Electronic Supporting Information

Synthesis and optimization of a reactive oxygen species responsive cellular delivery system

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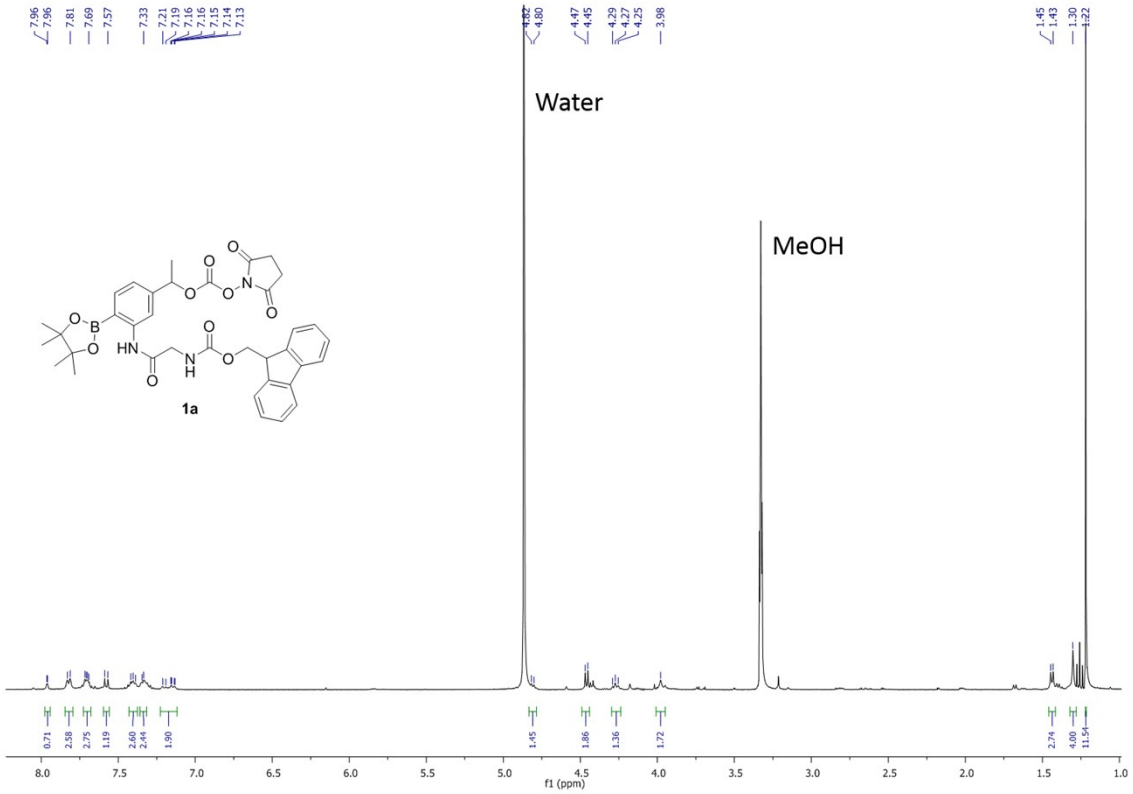
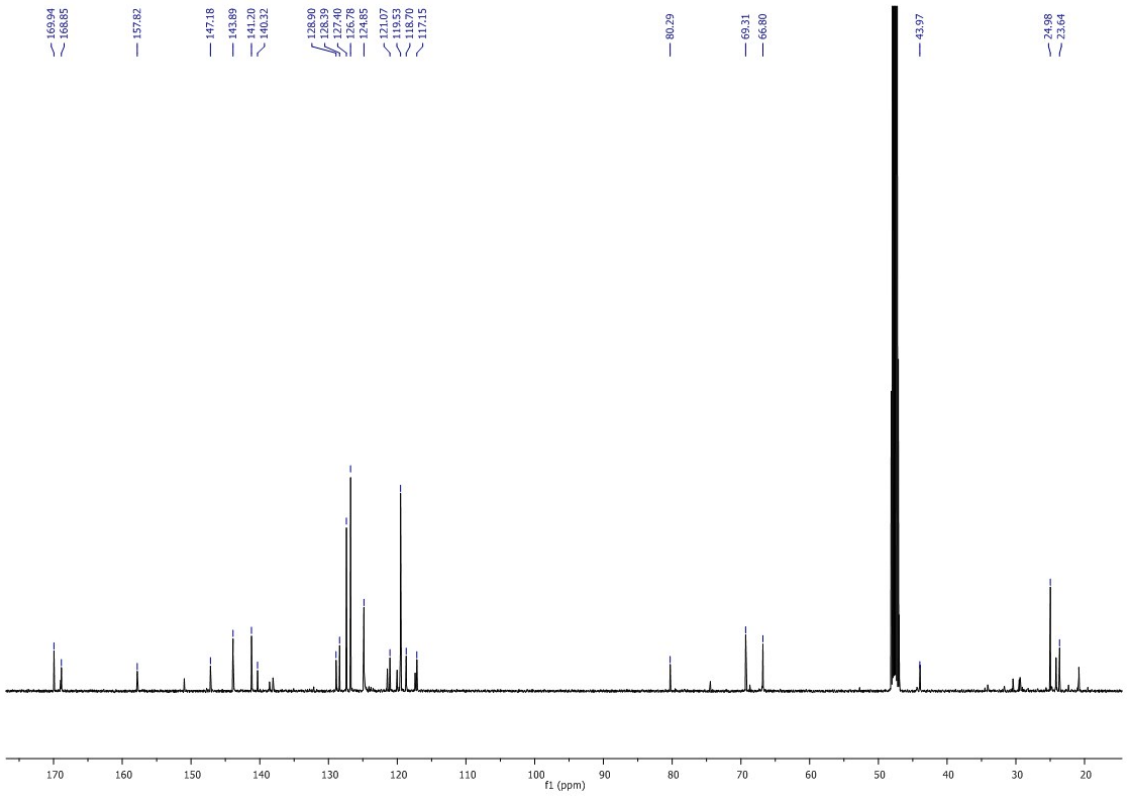
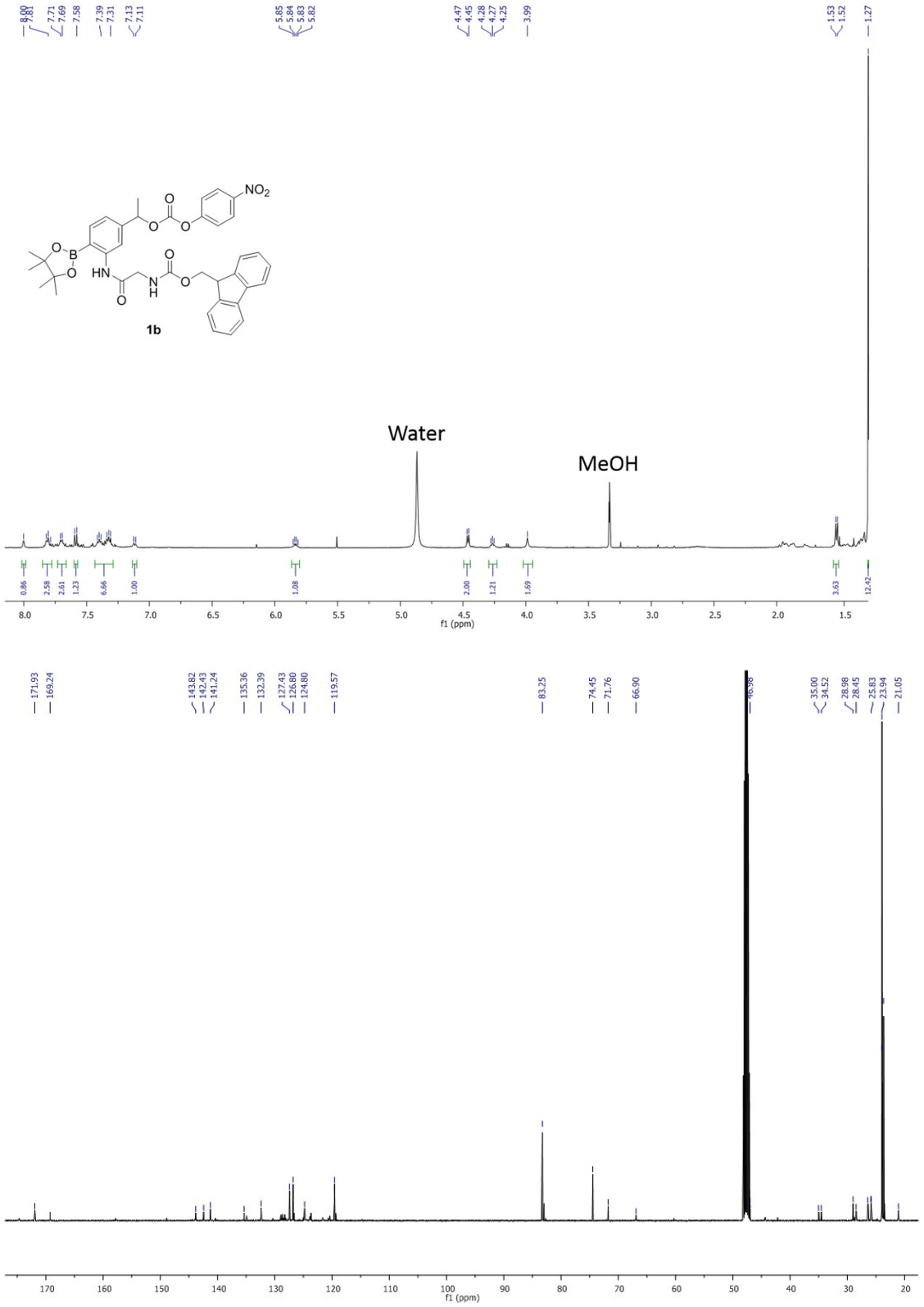
Table of Content

