Electronic Supplementary Material (ESI) for Chemical Communications

Supporting information for:

Synthesis and in vitro evaluation of a multifunctional and surface-switchable nanoemulsion platform

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Materials

All reagents, chemicals, materials and solvents were obtained from commercial sources, and were used as received: Biosolve, Merck and Cambridge Isotope Laboratories for (deuterated) solvents; and Aldrich, Acros, ABCR, Merck and Fluka for chemicals, materials and reagents. All solvents were of AR quality. Moisture or oxygen-sensitive reactions were performed under an atmosphere of dry Ar. Hygroscopic compounds (*e.g.* containing ethylene oxide chains) were stored under vacuum in a dessicator over P_2O_5. Bio-Beads S-X1 was obtained from Bio-Rad Laboratories. FMPB–AM resin (100–200 mesh, loading 0.98 mmol g^{-1}) was obtained from Novabiochem. DSPE was obtained from Lipoid GmbH. Alkynylated resin **1** was prepared following a literature procedure[1] by reductive amination of FMPB–AM resin using propargylamine. 3,6,9,12,15-Pentaoxa-17-azido-heptadecan-1-ol was prepared according to a literature procedure.[2] H-Gly-2-ClTrt resin (2-chlorotrityl resin pre-loaded with glycine, particle size 75-150 µm, loading 1.1 mmol g^{-1}) was obtained from Aldrich. *O*-(2-Azidoethyl)nonadecaethylene glycol was obtained from Polypure. c[RGDfK] was prepared according to a literature procedure.[3] 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-3000] ammonium salt (mPEG3000-DSPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-350] ammonium salt (mPEG350-DSPE), cholesterol and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl ammonium salt (Rho-DSPE) were all purchased from Avanti Polar Lipids. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-1000] Biotin (Biotin-PEG1000-DSPE) was purchased from Creative PEGWorks (NC, USA). MMP2p cleavable lipid (mPEG-MMP2p-DSPE) and c[RGDfK]-DSPE were synthesized by SyMO-Chem (Eindhoven, the Netherlands, www.symo-chem.nl). Human MMP2 and fluorogenic MMP2 substrate MCA-P-L-OH were purchased from R&D Systems. Simvastatin was purchased from AK Scientific (CA, USA). The oleic acid coated magnetite particles (Fe_3O_4) of 10 nm was purchased from NN-Labs (AR, USA). Chloroauric acid, 1-dodecanethiol, sodium borohydride, methyl trioctylammonium chloride, and cis-9-octadecene-1-thiol for gold nanoparticle synthesis were purchased

from Sigma-Aldrich (MO, USA). Human umbelical vein endothelial cells (HUVEC) and murine macrophage cell line (J774A1) were purchased from ATCC (Manassas, VA). Fetal bovine serum (FBS) and endothelial basal medium (EBM-2) were purchased from Lonza (Allendale, NJ). For atomic force microscopy (AFM) experiments, gold-coated silicon wafers were purchased from Sigma Aldrich, and magnetically actuated iDrive AFM cantilevers were obtained from Asylum Research (Santa Barbara, CA).

Abbreviations

Tetramethylsilane (TMS), evaporative light scattering detector (ELSD), room temperature (r.t.), *N*,*N*-dimethylformamide (DMF), 9-fluorenylmethoxycarbonyl (Fmoc), *N*-methylpyrrolidone (NMP), *O*-benzotriazole-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU), diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), total ion current (TIC), distearoylphosphatidylethanolamine (DSPE), (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO), 2,3,5,6-tetrafluorophenol (TFP), pyridinium *p*-toluenesulfonate (PPTS), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI·HCl), size-exclusion chromatography (SEC), polyethylene glycol (PEG), methoxypolyethyleneglycol (mPEG), hexaethyleneglycol (HEG), ethylene glycol (EG).

Methods

^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR and ^{31}P-NMR spectra were recorded on a Varian Mercury (400 MHz for ^{1}H-NMR, 100 MHz for ^{13}C-NMR, 376 MHz for ^{19}F-NMR and 162 MHz for ^{31}P-NMR) spectrometer at 298 K. Chemical shifts are reported in ppm downfield from TMS at r.t. using deuterated chloroform ($CDCl_3$) as a solvent and internal standard unless otherwise indicated. Abbreviations used for splitting patterns are s = singlet, t = triplet, q = quartet, m = multiplet, dd = double doublet and dm = double multiplet. IR spectra were recorded on a Perkin Elmer 1600 FT-IR (UATR). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a PerSeptive Biosystems Voyager-DE PRO spectrometer using an α-cyano-4-hydroxycinnamic acid matrix. Analytical thin layer chromatography was performed on Kieselgel F-254 precoated silica plates. Column chromatography was carried out on Screening Devices B.V. flashsilica gel (40-63 μm mesh). Automated peptide synthesis was carried out on a Protein Technologies Prelude. Preparative size exclusion chromatography was performed using Bio-Rad Bio-Beads S-X1 (200-400 mesh). GPC was measured on a Shimadzu LC-10AD VP system with a RID-10A detector and a SPD-M20A diode array detector using a PL gel 5μm mixed-D column and $CHCl_3$ as the eluting solvent (flow rate 1 mL min^{-1}). LC-MS was performed using a Shimadzu LC-10 AD VP series HPLC coupled to a diode array detector (Finnigan Surveyor PDA Plus detector, Thermo Electron Corporation) and an Ion-Trap (LCQ Fleet, Thermo Scientific). Analyses were performed using a Alltech Alltima HP C18 3μ column using an injection volume of 1-4 μL, a flow rate of 0.2 mL min^{-1} and typically a gradient (5% to 100% in 10 min, held at 100% for a further 3 min) of CH_3CN in H_2O (both containing 0.1% formic acid) at 298 K. Preparative RP-HPLC (CH_3CN / H_2O with 0.1% formic acid) was performed using a Shimadzu SCL-10A VP coupled to two Shimadzu LC-8A pumps and a Shimadzu SPD-10AV VP UV-vis detector on a Phenomenex Gemini 5μ C18 110A column. The gradient for 8 comprised, % CH_3CN (min): 5 (0-1), 5 to 100 (1-11), 100 (11-13), 100 to 5 (13-14), 5 (14-15) whereas the gradient for **10** comprised, % CH_3CN (min): 18 to 20 (0-11), 20 to 100 (11-12), 100 (12-13), 100 to 18 (13-14), 18 (14-15).. Analytical RP-HPLC (THF / H_2O with 0.1% TFA) was performed using a Shimadzu LC-10AD VP

