Supplementary Material (ESI) for *PCCP*

Peptide Modified Mesoporous Silica Nanocontainers

Fabiola Porta[†], Gerda E. M. Lamers*, Jeffrey I. Zink[‡] and Alexander Kros[†]

[†]*Leiden Institute of Chemistry, Leiden University Einsteinweg 55 2300 RA Leiden The Netherlands*
a.kros@chem.leidenuniv.nl
**Leiden Institute of Biology, Leiden University Wassenaarseweg 72, 2333 BE Leiden The Netherlands*
‡*Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095US*

1. Materials and Methods.

Abbreviations

ACH, α-Cyano-4-hydroxycinnamic acid; APTES, (3-Aminopropyl)triethoxysilane; AAPTMS, γ-(2-Aminoethyl)aminopropyl trimethoxysilane, AEPTMS, (3-[2-(2-Aminoethylamino) ethylamino]propyltrimethoxysilane), CD, α-Cyclodextrin; CTAB, Hexadecyltrimethyl-ammonium bromide; DCC, Dicyclohexylcarbodiimide; DIPEA, N,N-diisopropylethylamine; DTT, DL-Dithiothreitol; EDT, 1,2-Ethanedithiol; FT-IR, Fourier-transform infrared spectroscopy; HCTU, 1H-Benzotriazolium 1-[bis(dimethylamino)methylene]-5-chloro-hexafluorophosphate Novabiochem; HeLa cells, Henrietta Lacks cells; ^{1}H-NMR, ^{1}H nuclear magnetic resonance; MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight; NMP, N-methylpyrrolidone, PBS, phosphate buffered saline; SEM, scanning electron microscope; TEA, triethylamine; TEM, transmission electron microscopy; TEOS, Tetraethyl orthosilicate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TRIS, tris(hydroxymethyl) aminomethane; XRD, X-Ray diffraction.

Materials. FMOC-protected amino acids and Sieber Amide resin (0.62 mmol of NH_2/g of resin) were purchased from Novabiochem. All other reagents and solvents were

obtained at the highest purity available from Sigma-aldrich, ABCR or Biosolve Ltd. And used without further purification. Milli-Q water with a resistance of more than 18.2 MΩ/cm was provided by a Millipore Milli-Q filtering system with filtration through a 0.22 μm Millipak filter.

Methods. ^{1}H-NMR spectra were recorded on a Bruker AV-500 spectometer or a Bruker DPX300 spectrometer using the residual proton resonance of deuterated water, acetonitrile, or chlorofom for calibration. FT-IR spectra were acquired with a BIORAD Excalibur Series FTS 4000 instrument. MALDI-TOF mass spectra were acquired using an Applied Biosystems Voyager System 6069 MALDI-TOF spectrometer. Samples were dissolved in 1:1 (v/v) 0.1% TFA in water:acetonitrile (TA), at concentrations of ~0.3 mg ml^{-1}. Solutions for spots consisted of (v/v) 1:10 sample solution: 10 mg ml^{-1} ACH in TA. TEM was conducted on a JEOL 1010 instrument with an accelerating voltage of 60 kV. Samples for TEM were prepared by placing a drop of each solution on carbon-coated copper grids. After ~ 10 minutes the droplet was removed from the edge of the grid. UV-VIS absorbance spectra were obtained with a PerkinElmer precisely Lambda 25 UV-VIS spectrometer. The particle shape was analyzed with a NovaSEM microscope. Flow cytometry was performed with a Beckham Coulter Cell Lab Quanta SC. Confocal laser scanning microscopy (CLSM) was performed using a Carl Zeiss Observer, LSM 5 exciter.

1.2 TAT Peptide synthesis

The TAT-peptide was synthesized with standard solid phase peptide synthesis protocols using FMOC-chemistry on an Applied Biosystems 431A automated peptide synthesizer,