1. ^{1}H- and ^{13}C-NMR spectra of the linkers 1a and 1b

All NMR-spectra were recorded at ambient temperature in MeOD as solvent.







2. Synthesis of Cy5.5 and Cy7. Cyanine dyes were synthesized following the methodology reported before.[1]

3. Synthesis of ROS-responsive delivery systems

Solid-phase synthesis using orthogonal chemistry based on Dde and Fmoc protecting groups was used to allow the coupling of the cargos and the attachment of the ROS-cleavable linker for the synthesis of ROS-responsive delivery systems. Chemical abbreviations for N,N'-diisopropylcarbodiimide (DIC), ethyl (hydroxyimino)cyanoacetate (Oxyma), N-hydroxysuccinimide (NHS), 4-dimethylaminopyridine (DMAP), methyl red (MR), 5(6)-carboxyfluorescein (FAM), 6-aminohexanoic (Ahx), glutamic (Glu), lysine (Lys), trifluoroacetic acid (TFA), triisopropylsilane (TIS), 8-amino-3,6-dioxaoctanoic (PEG), diisopropylethylamine (DIPEA). The coupling/deprotection were monitored by a ninhydrin/chloranil test until completion.

Coupling of Fmoc-amino acids and carboxylic acids

The Rink amide resin (1eq) was pre-swollen in DCM and washed with DMF. For the amino functionalised microspheres (1mL, 1eq), were washed in DMF (3 x 1mL) and suspended in DMF (1mL). It was added a pre-activated mixture of the carboxylic acid (3eq) and DIC/oxyma (3eq of each) in DMF. This reaction mixture (0.1M in amino acid) was stirred in the microwave for 20 minutes at 60°C for Rink amide resin, and in the ThermoMixer® at 700 rpm for 2 hours at 60°C for polymeric microspheres.

Coupling of Cy5.5 and Cy7

Cyanine dyes (2eq) were activated using a mixture of DIC (2eq), N-hydroxysuccinimide (2eq) and DMAP (0.2eq) in DMF at a concentration of 0.2M overnight. The solution containing the NHS-activated cyanine dye was added to the Rink amide resin or the microspheres, and stirred on a rotary wheel for 4h. The mixture was protected from the light by aluminium foil.

Coupling of ROS cleavable linker **1a-1b**

ROS cleavable linker **1a or 1b** (3eq) was dissolved in 1mL of DMF, and DIPEA (for microspheres) or Et_3N (for Rink amide resin) (6eq) was added to this solution. This reaction mixture (0.1M of **1a or 1b**) was added to the polymeric microspheres or Rink amide resin (1eq), and they were shaking on a rotatory wheel overnight at room temperature.

Fmoc deprotection

The Rink amide resin or the polymeric microspheres were stirred with 20% piperidine in DMF for 10 minutes on a rotary wheel, washed with DMF and treated again with 20% of piperidine in DMF. The reaction mixture was again stirred on a rotary wheel for 10 minutes at room temperature. Resin was washed with DMF, DCM and MeOH, and microspheres were obtained by centrifugation and subsequently washed with DMF (3 x 1mL), MeOH (3 x 1mL), deionised water (3 x 1mL) and finally DMF (3 x 1mL).

Dde deprotection

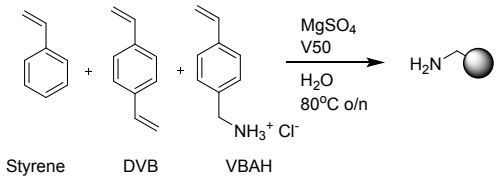
Dde deprotection was facilitated by treating resin or the microspheres with the solution mixture of $NH_2OH{\cdot}HCl$ 1.25g (1.80mmol) and 0.918g (1.35mmol) of imidazole suspended in 5mL of NMP, and the mixture was diluted in DMF/NMP (1:5) (1mL) for treating during 1 hour at r.t. on a rotary wheel, then resin or the microspheres were washed with DMF (1mL) and the entire process repeated under the same conditions. The resin was then washed using DMF, DCM and

MeOH, and microspheres were obtained by centrifugation and subsequently washed with DMF (3 x 1mL), methanol (3 x 1mL), deionised water (3 x 1mL) and finally DMF (3 x 1mL).

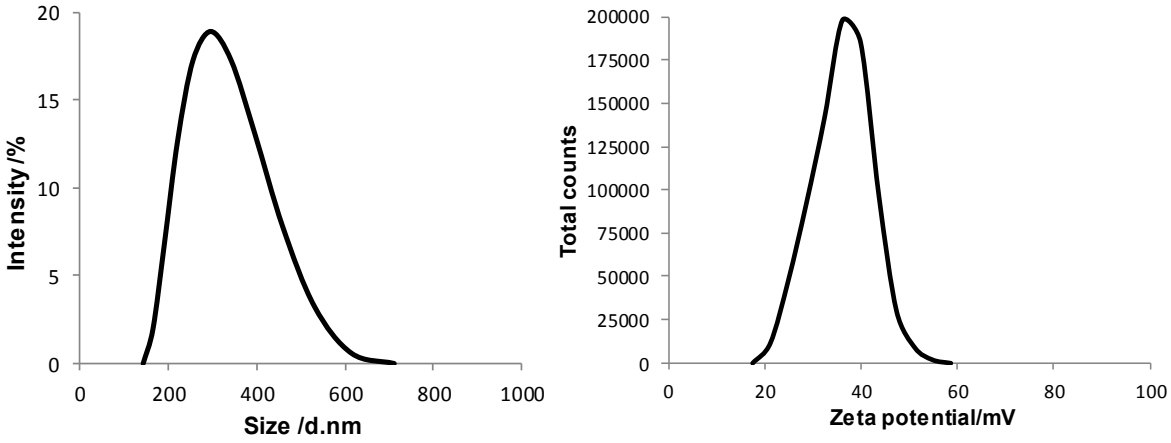
Rink amide resin cleavage with trifluoroacetic acid

A solution of TFA/TIS/DCM (90:5:5) was added to the resin (20 µL of the cleavage cocktail per mg of resin) and left to shake for 4 hours before washing with DCM. The reaction mixture was evaporated under reduced pressure and the desired compound precipitated using diethyl ether.