coupled to a Shimadzu SPD-10AV VP UV-vis detector and an Alltech ELSD 2000ES on a GL sciences Inertsil ODS-3 5µ column. The gradient comprised, % THF (min): 20 (0-1), 20 to 95 (1-18), 95 (18-24), 95 to 20 (24-26), 20 (26-35).

Synthesis of mPEG-MMP2p-DSPE

The synthesis of **mPEG-MMP2p-DSPE** is shown in Schemes S.1-S.3. An alternative route using tosyl (Tos) protected arginine was also explored (*i.e.* after cleavage from the resin, the arginine in GPLGVRGG still bears a protecting group). This procedure yields a very clean coupling to mPEG2000 (Scheme S.3) but, following the subsequent successful click reaction, the harsh conditions for cleaving the Tos group are incompatible with the DSPE unit.

*Fmoc-GPLGVRGG(-≡)-resin (**2**)*

The GPLGVRGG sequence was synthesized using standard Fmoc coupling chemistry[4] on alkynylated resin **1**[1]. After swelling the resin in NMP for 20 min, subsequent amino acid couplings were carried out by adding fmoc-protected amino acid (4 eq to the resin loading), HBTU (8 eq) in NMP and DIPEA (16 eq) in NMP to the reaction vessel. The contents of the reaction vessel was mixed for 20 min under a nitrogen atmosphere. After draining the contents and washing with NMP, the amino acid coupling step was repeated with fresh reactants. The best results for obtaining pure peptide were obtained when the R-coupling step was performed 6 times and when both the R-coupling as well as the initial two G-couplings were followed by a capping step using acetic anhydride (30 eq) and pyridine (36 eq) in NMP. Fmoc deprotection during SPPS was performed by mixing the resin in 20 v/v % piperidine in NMP for 5 min with two repetitions, followed by washing the resin with NMP (3×). After the sequence was completed, the resin was washed with NMP (4×) and CH_2Cl_2 (4×) and allowed to dry under vacuum. Fmoc-GPLGVRGG(-≡)-resin **2** was stored at 5 °C under an Ar atmosphere for further functionalization. For analysis of **2**, peptide cleavage from the resin and removal of side-chain protecting groups was accomplished by stirring the resin (typically 5 mg) in a mixture of TFA and H_2O (95:5 v/v %, 1 mL) for 3 h at r.t. The resin was removed by filtration and washed with a small amount of TFA, after which the peptide was precipitated out of the filtrate by the addition of cold diethyl ether (25 mL). After centrifugation and decantation, the crude peptide was dissolved in H_2O and lyophilized to yield a white fluffy solid. Due to the optimized reaction conditions in SPPS, the peptide was obtained pure enough (~95%) to continue synthesis without additional purification. ESI-MS: *m/z* Calc. 970.50 g/mol; Obs. $[M+H]^+$ 971.8, $[M+2H]^{2+}$ 486.3 g/mol. For LC-MS characterization (TIC, positive mode) see Figure S.3.

*NH_2-GPLGVRGG-≡ (**3**)*

In a 25 mL round-bottom flask, 20 v/v % piperidine in CH_2Cl_2 (10 mL) was added to **2** (340 mg, theoretically 347 µmol peptide) and the mixture was gently stirred at r.t. for 2 h. The solution was filtrated, the resin was washed extensively with CH_2Cl_2 and transferred to a 25 mL round-bottom flask. TFA / H_2O (95:5 v/v %, 10 mL) was added and the solution was gently stirred at r.t. for 3 h. The resin was removed by filtration and washed with a small amount of TFA, after which the peptide was precipitated out of the filtrate by the addition of cold diethyl ether (25 mL). After centrifugation and decantation, the crude peptide was dissolved in H_2O and lyophilized yielding a white fluffy solid. ESI-MS: *m/z* Calc. 748.43; Obs. $[M+H]^+$ 749.50, $[M+2H]^{2+}$ 375.33.

