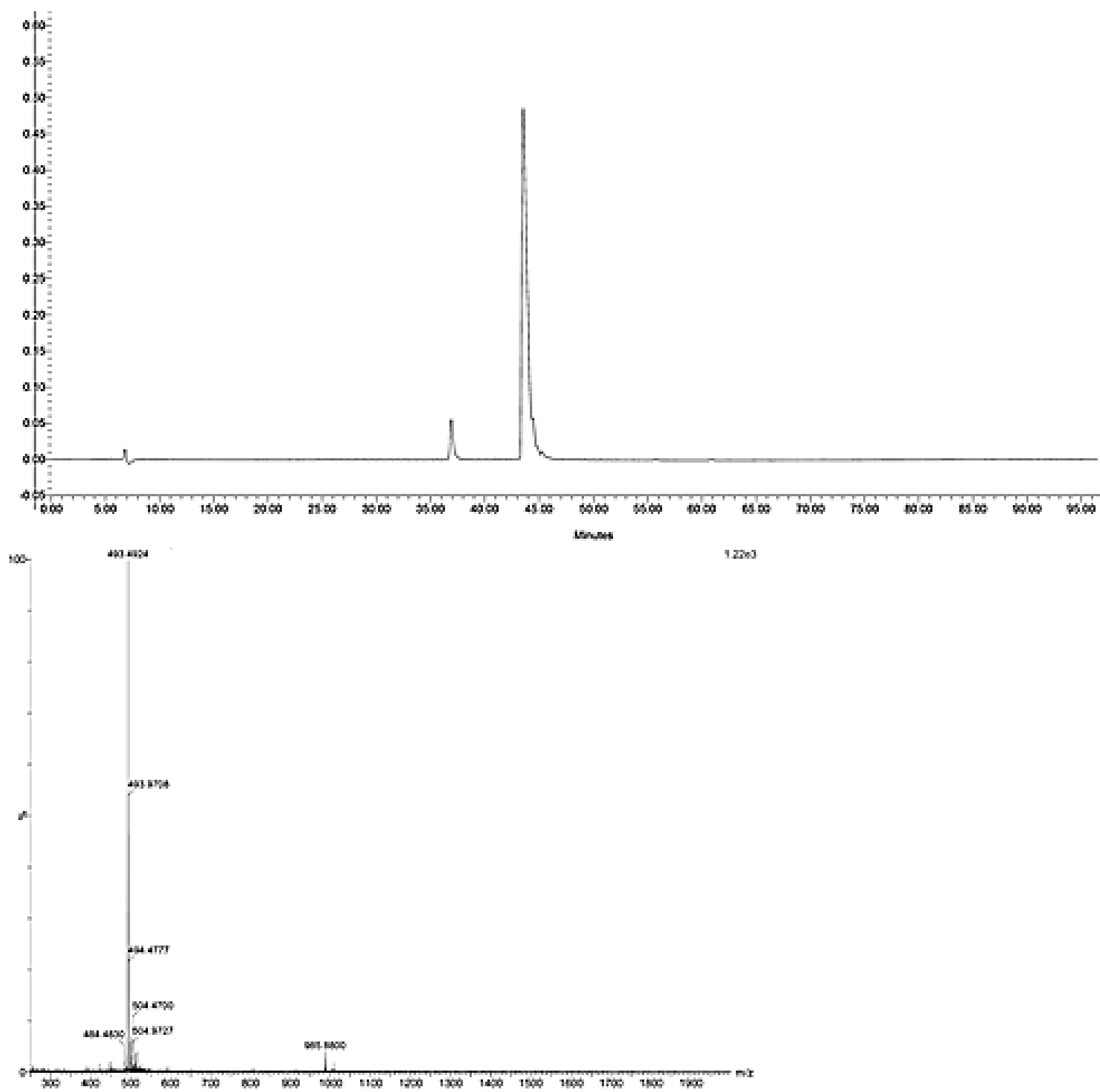


Fig. S1 Analytical HPLC chromatogram (top) and ESI-ToF MS spectrum (bottom) of the purified AzoRGD.