3.1. Characterization of ROS-responsive microspheres probe 15

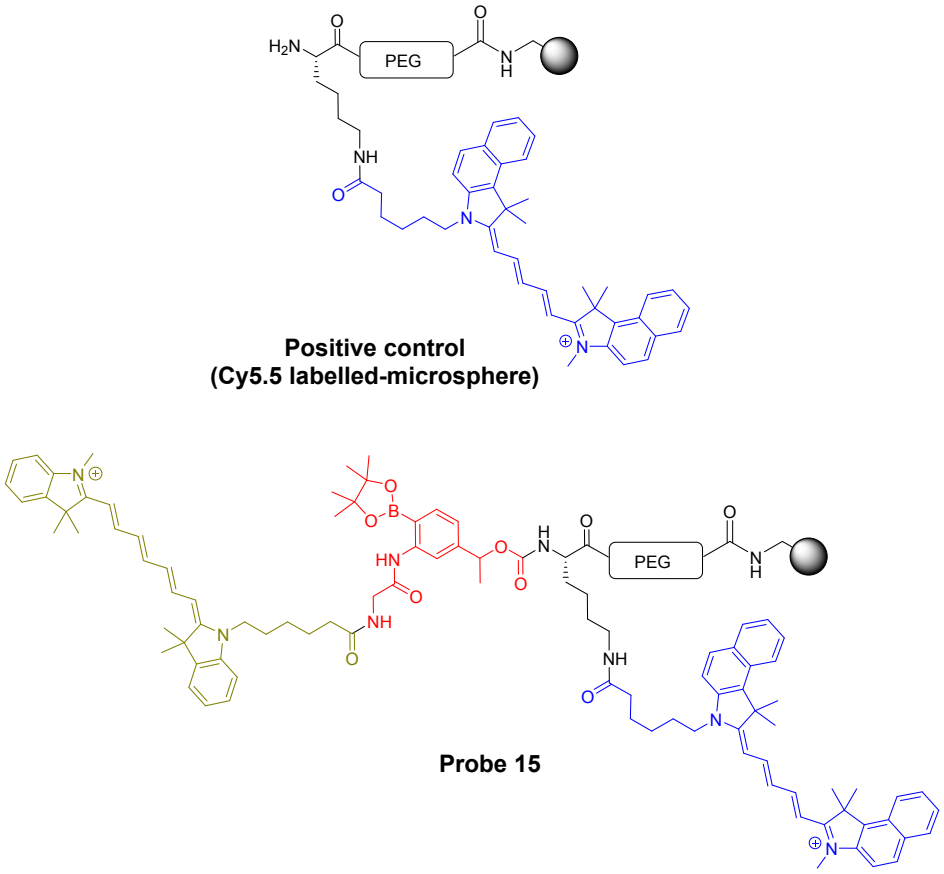


Supplementary Scheme 1. *Polymeric Microspheres (200nm) synthesis.*

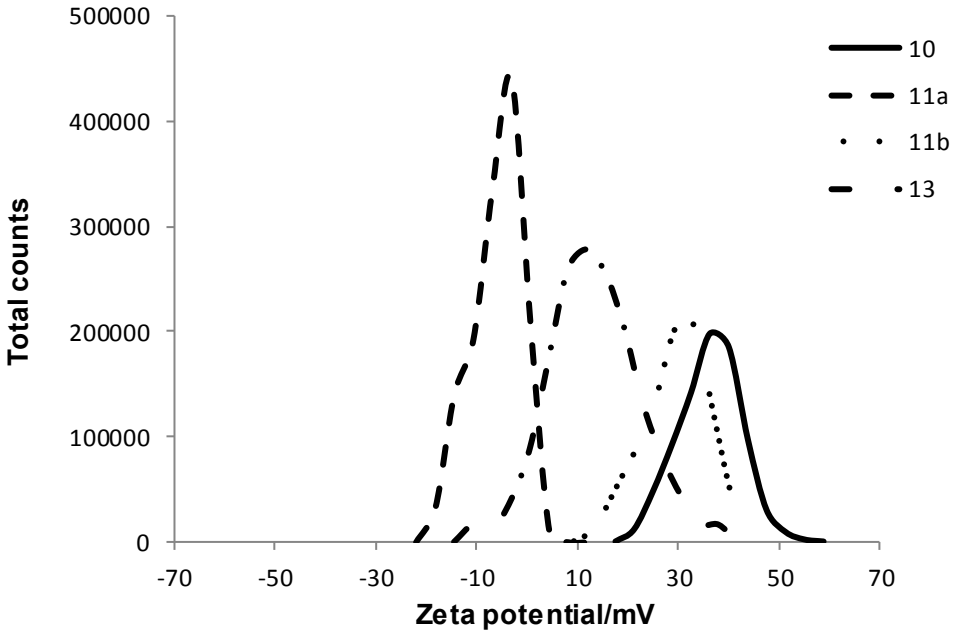


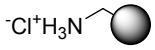
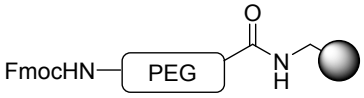
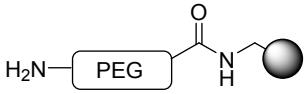
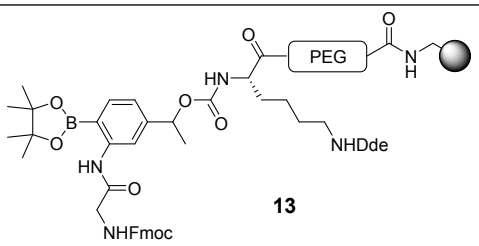
Sample	Mean diameter (nm)	Coefficient of variation (%)	Standard desviation (µm)	Ninhydrin test	Mean Zeta potential/mV
Microspheres	298	29.36	0.087	Positive	+ 40