with a PL-Sieber Amide resin on a 0.25-mmol scale. The amino acids (4 eq.) were activated with HCTU (4 eq.) and DIPEA (8 eq.) in NMP. The N-terminal FMOC-group was removed with 20% (v/v) piperidine in NMP. Every amino acid coupling was followed by capping non-reacted amino groups using 0.05 M acetic anhydride, 0.125M DIPEA in NMP. Peptides were cleaved from the resin for 5 h using a mixture of TIS:EDT:H2O:TFA (2.5:2.5:2.5:92.5 v/v%), followed by precipitation in cold diethyl ether. Deprotection of the cysteine was done with a standard procedure. A solution with the synthesized peptide in ammonium bicarbonate buffer 0,1M, with a concentration of 3 mg ml^{-1} of peptide, was added 20 equivalents of DTT for each mmole of peptide. The solution was blanket with Argon for 12 hours to remove the StBut group. Lyophilization resulted in a white powder at 80% yield. Maldi-TOF MS (Cys-Gly-Arg-Arg-Arg-Gln-Arg-Arg-Lys-Lys-Arg-Gly) = 1598.47 m/z.

2. General synthetic procedures of the nanovalves and characterization.

2.1 Synthesis of functionalized Silica Nanovalves. In a flask 2g Hexadecyltrimethyl-ammonium bromide (CTAB), was dissolved in 960 ml of Milli-Q water and 7 ml of 2N NaOH. The solution was heated at 80 °C for 30 min and 9 g of TEOS was added. After two hours, filtration of the reaction mixture yielded a white solid with a yield of 58%.

The surfactant was removed from the particles (5.2g) by refluxing overnight in a mixture of methanol (310 ml) and fuming hydrochloric acid 37% (31 ml) under an inert atmosphere. Filtration resulted in a white powder with a yield of 60%. The removal of CTAB from the silica particles was confirmed using FT-IR.

2.2 Characterization

SEM analysis of the silica nanoparticles showed that the particles were spherical (Figure S1) TEM microscopy revealed the mesoporous nature of the silica particles in order to check the mesoporosity (Figure S2).

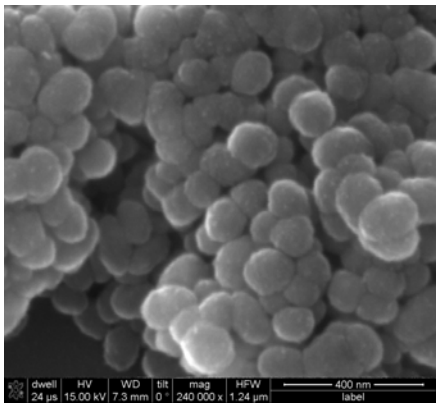


Figure S1. SEM image of SNP after CTAB removal.

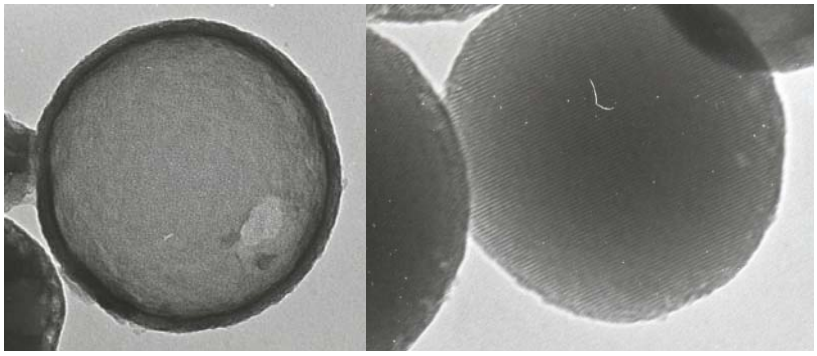


Figure S2. TEM images for SNP after CTAB removal from different sides.

The mesoporosity of the silica nanoparticles was confirmed with XRD and the 1.0.0 peak at $2\theta=2.19°$ corresponds, according to the Brag law, to a 2D-hexagonal mesostructure with lattice spacing of 2.8 nm (figure S3).

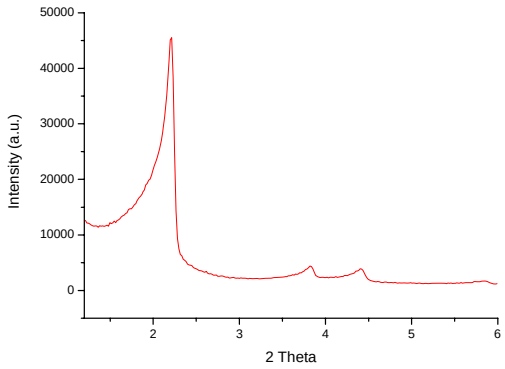


Figure S3. XRD-spectrum of mesoporous SNP after CTAB removal.