Nanoparticles preparation and characterization

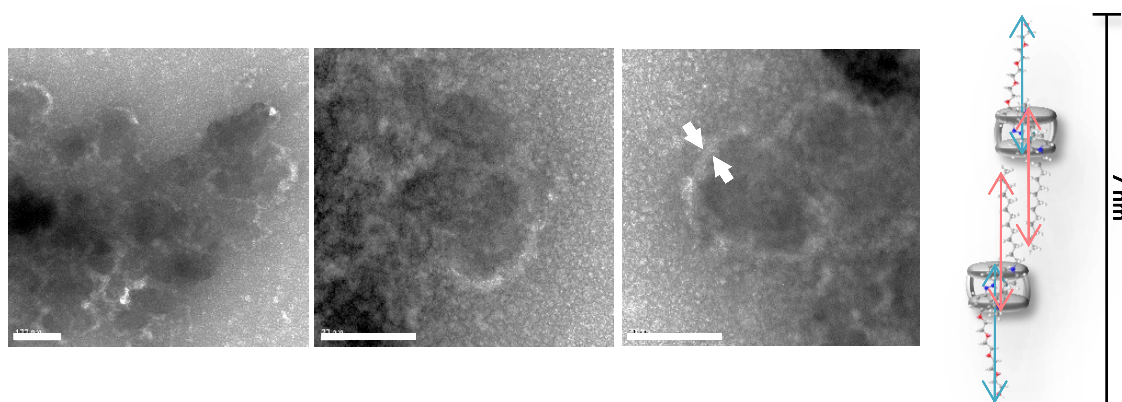


Fig. S2. TEM images of particles formed at equimolar ratios of MV, CB[8] and Azo (16 µM). Scale bars 100 nm, negative staining with 1% uranyl acetate. The schematic representation of two supramolecular ternary complexes in their extended conformation is also reported.

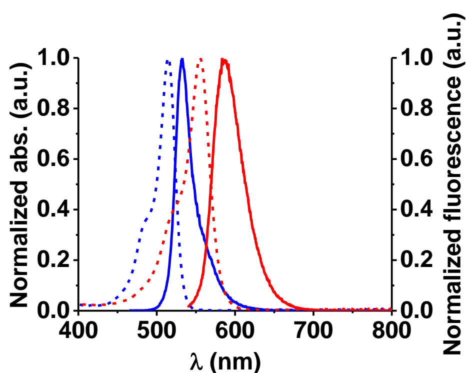


Fig. S3. Normalized absorption (dashed lines) and fluorescence (solid lines) spectra of YFP (blue, 18 µM, λ_{ex} = 440 nm) and TagRFP (red, 18 µM, λ_{ex} = 500 nm).

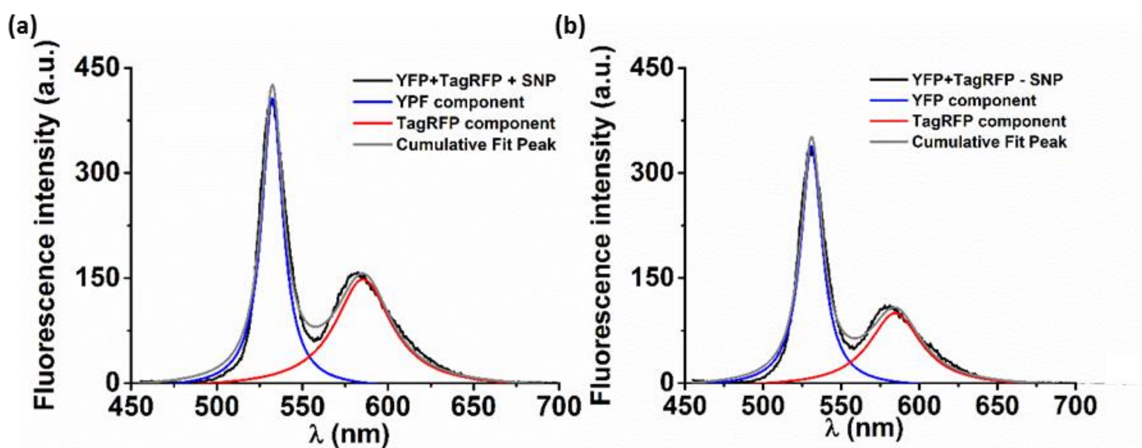


Fig. S4. (a) Lorentzian fits of the non-normalized fluorescence spectra (λex = 440 nm) of YFP/TagRFP mixtures (YFP+TagRFP) with particles (SNP) and (b) without SNPs Each condition was measured for proteins (18 µM) mixed with MV, CB[8] and Azo (+SNP) and only proteins (-SNP).