*2-((17-Azido-3,6,9,12,15-pentaoxaheptadec-1-yl)oxy) acetic acid (**4**)*

In a 100 mL round-bottom flask, a suspension was prepared by adding powdered NaOH (2.2 g, 54 mmol, 12 eq) to a solution of 3,6,9,12,15-pentaoxa-17-azido-heptadecan-1-ol (1.43 g, 4.65 mmol) in DMSO (20 mL). After stirring at r.t. for 15 min, bromoacetic acid (3.9 g, 27.2 mmol, 5.9 eq) in DMSO (8 mL) was added to the stirred and water cooled suspension (modest exothermic effect). The thick white suspension was stirred at r.t. under an Ar atmosphere for 22 h. Then H_2O (20 mL) was added and the clear solution was stirred for another 2 h. More H_2O (60 mL) was added and the basic aqueous layer was washed with $CHCl_3$ (4 × 25 mL). The aqueous layer was acidified to pH = 0 using concentrated HCl and extracted with $CHCl_3$ (4 × 25 mL) after which the combined organic layers were evaporated to dryness *in vacuo*. In order to lose the DMSO the residue was redissolved in 1 M HCl (100 mL) and the aqueous layer was extracted with $CHCl_3$ (4 × 25 mL). This procedure was then repeated and the combined organic layers were dried using $MgSO_4$. After filtration and removal of the solvent *in vacuo*, **4** was obtained as a colorless oil (1.05 g, 2.87 mmol, 62%). ^{1}H NMR: δ = 4.2 (s, 2H, CH_2COOH), 3.7 (m, 2H, OCH_2), 3.6 (m, 20H, OCH_2), 3.4 (t, 2H, CH_2N$_3$). ^{13}C-NMR: δ = 172.2 (*C*OOH), 71.1, 70.6-70.2, 69.9 (*C*H$_2$O), 68.9 (*C*H$_2$O), 50.6 (*C*H$_2$N$_3$). ESI-MS: *m/z* Calc. 365.18 g/mol; Obs. $[M+H]^+$ 365.9 g/mol. FT-IR (ATR): υ (cm^{-1}) = 3478, 2871, 2101 (N$_3$), 1736, 1639, 1452, 1348, 1284, 1247, 1092, 944, 850, 672.

*N_3-HEG-DSPE (**5**)*

In a 50 mL round-bottom flask, **4** (0.60 g, 1.64 mmol) was coevaporated with toluene (3×) and then dissolved in dry DMF (4 mL). DIPEA (0.85 g, 6.51 mmol, 4 eq) and HBTU (0.57 g, 1.52 mmol, 0.93 eq) were subsequently added and the mixture was stirred at r.t. for 15 min. Dry $CHCl_3$ (on 4Å molsieves, 12 mL) and DSPE (1.02 g, 1.60 mmol, 0.98 eq) were added and the resulting suspension was stirred at 50 °C for 1½ h, yielding a clear solution after ~20 min. $CHCl_3$ (120 mL) was added and the organic layer was washed with 0.5 M citric acid (pH = 2, 2 × 40 mL, second time with added NaCl) and H_2O (50 mL). The combined organic layers were dried using Na_2SO_4, filtrated and the solvent was removed *in vacuo*. After coevaporation with toluene (3×) and DMF removal *in vacuo* using an oil pump (40 °C) the residue was stirred in cold CH_3CN (150 mL) for 30 min. The suspension was centrifuged, the supernatant was decanted and the solid was washed with cold CH_3CN, after which centrifugation, decantation and drying of the solid afforded a beige waxy product. Purification using column chromatography (flash SiO_2, gradient CH_2Cl_2 to 20% MeOH in CH_2Cl_2) yielded almost pure product. Final purification was achieved by dissolving the compound in $CHCl_3$ (280 mL) and washing with sat. $NaHCO_3$, brine, 1 M HCl (2×) and brine (all 100 mL), respectively. Drying with $MgSO_4$, filtration and removal of the solvent *in vacuo* yielded **5** as a waxy off-white solid (1.32 g, 1.18 mmol, 74% based on DSPE). ^{1}H-NMR: δ = 7.79 (t, 1H, N*H*), 5.22 (m, 1H, C*H*), 4.35 (dd, 1H, C*H*HO), 4.19-4.08 (m, 5H, CH*H*O, POCH_2), 4.04 (s, 2H, CH_2CONH), 3.72-3.61 (m, 22H, CH_2O), 3.58 (q, 2H, CH_2NHCO), 3.40 (t, 2H, CH_2N$_3$), 2.32 (m, 4H, CH_2COO), 1.61 (m, 4H, CH_2CH$_2$COO), 1.34-1.21 (m, 56H, CH_2), 0.88 (t, 6H, CH_3).^{13}C-NMR: δ = 173.3 (*C*=O), 172.9 (*C*=O), 171.2 (*C*=O), 70.8-69.5, 66.0, 64.7, 62.0, (*C*H$_2$O), 50.6 (*C*H$_2$N$_3$), 39.6 (CONH*C*H$_2$), 34.2, 34.0, 31.9, 29.7-29.1, 24.8, 22.5 (aliphatic *C*H$_2$), 14.1 (*C*H$_3$). ^{31}P-NMR: δ = -0.4. FT-IR (ATR): υ (cm^{-1}) = 2955, 2916, 2873, 2849, 2104 (N$_3$), 1737, 1665, 1546, 1467, 1346, 1329, 1290, 1273, 1254, 1236, 1215, 1194, 1175, 1099, 1042, 992, 949, 852, 759, 720, 673. MALDI-TOF-MS: *m/z* Calc. (Na-salt of **5**) 1116.73 g/mol; Obs. $[M+H]^+$ 1117.7 g/mol, $[M+Na]^+$ = 1139.7 g/mol.

*mPEG2000-COOH (**6**)*

In a 500 mL round-bottom flask, mPEG2000 (2.50 g, 1.25 mmol), TEMPO (0.18 g, 1.13 mmol, 0.9 eq) and NaBr (0.18 g, 1.73 mmol, 1.4 eq) were dissolved in 0.25 M Na_2CO_3 (180 mL, pH = 11). On an ice bath, NaOCl (6.2 mL (13% active chorine), 13.0 mmol, 10.4 eq) was added dropwise and the mixture was stirred at r.t. for 45 min. 1 M $NaHSO_3$ (150 mL) was added and the solution was acidified to pH = 2 using concentrated HCl. The aqueous layer was extracted with $CHCl_3$ (3 × 100 mL) and the combined organic fractions were dried using $MgSO_4$. After filtration, the solvent was removed *in vacuo* yielding **1** as a white solid (2.50 g, 1.24 mmol, 99%). ^{1}H-NMR: δ = 4.16 (s, 2H, C*H*$_2$COOH), 3.83-3.45 (m, 180H, OC*H*$_2$), 3.38 (s, 3H, OC*H*$_3$). ^{13}C-NMR: δ = 171.8 (*C*OOH), 77.4, 71.8, 70.8, 71.0-70.0, 68.4 (*C*H$_2$COOH), 58.9 (O*C*H$_3$). FT-IR (ATR): υ (cm^{-1}) = 2883, 2860, 2741, 2694, 1744, 1466, 1454, 1413, 1359, 1340, 1279, 1240, 1199, 1146, 1103, 1059, 946, 841, 732, 673. ESI-MS: *m/z*Calc. (n = 45) 2028.41 g/mol; Obs. $[M+2H]^{2+}$ 1014.75, $[M+3H]^{3+}$ 676.75, $[M+4H]^{4+}$ 507.92 g/mol (mass envelopes with $\Delta m/z = 44/z$).