3. Organic Functionalization of the surface

3.1 Functionalization of SNP surfaces with APTES. To functionalise the surface of the particles, 100 mg of SNP was suspended in dried toluene with 0.1 mmol of APTES and refluxed overnight in an inert atmosphere. The suspension was filtered to get a white solid. ^{1}H-NMR was acquired with a Bruker Av-liq 400MHz, calibrated on the residual peaks of the deuterated TMS. AAPTMS and AEPTMS were coupled using the same procedure.

3.2 Functionalization of SNP with N-(2-Aminoethyl)-3-aminopropyltriethoxysilane (APTES-SNP). A suspension of 100 mg of mesoporous SNP was suspended in 10 ml of toluene with 0.1 mmol of N-(2-Aminoethyl)-3-aminopropyltriethoxysilane. The reaction was refluxed overnight; then the white solid was recovered by filtration and dried overnight at ambient pressure.

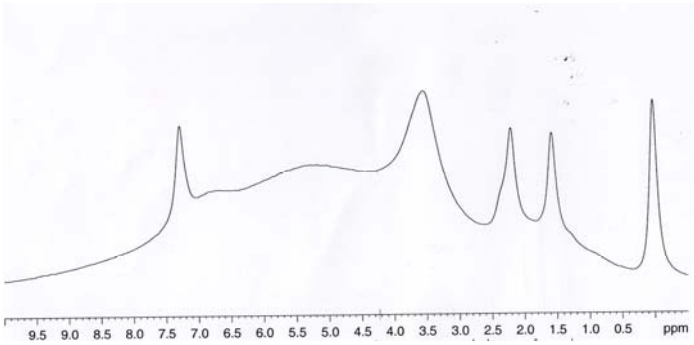
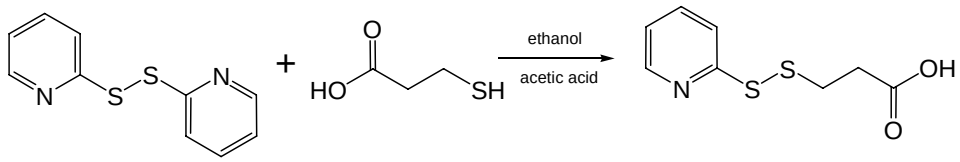


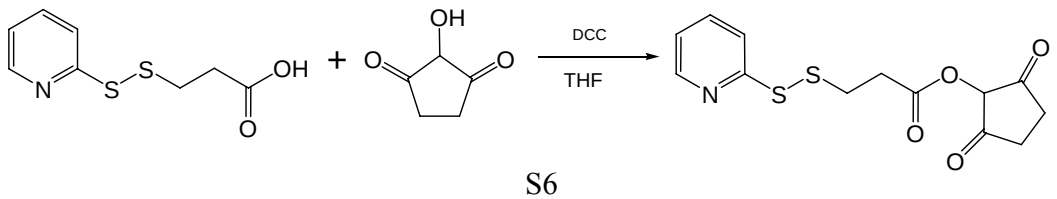
Figure S3. ^{1}H-NMR of SNP functionalized with APTES, the spectrum was done with LB=15.00 MHZ.

3.3 Synthesis of 2-Carboxyethyl 2-pyridil disulfide.[1]



2,2'-Dipyridyl disulfide (2.21 g) was dissolved in 10 ml of absolute ethanol and 0.4 ml of glacial acetic acid was added. The solution was stirred vigorously to dissolve the 2,2'-dipyridyl disulphide and 0.53 g of 3-mercaptopropionic acid in 5 ml of absolute ethanol was added drop wise. The reaction was stirred for 20 h, and after solvent removal a yellow product was recovered. Purification by column chromatography (eluent: CH_2Cl_2/MeOH 90:10 with 10% of glacial acetic acid) yielded a yellow oil (0.85 g, 80%). ^{1}H NMR ($CDCl_3$ δ) 2.80 (t, J=6.6 Hz, 2H). 3.07 (t, J=6.7 Hz, 2H), 7.15 (m, 1H), 7.65 (m, 2H), 8.48 (d, J=5.0 Hz, 1H).