Cell experiments

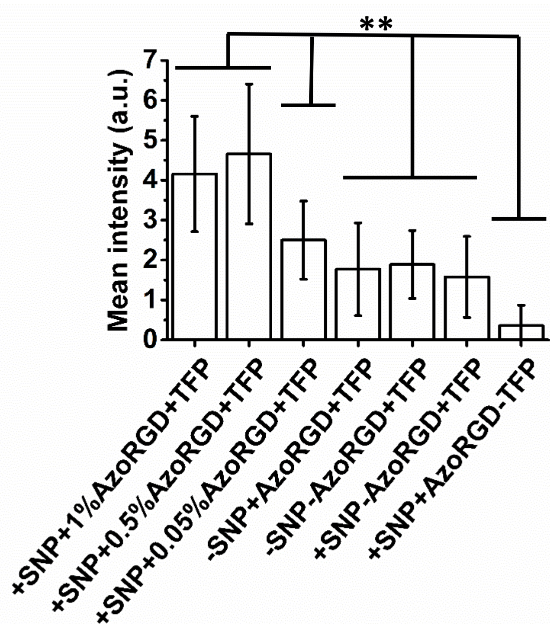


Fig. S5. The green fluorescence intensity from C2C12 cells threated with nanoparticles (SNP) loaded with TFP and functionalized with different amounts of AzoRGD ligands (0.05-1% of the total content of Azo) was measured by ImageJ software for all conditions and plotted as the average over $25 \le n \le 220$ cells collected over three independent experiments with the exception of 0.5 and 0.05% AzoRGD for which the averages are collected over two independent experiments. The error bars are the standard deviations of the distributions. The ** indicates a significant difference with a 95% level of confidence.

Calculation of the theoretical encapsulated protein fraction

To estimate the total volume fraction of proteins encapsulated inside the supramolecular nanoparticles (SNPs) ($V_{SNP,tot}$), a spherical vesicular nanoparticle was considered with an outer diameter of 204 nm and an inner diameter of 190 nm. The corresponding inner and outer spheres delimit the shell of the SNP corresponding to the walls, which thickness is set at 7 nm considering the bilayer structure of the supramolecular amphiphiles. For one SNP this yields to:

Outer surface area = $A_{out} = 4\pi r^2 = 1.31\times10^5$ nm^2

Inner surface area = $A_{in} = 4\pi r^2 = 1.13\times10^5$ nm^2

Total particle volume (including walls) = $V_{out} = (4/3)\pi r^3 = 4.45\times10^6$ nm^3

Enclosed particle volume (excluding walls) = $V_{in} = (4/3)\pi r^3 = 3.59\times10^6$ nm^3

Walls volume = $V_{walls} = V_{in} - V_{out} = 0.86\times10^6$ nm^3

Assuming the highest possible density in the plane, *i.e.* an hexagonal packing with a packing density equal to 0.907, and approximating that the footprint of each ternary complex projected onto respective sphere's surface to be the maximized cross section through the CB[8] molecule ($A_{CB[8]}$ = 0.61 nm^2), then the number of molecules comprising the wall of one nanoparticle is:

Molecules forming the outer sphere = $(A_{out} / A_{CB[8]}) * 0.907 = 1.95\times10^5$

Molecules forming the inner sphere = $(A_{in} / A_{CB[8]}) * 0.907 = 1.68\times10^5$

Thus, their sum (bilayer) gives:

Molecules per particle = 3.63×10^5

Next, the number of molecules forming ternary complexes MV·CB[8]·Azo at the equilibrium (at 1:1:1 ratios) in solution needs to be calculated. The overall equilibrium binding constant K_{ter} is:

$$K_{ter} = \frac{[\mathrm{MV{\cdot}CB[8]{\cdot}Azo}]}{[\mathrm{MV}]*[\mathrm{CB[8]}]*[\mathrm{Azo}]}$$

and, according to literature:[S5]

$K_{ter} = K_{MV\bullet CB[8]} * K_{(MV\bullet CB[8])\bullet Azo} = 8.5\times10^5\ M^{-1} * 1.4\times10^4\ M^{-1} = 1.2\times10^{10}\ M^{-2}$

Assuming that the azobenzene is fully present as *trans* isomer, the initial concentrations of the host and guests are known:

$[MV]_i = [CB[8]]_i = [Azo]_i = 16.6\times10^{-6}$ M

and therefore the problem can be solved analytically, setting the equilibrium concentration of the complex to [MV·CB[8]·Azo] = x and the equilibrium concentrations of the free reagents as [MV] = [CB[8]] = [Azo] = (16.6×10^{-6})-x. Substituting these equilibrium concentrations in the formula for K_{ter}, a cubic equation in x is obtained with only one real solution for x = 7.87 µM which is the concentration of the ternary complex at the equilibrium, *i.e.* [MV·CB[8]·Azo]. Therefore, having a known total volume of the solution of V_{tot} = 0.6 mL, the number of ternary complexes for x = 7.87 µM is equal to 2.84×10^{15}.