*mPEG2000-COOTFP (**7**)*
In a 10 mL round-bottom flask, **6** (0.47 g, 0.23 mmol), TFP (65 mg, 0.38 mmol, 1.6 eq) and PPTS (6 mg, 24 µmol, 0.1 eq) were dissolved in CH_2Cl_2 (2 mL). EDCI·HCl (63 mg, 0.32 mmol, 1.4 eq) was added and the solution was stirred at r.t. for 15 h. $CHCl_3$ (50 mL) was added and the solution was subsequently washed with sat. $NaHCO_3$ (20 mL) and brine (2 × 20 mL). After drying with $MgSO_4$ and filtration, removal of the solvents *in vacuo* afforded **7** (0.49 g, 0.23 mmol, 98%) as a white solid (**7** was used immediately in the next step to avoid any deactivation by hydrolysis). ^{1}H-NMR: δ = 7.03 (m, 1H, TFP*H*), 4.56 (s, 2H, C*H*$_2$COOTFP), 3.85-3.45 (m, 176H, OC*H*$_2$), 3.38 (s, 3H, OC*H*$_3$). ^{13}C-NMR: δ = 166.6 (*C*OOTFP), 146.0 (dm), 141.6 (dm), 104.5 (t), 77.6, 71.9, 71.1, 71.0-69.6, 67.8 (*C*H$_2$COOTFP), 58.9 (O*C*H$_3$), the signal from *C*O (TFP group) is not visible. ^{19}F-NMR: δ = -138.7, -152.8.

*mPEG2000-GPLGVRGG-≡ (**8**)*
In a 25 mL round-bottom flask under an Ar atmosphere, **3** (91 mg, 93 µmol) was dissolved in DMF (2 mL). DIPEA (46 µL, 0.26 mmol, 2.8 eq) and freshly prepared **7** (147 mg, 68 µmol, 0.73 eq)* were added and the mixture was stirred at r.t. for 2 h. After removal of DMF *in vacuo* using an oil pump (40 °C), preparative size-exclusion chromatography (BioBeads SX-1, 20% MeOH in $CHCl_3$) yielded still impure **8** (191 mg). The compound was purified by means of preparative RP-HPLC using an elution gradient of 5% to 100% CH_3CN in H_2O containing 0.1% formic acid (detection at λ = 190 and 200 nm) to give **8** (92 mg, 34 µmol, 50% based on **7**) as a colorless solid. ESI-MS: *m/z* Calc. (n = 44) 2759.28 g/mol; Obs. $[M+3H]^{3+}$ 920.33, $[M+4H]^{4+}$ 690.58, $[M+5H]^{5+}$ 553.00 g/mol (mass envelopes with $\Delta m/z = 44/z$). For LC-MS characterization (TIC, positive mode) see Figure S.4.

* The amount of added **7** is optimized per batch of peptide, since the purity of the peptide, *i.e.* the amount of remaining TFA, is not exactly known. Upon increasing the amount of added **7**, additional byproducts are formed.

mPEG2000-GPLGVRGG-HEG-DSPE or mPEG-MMP2p-DSPE
In a 5 mL tube flask, **8** (104 mg, 38 µmol) and **5** (47 mg, 42 µmol, 1.1 eq) were dissolved in CH_2Cl_2 (3 mL). Under an Ar flow, solvent was removed until the volume was ~1½ mL. Under an Ar atmosphere, Na-ascorbate (190 µL of a 0.4 M solution in H_2O, 76 µmol, 2 eq) and $CuSO_4·5H_2O$ (190 µL of a 0.2 M solution in H_2O, 38 µmol, 1 eq) were added and

the opaque two-phase system was vigorously stirred at r.t. for 18 h. Stirring was halted and after 15 min, the organic layer was separated using a pipette. To the remaining aqueous layer, H_2O (50 mL) was added and the aqueous layer was gently extracted with $CHCl_3$ (4 × 10 mL). The combined organic layers were dried using $MgSO_4$ and after filtration, the solvent was removed *in vacuo*. Preparative size-exclusion chromatography (BioBeads SX-1, 20% MeOH in $CHCl_3$) followed by precipitation (1½ mL CH_2Cl_2→ 40 mL diethyl ether at 0 °C) yielded mPEG-MMP2p-DSPE (126 mg, 33 µmol, 86%) as a white sticky solid. For ^{1}H-NMR, LC-MS (TIC, positive mode) and GPC characterization see Figures S.5, S.6a and S.6b, respectively. From Figure S.5b: ^{1}H-NMR: δ = 3.72-3.53 (m, OCH_2), 3.38 (s, 3H, OCH_3), 1.30-1.23 (m, 56H, CH_2), 0.98-0.88 (m, 18H, CH_3). MALDI-TOF MS: *m/z* Calc. (n = 44, main isotope) 3853.36 g/mol; Obs. (reflector mode) $[M+Na]^+$ 3877.03, $[M+K]^+$ 3893.18 g/mol (mass envelope with Δm = 44, see Figure S.7). For HPLC-ELSD characterization in THF / H_2O, see Figure S.8 and S.9.