3.4 Synthesis of N-succinimidyl 3-(2-pyridyldithio)-propionate.



A mixture of 2-Carboxyethyl 2-pyridil disulfide (1 g) and N-Hydroxysuccinimide (0.5 g) was dissolved in CH_2Cl_2 followed by the addition of Dicyclohexylcarbodiimide (0.9 g) was added to the solution at 0 °C. After 10min the ice bath was removed and the solution was stirred for 3.5 h at room temperature. The yellow solution was filtered through celite and a yellow oil was obtained after solvent removal (yield = 60%) . ^{1}H NMR ($CDCl_3$ δ) 2.85 (m, 4H), 3.20 (m, 4H), 6.78 (m, 1H), 7.31 (m, 1H), 7.45 (m, 1H), 7.68 (m, 1H).

3.5 SPDP coupling on the surface of SNP.

A mixture of 100 mg of APTES-SNP and 30 mg of SPDP was suspended in 10 ml of DMF and stirred overnight under an inert atmosphere. The reaction was filtered and a white-yellow solid was obtained. The same reaction was done with the SNP functionalized with the N-(2-Aminoethyl)-3-aminopropyltriethoxysilane and N^1-[3-(Trimethoxysilyl)-propyl]diethylenetriamine using the same experimental conditions. Figure S6 shows a ^{1}H-NMR of SNP after SPDP modification. The multiplicity of these spectra was lost due to the modification of the LB parameter, and the high amount of SNP in the suspension.

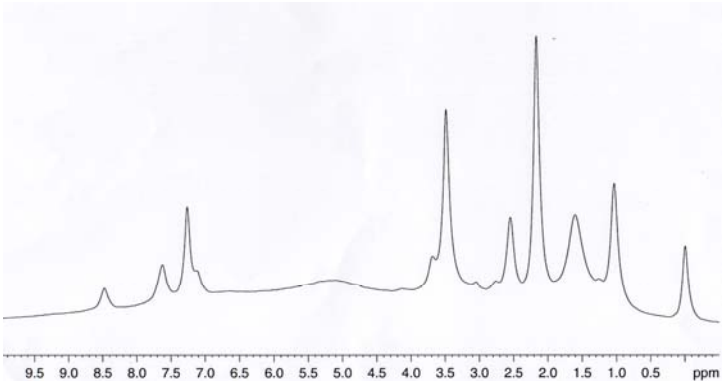


Figure S5. ^{1}H-NMR of SNP with SPDP modification, the spectrum was done with LB=15.00MHZ.

3.6 General preparation of peptide modified mesoporous silica nanocontainers. SPDP-functionalized silica nanoparticles were dispersed in an aqueous solution of sodium fluorescein (2.5mM) and stirred for overnight. The suspension was cooled down to 5 °C and α-CD (480 mg, 0.493 mmol) was added to the suspension and stirred overnight. An aqueous solution of the peptide stopper (0.1 mmol) was added to the suspension, followed by raising the pH to 9.00 using 2N NaOH. The mixture is stirred for 3 days at 5 °C under an inert atmosphere. The resulting orange nanoparticles were filtered, washed 3 times with water and dried under high vacuum.

3.8 General procedure for DTT activation of the Peptide functionalized system. In an eppendorf tube, 5 mg of Peptide functionalized SNP were dispersed in 1 ml of TRIS buffer (pH 8.5) and DTT (0.6 mmol) was added to reduce the disulfide bond. Release of fluorescein was measured as a function of time using UV-VIS spectroscopy. Release of fluorescein in the absence of DTT was also measured.

4. Fluorescein release graphs

4.1 Fluorescein release from nanovalves systems in the presence and absence of α-cyclodextrin

All the release experiments reported in this article have been performed in triplo, and each point is the result of the average.

In Figure S6 the release of fluorescein from PMSNs without cyclodextrins tethered on the aliphatic chain is shown. This graph highlights how the release is influenced by the presence of the cyclodextrin, when this component is not present on the chain the release starts at t=0 without any lag time. In Figure S8 the release (i.e. leakage) of fluorescein from PMSNs with [2] rotaxane tethered on the chain is shown.