Supplementary Figure 1*. Particle size and Zeta-potential.*



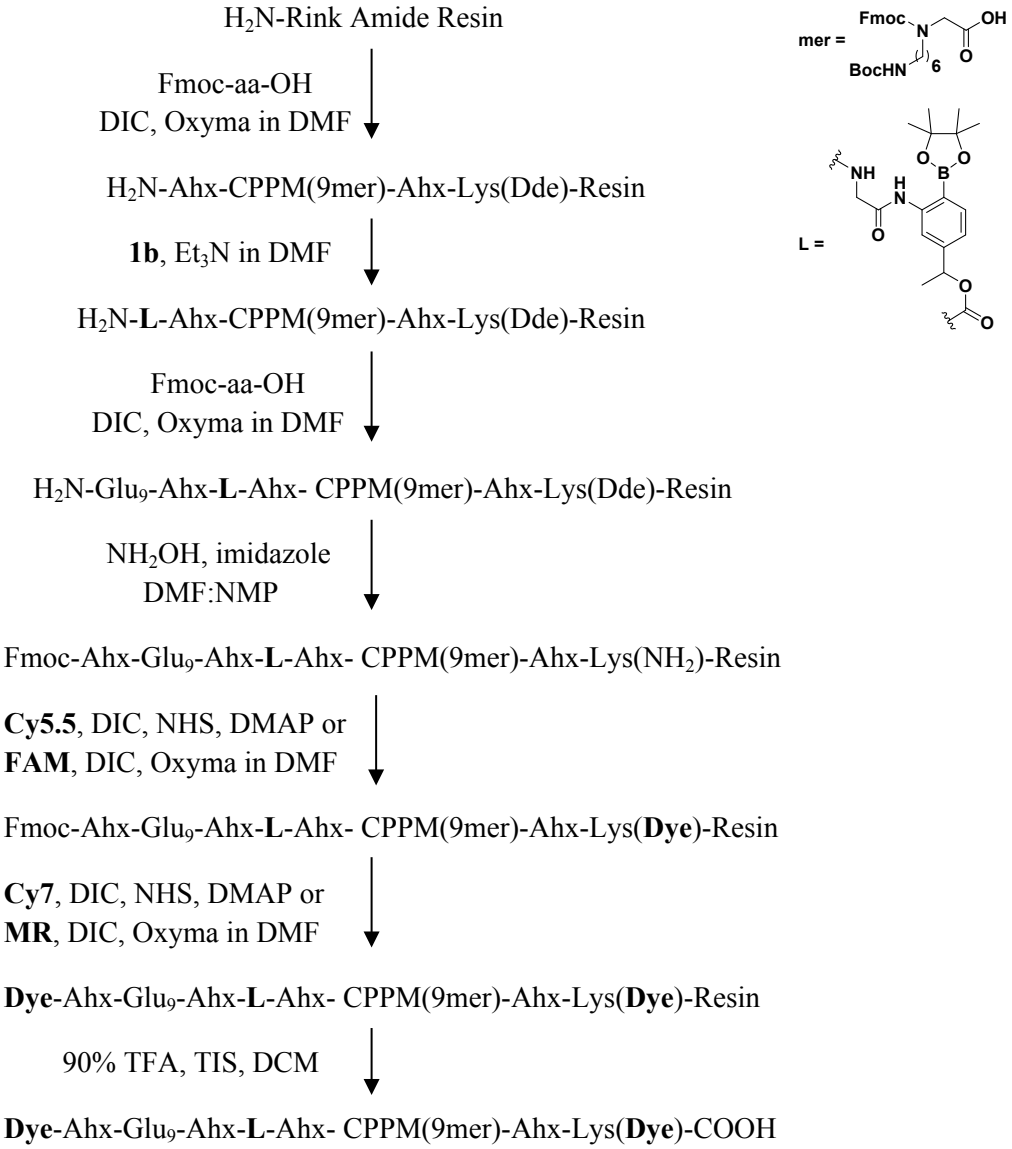
Supplementary Figure 2. *Cy5.5 labelled-microspheres (positive control) and ROS-responsive polymeric microspheres probe* ***15****.*



Particles	*Zeta Potential/mV*
 **10**	+40
 **11a**	-3
 **11b**	+30
 **13**	+11

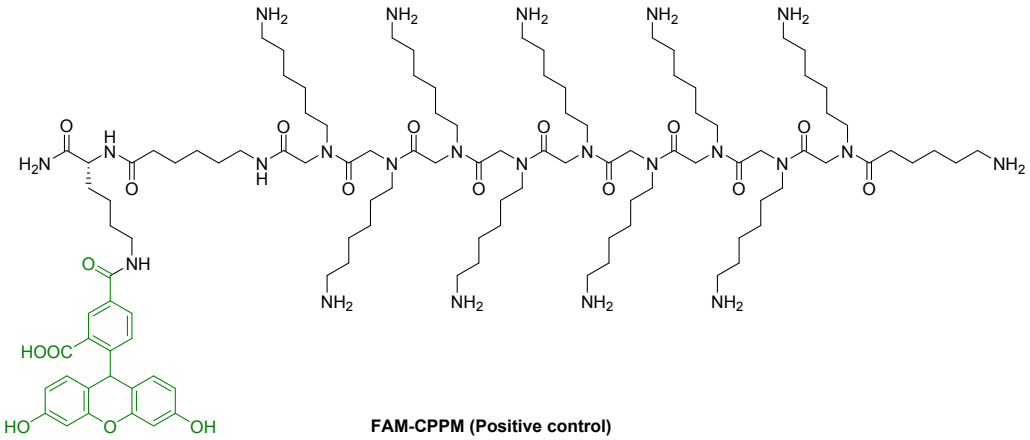
Supplementary Figure 3. *Colloidal stability and reaction control of 200 nm amino-functionalized beads. Zeta potential of amino beads and subsequently functionalized polystyrene microparticles.*