Synthesis of c[RGDfK]-DSPE

The synthetic route to **c[RGDfK]-DSPE** is shown in Scheme S.4.

*2,3,5,6-Tetrafluorophenol 5-hexynoate (**9**)*

In a 25 mL round-bottom flask, 5-hexynoic acid (116 mg, 1.0 mmol), TFP (189 mg, 1.10 mmol, 1.1 eq) and PPTS (26 mg, 0.10 mmol, 0.1 eq) were dissolved in CH_2Cl_2 (1½ mL). EDCI·HCl (216 mg, 1.10 mmol, 1.1 eq) and CH_2Cl_2 (1 mL) were added and the solution was stirred at r.t. for 16 h. $CHCl_3$ (60 mL) was added and the solution was subsequently washed with sat. $NaHCO_3$ (2 × 20 mL) and brine (2 × 20 mL). After drying with $MgSO_4$ and filtration, removal of the solvents *in vacuo* afforded **9** (285 mg, >1.0 mmol, >100%) as a colorless oil (**9** was used immediately in the next step to avoid any deactivation by hydrolysis). ^{1}H-NMR: δ = 7.00 (m, 1H, ArH), 2.83 (t, 2H, CH_2CO), 2.38 (t, 2H, CH_2C≡C), 2.05-1.98 (m, 3H, $CH_2CH_2CH_2$, C≡CH). ^{19}F-NMR: δ = -139.0, -153.0.

*c[RGDfK]-≡ (**10**)*

In a 5 mL tube flask, c[RGDfK] (max. 136 µmol) and DIPEA (300 µL, 223 mg, 1.71 mmol, 12.5 eq)* were dissolved in DMF (2 mL). Dropwise, **9** (35 mg, 124 µmol, 0.91 eq)** was added and the mixture was stirred at room temperature for 1½ h. DMF was removed *in vacuo* using an oil pump (40 °C) and the product was purified using preparative RP-HPLC using an elution gradient of 18% to 20% CH_3CN in H_2O containing 0.1% formic acid (detection at λ = 200 and 254 nm) to give **10** (53 mg, 76 µmol, 56 % based on **9**) as a white fluffy solid after lyophilization. ^{1}H-NMR (D_2O / acetonitrile-d_3 3:1):*** δ = 8.21 (t, 1H, NHCOCH$_2$CH$_2$CH$_2$), 7.46-7.30 (m, 5H, ArH), 4.80 (t, 1H, αCH_{Asp}), 4.69 (m, 1H, αC$H_{D\text{-}Phe}$), 4.38 (m, 1H, αCH_{Arg}), 4.26 (d, 1H, αCHH$_{Gly}$), 3.97 (m, 1H, αCH_{Lys}), 3.54 (d, 1H, αCHH_{Gly}), 3.24 (q, 2H, εC$H_{2\ Lys}$), 3.15 (t, 2H, δC$H_{2\ Arg}$), 3.16-3.03 (m, 2H, βC$H_{2\ D\text{-}Phe}$), 2.96 (dd, 1H, βCHH$_{Asp}$), 2.76 (dd, 1H, βCHH_{Asp}), 2.48 (t, 1H, C≡CH), 2.40 (t, 2H, NHCOCH_2CH$_2$CH$_2$), 2.30 (dt, 2H, NHCOCH$_2$CH$_2$CH_2), 1.87 (qn, 2H, NHCOCH$_2$CH_2CH$_2$), 1.98-1.83 (m, 1H, βCHH$_{Arg}$), 1.76-1.66 (m, 2H, βCHH_{Arg}, βCHH$_{Lys}$), 1.67-1.51 (m, 3H, βCHH_{Lys}, δC$H_{2\ Lys}$), 1.48-1.39 (m, 2H, γCH$_{2\ Arg}$), 1.13-1.00 (m, 2H, γC$H_{2\ Lys}$), the NH signals of the constituent amino acids are not visible. The assignments were confirmed by 2D (^{1}H,^{1}H) correlation spectroscopy (gCOSY). ^{13}C-NMR (D_2O / acetonitrile-d_3 3:1): δ = 175.4, 174.4, 174.2, 172.9, 172.7, 171.30, 171.28, 156.8, 136.3, 129.4, 128.9, 127.3, 84.7, 70.2, 55.5, 55.1, 52.5, 49.7, 43.6, 40.7, 39.0, 37.2, 34.9, 34.6, 30.2, 27.8, 27.5, 24.7, 24.5, 22.8, 17.3. FT-IR (ATR): υ (cm^{-1}) = 3276, 3073, 2936, 2865, 1634, 1547, 1434, 1369, 1285, 1201, 1184, 1131, 1031, 959, 913, 838, 800, 743, 721,

699. ESI-MS: *m/z* Calc. 697.35; Obs. $[M+H]^+$ 698.58, $[M+2H]^{2+}$ 349.83. For LC-MS characterization (TIC, positive mode) see Figure S.16.

* **c[RGDfK]** still contained an unknown amount of TFA.
** increasing the amount of **9** increases the amount of byproducts.
*** ^{1}H-NMR assignment partly based on: (a) P. Y. W. Dankers, P. J. H. M. Adams, D. W. P. M. Löwik, J. C. M. van Hest, E. W. Meijer, *Eur. J. Org. Chem.* **2007**, 3622–3632 (b) J. Paleček, G. Dräger, A. Kirschning, *Synthesis* **2011**, *4*, 653–661.