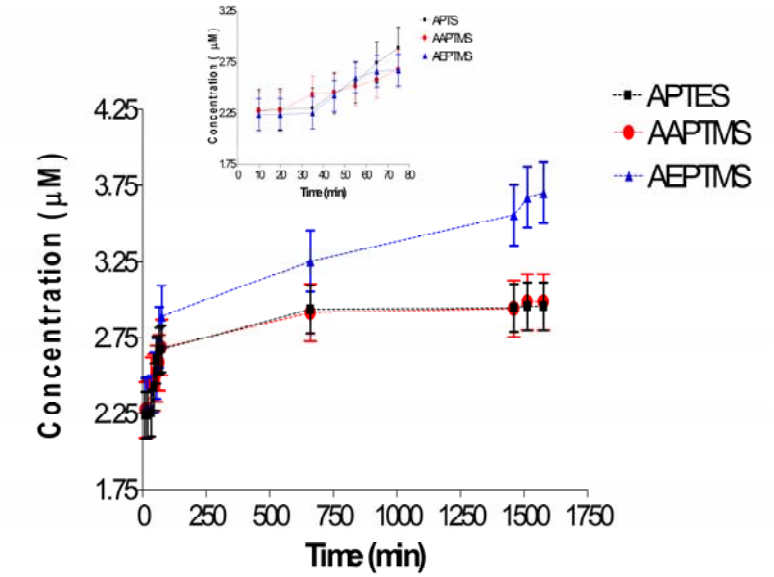


Figure S6. Release of fluorescein from PMSN as a function of the spacer composition. In all cases cyclodextrin was immobilized on an APTS (■), AAPTMS (●) and AEPTMS (▲) spacer. At t=0 DTT was added and typically a lag time of 30 minutes was observed.

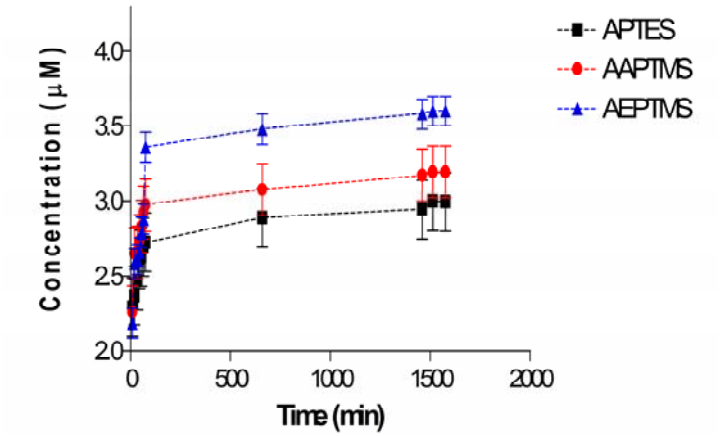


Figure S7. Release of fluorescein from PMSNs without α-cyclodextrin tethered on the aliphatic chain upon the addition of DTT (t = 0 min).

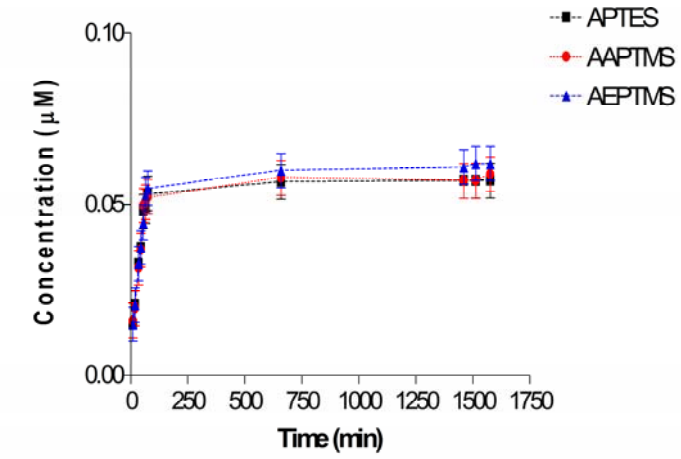


Figure S8. Release of fluorescein from PMSN as a function of the spacer composition in the absence of DTT. In all cases cyclodextrin was immobilized on the tether.

4.2 Fluorescein release experiment with glutathione.

5 mg of PMSNs with α-CD tethered on the medium linker AAPTMS, was suspended in a PBS solution (pH=7.2). Next, glutathione (0.3 mmol) was added and the fluorescein release was measured with UV-VIS spectroscopy as a function of time.

5. Flow cytometry

The cellular uptake of PMSNs was monitored with flow cytometry; the discrimination between internalized PMSNs and PMSNs only adsorbed on the surface, was examined using confocal laser scanning microscopy.