Next, the average number of particles in the solution can be calculated from the ratio of the total number of ternary complexes in solution at the equilibrium (2.84×10^{15}) and the average number of ternary complexes per particle (3.63×10^{5}), yielding $n_{tot} = 7.82\times10^{9}$.

Finally the total volume enclosed in the particles is calculated as:

$V_{in, tot} = V_{in} * n_{tot} = 2.81\times10^{16}$ nm^3 = 0.028 µl

The total volume occupied by the walls of the nanoparticles results:

$V_{walls, tot} = V_{walls} * n_{tot} = 6.72\times10^{15}$ nm^3 = 0.007 µl

Assuming a uniform distribution of the protein molecules in the solution, which means roughly excluding interactions of the proteins with the walls of the particles, the total volume available for protein encapsulation, arbitrarily defined by the walls and the cavity of the particles, yields:

$V_{SNP,tot} = V_{in, tot} + V_{walls, tot} = 3.48\times10^{16}$ nm^3 = 0.035 µl

This corresponds to approx. 0.01% of the total volume (V_{tot} = 0.6 mL) of the solution that is available for protein encapsulation.

Calculations of the Förster radius

Non-radiative fluorescence resonance energy transfer (FRET), between a donor molecule in its excited state (D*) and an acceptor molecule (A) in the ground state, can be characterized by the Förster radius R_o, defined by:

$$R_0 = \sqrt[6]{\frac{9000\, ln10}{128 * \pi^5 * N} \frac{\kappa^2 * \, Q_D * J(\lambda)}{n^4}} = 0.211 \sqrt[6]{\frac{\kappa^2 * \, Q_D * J(\lambda)}{n^4}}$$

where κ^2 describes the relative orientation of the transition dipole moments of the donor and the acceptor, which is assumed to be equal to 2/3 for randomly oriented molecules in solution. Q_D is the quantum yield of the donor (= intensity of fluorescence/intensity of the absorbed radiation) and for YFP equal to 0.61. N is Avogadro's number and the refractive index of the solvent is n = 1.333 for water at 25 ºC.

The degree of spectral overlap between the donor and the acceptor is described by the overlap integral ($J(\lambda)$). The absorption spectrum of the acceptor expressed as variations of the extinction coefficient as a function of λ ($\varepsilon_A(\lambda)$). The emission spectrum of the donor was normalized so that the value of the integral over λ from 0 to "infinity" was equal to 1 ($F_D(\lambda) = 1$). These spectra were used to calculate the overlap function and thus the overlap integral $J(\lambda)$:

$$J(\lambda) = \int_{\infty}^{0} F_D(\lambda) * \varepsilon_A(\lambda) * \lambda^4 d\lambda$$

where $\varepsilon_A(\lambda)$ is given in units of $M^{-1}cm^{-1}$ and λ is in nm, thus $J(\lambda)$ is in $M^{-1}cm^{-1}nm^4$. The overlap integral yielded $J(\lambda) = 4.39*10^{15}$ $nm^4M^{-1}cm^{-1}$, and the resulting Förster radius R_0 was determined to be 56.7 Å.

References

S1. M. C. Grenier, R. W. Davis, K. L. Wilson-Henjum, J. E. LaDow, J. W. Black, K. L. Caran, K. Seifert and K. P. C. Minbiole, *Bioorg. Med. Chem. Lett.*, 2012, **22**, 4055-4058.

S2. S. K. M. Nalluri, J. Voskuhl, J. B. Bultema, E. J. Boekema and B. J. Ravoo, *Angew. Chem. Int. Ed.*, 2011, **50**, 9747-9751.

S3. Y. Yan, H. Wang, B. Li, G. Hou, Z. Yin, L. Wu and V. W. W. Yam, *Angew. Chem. Int. Ed.*, 2010, **49**, 9233-9236.

S4. I. Coin, M. Beyermann and M. Bienert, *Nat. Protoc.*, 2007, **2**, 3247-3256.

S5. a) M. E. Bush, N. D. Bouley and A. R. Urbach, *J. Am. Chem. Soc.*, 2005, **127**, 14511-14517. b) F. Tian, D. Jiao, F. Biedermann and O. A. Scherman, *Nat. Commun.*, 2012, **3**.