O-(2-Azidoethyl)-O'-(2-carboxyethyl)nonadecaethylene glycol (**11**)
In a 100 mL round-bottom flask, *O*-(2-azidoethyl)nonadecaethylene glycol (150 mg, 0.16 mmol), TEMPO (23 mg, 0.14 mmol, 0.9 eq) and NaBr (23 mg, 0.22 mmol, 1.4 eq) were dissolved in 0.25 M Na_2CO_3 (25 mL, pH = 11). On an ice bath, NaOCl (13% active chorine, 800 µL, 1.67 mmol, 10.5 eq) was added dropwise and the mixture was stirred at r.t. for 3 h. 1 M $NaHSO_3$ (20 mL) was added, the solution was acidified to pH = 1 using concentrated HCl and the aqueous layer was extracted with $CHCl_3$ (3 × 20 mL). The combined organic fractions were dried using $MgSO_4$, filtrated and the solvent was removed *in vacuo*. Purification was achieved using column chromatography (flash SiO_2, gradient $CHCl_3$ to 8% MeOH in $CHCl_3$) yielding pure **11** as a white waxy solid (139 mg, 0.15 mmol, 91%). ^{1}H-NMR: δ = 4.17 (s, 2H, CH_2COOH), 3.83-3.44 (m, 74H, OCH_2), 3.40 (t, 2H, CH_2N$_3$). ^{13}C-NMR: δ = 171.8 (*C*OOH), 70.9, 70.7, 70.62, 70.59, 70.56, 70.53, 70.48, 70.4, 70.3, 70.0, 68.8 (*C*H$_2$COOH), 50.6 (*C*H$_2$N$_3$). FT-IR (ATR): υ (cm^{-1}) = 2866, 2101, 1757, 1456, 1347, 1324, 1297, 1249, 1094, 1039, 946, 846, 671. ESI-MS: *m/z* Calc. 937.52; Obs. $[M+H]^+$ 938.25, $[M+Na]^+$ 960.42.

*N_3-EG19-DSPE (**12**)*
In a 25 mL round-bottom flask, **11** (136 mg, 0.14 mmol) was coevaporated with toluene (3×) and then dissolved in dry DMF (0.5 mL). DIPEA (102 µL, 76 mg, 0.58 mmol, 4 eq) and HBTU (54 mg, 0.14 mmol, 0.98 eq) were subsequently added and the mixture was stirred at r.t. for 15 min. Dry $CHCl_3$ (on 4Å molsieves, 1½ mL) and DSPE (107 mg, 0.14 mmol, 0.98 eq) were added and the resulting suspension was stirred at 50 °C for 2 h, yielding a clear solution after ~30 min. DMF was removed *in vacuo* using an oil pump (40 °C) and $CHCl_3$ (60 mL) was added. The organic layer was washed with 0.5 M citric acid (pH = 2), H_2O, brine, sat. $NaHCO_3$, 1 M HCl (2×) and brine (all 20 mL). The organic layer was dried using Na_2SO_4, filtrated and the solvent was removed *in vacuo*. Purification was achieved using column chromatography (flash SiO_2, gradient $CHCl_3$ to 20% MeOH in $CHCl_3$) and preparative SEC (BioBeads SX-1, 20% MeOH in $CHCl_3$) yielding pure **12** as a waxy off-white solid (183 mg, 0.11 mmol, 77% based on DSPE). ^{1}H-NMR: δ = 5.20 (m, 1H, C*H*), 4.37 (dd, 1H, C*H*HO), 4.15 (dd, 1H, CH*H*O), 4.03 (s, 2H, CH_2CONH), 3.97 (m, 4H, POCH_2), 3.75-3.45 (m, 74H, CH_2O), 3.51 (q, 2H, CH_2NHCO), 3.39 (t, 2H, CH_2N$_3$), 2.28 (m, 4H, CH_2COO), 1.58 (m, 4H, CH_2CH$_2$COO), 1.32-1.20 (m, 56H, CH_2), 0.88 (t, 6H, CH_3). The signals due to N*H* and PO*H* are not visible. ^{13}C-NMR: δ = 173.5 (*C*=O), 173.0 (*C*=O), 70.7-69.6 (*C*H$_2$O), 63.5, 62.9, 50.7 (*C*H$_2$N$_3$), 34.3, 34.1, 31.9, 29.73, 29.68, 29.6, 29.38, 29.35, 29.2, 25.0, 24.9, 22.7, 14.1 (*C*H$_3$). ^{31}P-NMR: δ = 0.9. FT-IR (ATR): υ (cm^{-1}) = 2916, 2850, 2106, 1736, 1665, 1541, 1467, 1348, 1288, 1247, 1195, 1098, 987, 947, 844, 752, 721. MALDI-TOF-MS: *m/z* Calc. 1667.09; Obs. $[M+Na]^+$ 1690.18, $[M+K]^+$ 1706.18.

c[RGDfK]-EG19-DSPE or c[RGDfK]-DSPE
Alkyn **10** (64 mg, 73 µmol) was placed in a 25 mL round-bottom flask and a solution of **12** (122 mg, 52 µmol, 0.8 eq)* in THF / water 1:1 (3.2 mL) was added. The flask was