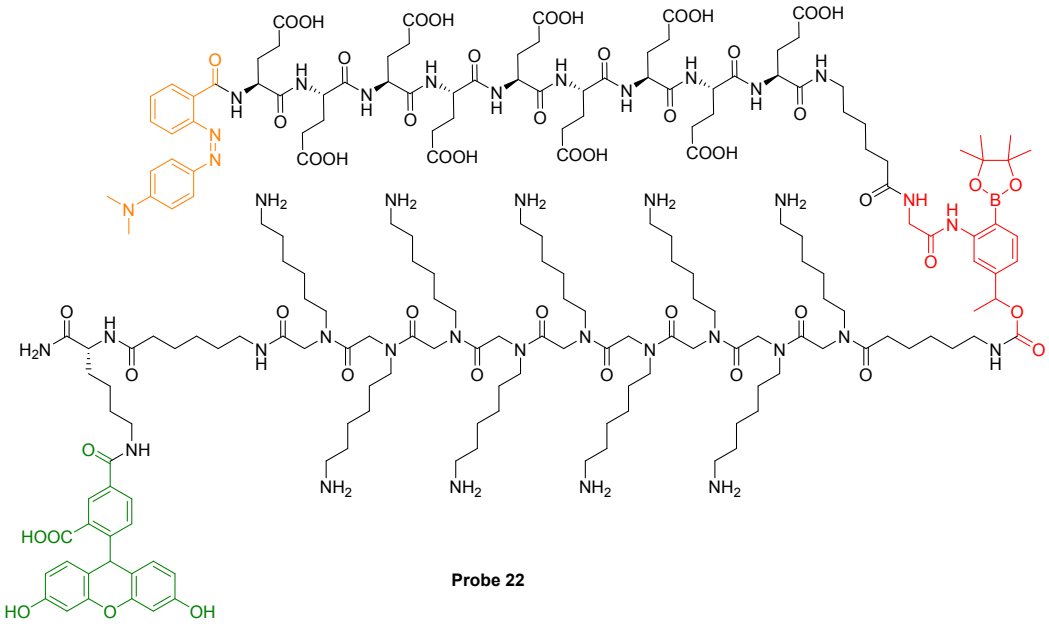
3.2. Solid-phase synthesis of ROS-responsive cell penetrating peptide mimetic (CPPM) probes 22 and 23.



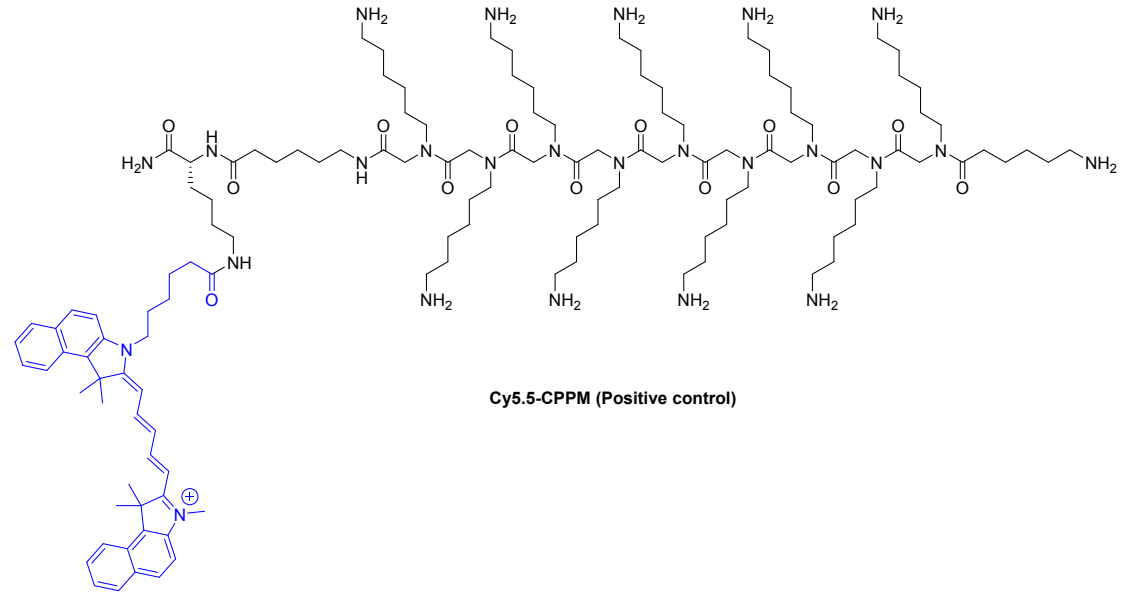
Supplementary Scheme 2. *General synthesis of ROS-responsive Cell Penetrating Peptide* ***22 and 23****.*