HeLa cells were cultured in DMEM medium at 37° C under a 5% atmosphere of CO_2. PMSNs were incubated with HeLa cells for one hour. The cells were washed with PBS/EDTA for 2 min and then with PBS for other 2 min. Next, the cells were trypsonized with a 5% solution of trypsin for 10 min at 37° C. Cells were harvested with DMEM and centrifuged to remove any residual amount of trypsin which could affect the results of the experiment. Therefore the cells were resuspended in DMEM and the suspension was analyzed using flow cytometry. The several washing steps ensures that particles that adhered to the outer membrane surface, if any, were removed.

Figure S9 shows the results of flow cytometry: 98% of the cells internalized the PMSNs while in a control experiment using bare silica particles the uptake was only 13%.

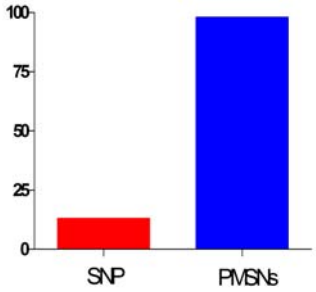


Figure S9. Percentage of HeLa cells with bare silica nanoparticles (SNP) and PMSNs internalized after a 2h incubation period.

Figure S10 shows a confocal laser scanning microscopy z-scan image of HeLa cells after incubation with PMSN (green) and with nuclei stained with DAPI (blue).

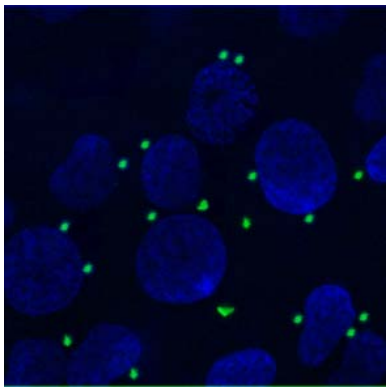


Figure S10. Confocal laser image of HeLa cells showing the effective uptake of PMSNs in the cytosol of the cell. PMSNs stained with fluorescein (green signal) and nuclei stained with DAPI (blue signal).

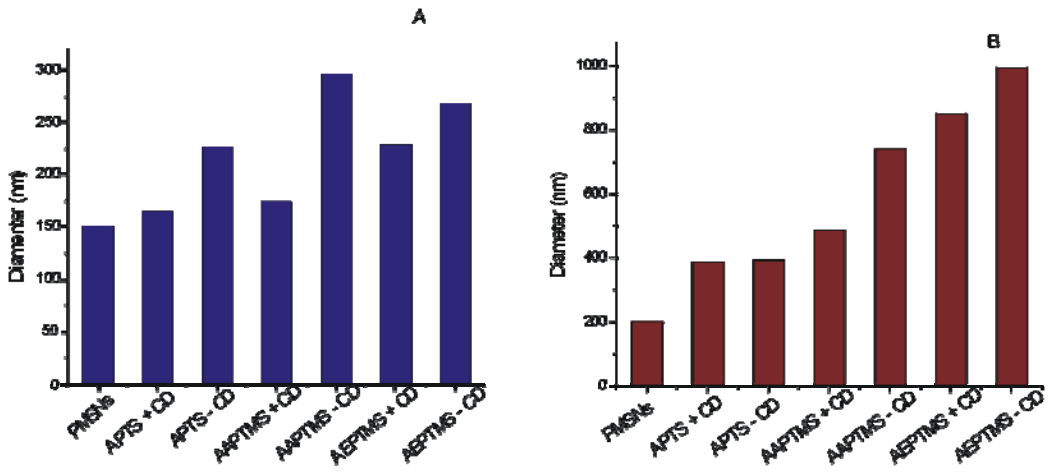


Figure S11. Observed hydrodynamic diameters of PMSN in MilliQ water (A) and in PBS buffer (B). The hydrodynamic diameter undergoes to a drastic change due to the functionalization of the surface. The presence of cyclodextrin influences the capacity of aggregation of particles due to the masking of positive charges of amino groups in the chain.

Reference

1. Carlssson Jan, D. H., Axen Rolf, Protein Thiolatio and Reversible Protein-Protein Conjugation. *Biochem. J.* **1977,** 173, 723-737.