flushed with Ar for 10 min while additional THF (1 mL) was added to keep **12** in solution. Under an Ar atmosphere, Na-ascorbate (400 µL of a fresh 0.4 M solution in H_2O, 160 µmol, 2.2 eq) and $CuSO_4 \cdot 5H_2O$ (400 µL of a fresh 0.2 M solution in H_2O, 80 µmol, 1.1 eq) were added and the solution was stirred at r.t. for 2 h. Initially, a yellow precipitate formed, whereas a clear yellow solution was obtained after 20 min and a dark green solution after 1½ h. At this point, HPLC indicated full conversion of **12**. THF was removed *in vacuo* and water (150 mL) was added. The aqueous phase was extracted with $CHCl_3$ (4 × 30 mL) and the combined organic layers were dried using Na_2SO_4. After filtration, the solvent was removed *in vacuo* yielding a yellowish solid. This solid was redissolved in $CHCl_3$ / MeOH 95:5 (160 mL) and gently washed with 0.07 M EDTA (2 × 40 mL). The organic layer was dried using Na_2SO_4, filtrated, and the solvent was removed *in vacuo* yielding a colorless solid. Preparative SEC (BioBeads SX-1, 20% MeOH in $CHCl_3$) followed by precipitation (1½ mL CH_2Cl_2 / MeOH 95:5 → 40 mL diethyl ether at 0 °C) yielded c[RGDfK]-DSPE (130 mg, 55 µmol, 75%) as a slightly brownish sticky solid. For ^{1}H-NMR ($CDCl_3$ / MeOD-d_4 95:5) characterization, see Figure S.17. ^{13}C-NMR ($CDCl_3$ / MeOD-d_4 95:5): δ = 174.0, 173.9, 173.6, 173.4, 172.8, 172.1, 171.1, 170.9, 170.8, 170.6, 157.1, 157.0, 147.2, 136.7, 129.3, 128.5, 126.9, 122.8, 70.7, 70.5, 70.44, 70.39, 70.36, 70.29, 70.2, 70.13, 70.10, 70.0, 69.5, 66.0, 64.3, 64.03, 63.98, 63.7, 63.6, 62.8, 55.6, 55.0, 52.0, 50.3, 43.9, 40.8, 40.05, 39.99, 38.6, 38.0, 35.5, 34.7, 34.3, 34.20, 34.18, 32.0, 30.4, 29.8, 29.75, 29.73, 29.63, 29.62, 29.43, 29.41, 29.3, 29.2, 28.6, 28.4, 27.4, 25.7, 25.6, 25.2, 25.0, 24.9, 24.7, 24.6, 22.84, 22.75, 15.2, 14.1. ^{31}P-NMR ($CDCl_3$ / MeOD-d_4 95:5): δ = -0.2. FT-IR (ATR): υ (cm^{-1}) = 3278, 3070, 2917, 2850, 1736, 1636, 1544, 1466, 1349, 1288, 1251, 1216, 1194, 1095, 948, 844, 805, 745, 720, 699, 663. MALDI-TOF-MS: *m/z* Calc. 2364.44; Obs. $[M+H]^+$ 2365.47, $[M+Na]^+$ 2387.46, $[M+EG1+H]^+$ 2409.46, $[M+EG1+Na]^+$ 2431.44. For MALDI-TOF MS analysis, see Figure S.18. For HPLC-ELSD characterization in THF / H_2O, see Figure S.19.

* The amount of added **12** has to be optimized per peptide batch, since **10** still contains an unknown amount of TFA.

Gold nanocrystals synthesis

1-dodecanethiol-coated gold nanocrystals were synthesized using Brust method.[5] These gold nanocrystals were dissolved in chloroform at a concentration of 10 mg/ml and filtered through a 0.2 µm syringe filter. Prior to nanoemulsion synthesis gold nanocrystals were incubated for 30 min with 0.1 M cis-9-octadecene-1-thiol in chloroform to exchange ligands. We have found cis-9-octadecene-1-thiol coated gold nanocrystals have better affinity for and higher incorporation into soybean oil.

Nanoemulsion synthesis

For the nanoemulsion synthesis all components were dissolved in chloroform. Each formulation was prepared using 20 mg of soybean oil and 7,13 µmole of coating lipids. The lipid mole percentages used to obtain the different nanoemulsions are shown in Tables S.1 and S.2. Theranostic nanoemulsions were obtained adding 0.5 mg Simvastatin, 1.875 mg iron oxide crystals (NNLabs, AR USA) and 10 mg Au to the chloroform mixture. After mixing the components, lipid films were obtained using a roto-evaporator. Films were then hydrated with 10 ml of Hepes Buffer containing 5 mM $CaCl_2$. Crude emulsions were sized by microfluidizer (Microfluidics, MS, USA) or sonicated for 15 minutes, and subsequently concentrated to 2 ml of final volume using Vivaspin filter tubes.

Phospholipid composition analysis by HPLC-ELSD

The nanoemulsion phospholipid composition was measured by HPLC. A set of samples was represented by frozen-dried nanoemulsions obtained after the entire nanoemulsion synthesis process. Mixtures composed of all the lipid ingredients dissolved in chloroform prior to the nanoemulsion synthesis were used as controls. Analytical RP-HPLC (THF / H_2O with 0.1% TFA) was performed using a Shimadzu LC-10AD VP coupled to a Shimadzu SPD-10AV VP UV-vis detector and an Alltech ELSD 2000ES on a GL sciences Inertsil ODS-3 5µ column. The gradient comprised, % THF (min): 20 (0-1), 20 to 95 (1-18), 95 (18-24), 95 to 20 (24-26), 20 (26-35). HPLC was measured on all separate components (mPEG-MMP2p-DSPE, mPEG3000-DSPE, mPEG350-DSPE, biotin-PEG1000-DSPE, cholesterol and soybean oil, the rhodamine-lipid was omitted because of the low amount present), their mixtures and resulting nanoemulsions (see 'Nanoemulsion synthesis' for details and Table S.2 for the intended lipid composition). On the basis of the reference measurements, the peaks in the mixtures and nanoemulsions spectra were identified. It could be shown that the phospholipid composition of the mixtures (prior to the nanoemulsion synthesis) and of the nanoemulsions was similar (see Table S.3 and Figure S.10 for a typical HPLC-ELSD chromatogram) and that both compositions agree well with the intended lipid composition. The depicted percentages are %area percentages, and these are therefore uncorrected for molecular weight. A reference sample containing a 1:1 molar ratio of mPEG-MMP2p-DSPE and mPEG350-DSPE showed an overestimation of the high molecular weight compound mPEG-MMP2p-DSPE by a factor of ca. 2. The intensity of the soybean oil peak was variable due to bad solubility in THF / H_2O. Furthermore, the set-up was not suitable for measuring cholesterol due to its hydrophobicity resulting in its insolubility in the aqueous eluent mixture.