Structures of the probe **22-23** and controls

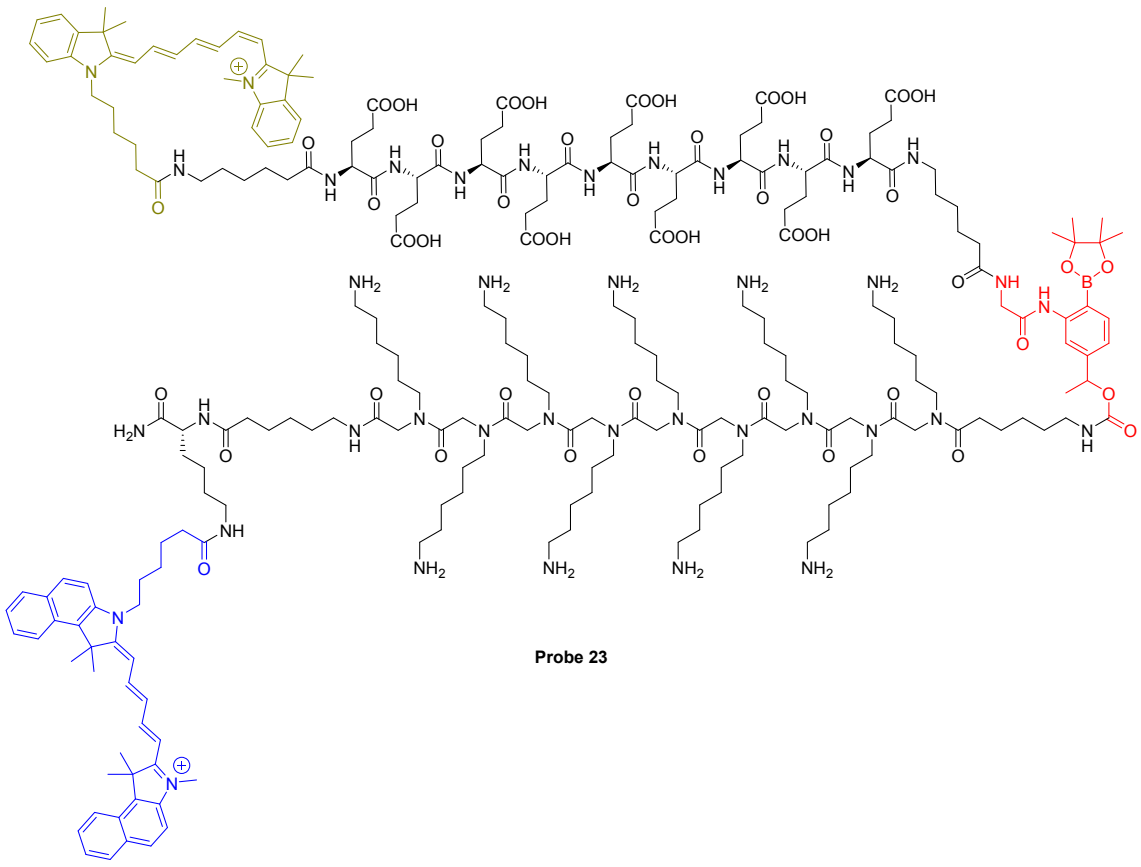




Supplementary Figure 4. *FAM positive control Cell Penetrating Peptide Mimetic (CPPM) and ROS-responsive Cell Penetrating Peptide probe* ***22****.*



Cy5.5-CPPM (Positive control)



Probe 23

Supplementary Figure 5. *Cy5.5 positive control Cell Penetrating Peptide Mimetic (CPPM) and ROS-responsive Cell Penetrating Peptide probe* ***23****.*

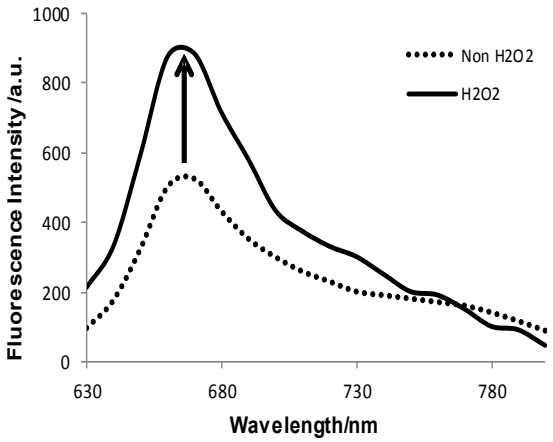
Supplementary Table 1. *MALDI-TOF-MS and HPLC (ELSD) analysis of positive controls and ROS-responsive Cell Penetrating Peptide probes* ***22 and 23****.*

Compound[a]	MW (calc.)	MALDI-TOF (*m/z*)	LCMS[b] MW (*m/z*)	ELSD Purity (%)	Structures
FAM-CPPM	2134.5	$(M)^+$ 2134.0	$(M+2)^{2+}/2$ 1068.8 $(M+3)^{3+}/3$ 713.0 $(M+4)^{4+}/4$ 535.0	100	Ahx-CPPM(9mer)-Ahx-Lys(FAM)
Cy5.5-CPPM	2343.2	$(M+H)^+$ 2344.5	$(M+4)^{4+}/4$ 585.3	100	Ahx-CPPM(9mer)-Ahx-Lys(Cy5.5)
22	4008.3	$(M+NH_4+H)^{2+}/2$ 2013.1 $(M+NH_4+Na)^{2+}/2$ 2024.2	$(M+4)^{4+}/4$ 1020.8 $(M+7)^{7+}/7$ 585.0 $(M+8)^{8+}/8$ 517.0	100	MR-Glu_9-Ahx-**Linker**-Ahx-CPPM(9mer)-Ahx-Lys(FAM)
23	4569.7	$(M+2ACN)^{2+}/2$ 2328.8	$(M+3)^{5+}/5$ 937.1 $(M+4)^{6+}/6$ 781.1 $(M+6)^{8+}/8$ 592.8	100	Cy7-Ahx-Glu_9-Ahx-**Linker**-Ahx-CPPM(9mer)-Ahx-Lys(Cy5.5)

[a] Cell Penetrating Peptide Mimetic (CPPM).

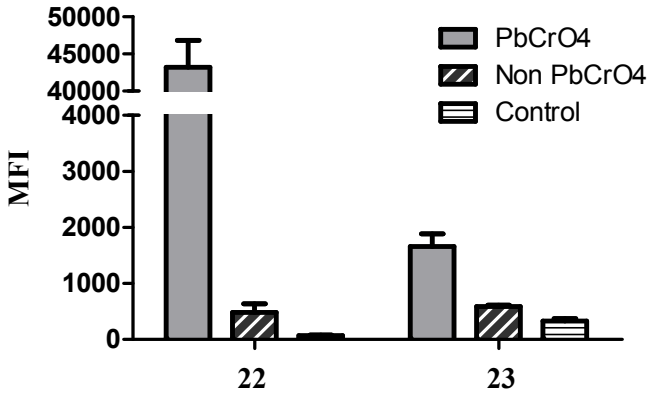
[b] MALDI-TOF and LCMS adducts were obtained in positive mode showing multicharges for **probe 22** (ions by addition of $Na^+/NH_4^+/H^+$) and for **probe 23** with positively charged cyanine dyes +2 (ions with acetonitrile (ACN) clusters or by addition of H^+).[2]

4. Sensitivity test.

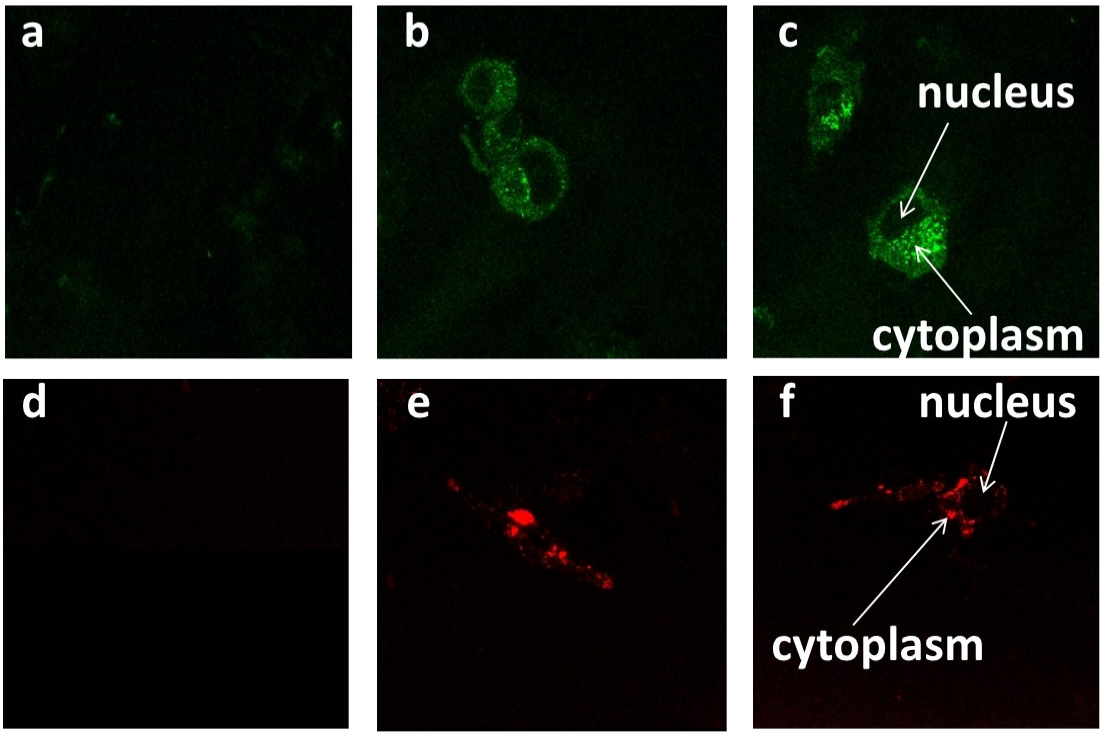


Supplementary Figure 6. *Fluorescent spectra of the ROS-responsive microspheres (**15**) before and after the addition of H_2O_2 (40mM).*

5. Cellular assays.



Supplementary Figure 7. *Flow cytometry assay of RAW cells incubated with the **probe 22 and 23** (5μM) before and after the addition of $PbCrO_4$ (150 μM) showing a large shift to higher fluorescent intensity (x10-20 fold). Mean Fluorescence Intensity (MFI) for **22** in the FITC channel and for **23** in the APC channel.*



Supplementary Figure 8. *Confocal microscopy of RAW cells after incubation with ROS-probe* ***22 and 23*** *(5μM) without (**a, d**) and with (**b, e**) $PbCrO_4$. (150 μM) (**c, f**) Images of FAM-CPPM and Cy5.5-CPPM (**positive controls**) in RAW cells in the GFP and RFP channel. Objective: HCX PLAPO x63/1.40-0.6 Oil CS.*

6. References

1. M. Lopalco, N.E. Kioni, J.K. Cho and M. Bradley. *Org. Biomol. Chem.,* 2009, **7**, 856-859.

2. H. Tong, D. Bell, K. Tabei and M.M. Siegel, *J. Am. Soc. Mass Spectrom.,* 1999, **10**, 1174-1187.