MMP2 activation

hMMP2 was activated with APMA 1 mM in HBS Buffer for 1 h at 37 °C. The enzyme activation was tested using 10 µM of fluorogenic MMP2 substrate MCA-P-L-OH at 37 °C. The substrate cleavage was followed in a kinetic mode using a plate reader (Ex = 340/20 nm, Em = 380/20 nm).

Aggregation experiments

For setting experiments 1 µl of nanoemulsions containing 1% or 2.5% of biotin-PEG1000-DSPE and 0%, 10% or 20% mPEG3000-DSPE were incubated with 1.5 µl avidin 10 uM for 30 min, 2 and 4 hours. The nanoparticle effective diameter was measured by DLS device (Brookhaven Instrument, NY, USA). The nanoparticle aggregation was evaluated through the ratio: effective diameter $_{t=Xmin}$ / effective diameter $_{t=0min}$. For the main experiments 3 µl of nanoemulsions, corresponding to 1065 pmole of mPEG-MMP2p-DSPE or PEG3000-DSPE and 266 pmole biotin-PEG1000-DSPE, were diluted in 100 µl HEPES Buffer and incubated 12 hours at 37 °C with 525ng hMMP2 previously activated. Samples were incubated for 30 minutes with 40 pmole avidin at room temperature. Effective diameter was measured in water by DLS and the nanoparticle aggregation was evaluated through the ratio effective diameter $_{t=30min}$ / effective diameter $_{t=0min}$.

MMP2 dose dependent aggregation experiments

Three µl of nanoemulsions, corresponding to 1065 pmole of mPEG-MMP2p-DSPE or PEG3000-DSPE and 266 pmole biotin-PEG1000-DSPE, were diluted in 100 µl HEPES buffer and incubated 12 hours at 37 °C with 0, 65, 131, 262 and 525ng hMMP2

previously activated. Samples were incubated for 30 minutes with 40 pmole avidin at room temperature. Effective diameter was measured in water by DLS and the nanoparticle aggregation was evaluated through the ratio effective diameter $_{t=30min}$ / effective diameter $_{t=0min}$. See Figure S.12.

Binding on streptavidin coated plate

Binding experiments were performed using 1 μl of nanoemulsions containing 1% or 2.5% of biotin-PEG1000-DSPE and 0%, 10% or 20% mPEG3000-DSPE diluted in 50 μl HEPES Buffer and incubated on streptavidin coated 96 well plate for 30 min. The same setting was adopted for samples containing 0-10% mPEG-MMP2p-DSPE or mPEG3000-DSPE and treated/not treated with activated MMP2. Fluorescence at 590 nm was measured with a plate reader before and after well washing. The nanoparticle binding on the plate surface was evaluated through the ratio of emitted fluorescence after washing / emitted florescence before washing.

Avidin self assembled monolayer on gold silicon wafers

Gold silicon wafers were thiol functionalized with 2 mM of 11-mercaptoundecanoic acid (MUA) solution in isopropanol for 2 hours. After 1 hour soaking in MES buffer, the wafers underwent to EDC/NHS reaction for 1 hour to enable the carboxylic derivate formation. The avidin covalent binding to wafers was obtained after incubation with 1 mg/ml protein solution in PBS for 2 hours.

Atomic force microscopy

Nanoemulsions diluted 1/10 in water were incubated for 30 minutes on avidin functionalized gold silicon wafers. After several washings, the wafers were mounted on the stage of an MFP-3D-BIO atomic force microscope (Asylum Research, Santa Barbara, CA). Imaging was performed at room temperature with the sample fully hydrated in water, using AC-mode with magnetically actuated iDrive cantilever probes having a tip radius of approximately 40 nm. Each sample was scanned in multiple randomly selected 4x4-μm sub-regions at a rate of 5.0 μm/sec and an image resolution of 512x256 pixels, with the resulting height images pseudo-colored using a linear scale. The AFM operator was blind to the sample conditions.

Cleavage experiment of mPEG-MMP2p-DSPE with MMP2

To test the enzymatic cleavage of mPEG-MMP2p-DSPE, the lipid was solubilized in water and incubated with or without activated MMP2, for 12 hrs at 37 °C. After incubation the samples were analyzed with MALDI-TOF mass spectrometry to possibly identify the presence of the fragments. Only in the sample that was incubated with activated MMP2, the cleaving fragment mPEG-GPLG-COOH was traced. See Figure S.13.

PEG cleavage kinetic experiments

Three μl of nanoemulsions, corresponding to 1065 pmole of mPEG-MMP2p-DSPE or mPEG3000-DSPE and 266 pmole biotin-PEG1000-DSPE, were incubated for 0, 1, 2, 4, 6 and 12 hours at 37 °C with 525ng MMP2 previously activated with APMA. Samples were incubated for 30 minutes with 40 pmole avidin at room temperature. Effective diameter was measured in water by DLS and the nanoparticle aggregation was evaluated through the ratio effective diameter $_{t=30min}$ / effective diameter $_{t=0min}$. See Figure S.14b.

Nanoparticle stability in presence of FBS

The nanoparticle stability in presence of serum proteins was established incubating 3 μl of 0% and 10% mPEG3000-DSPE-2.5% biotin containing nanoemulsions and 10% mPEG-MMP2p-DSPE-2.5% biotin containing nanoemulsions with 0, 5, 20, 40, 60, 80, 100% of FBS in HBS buffer at 37 °C for 12 hours. The nanoparticle effective diameter was measured by DLS device (Brookhaven Instrument, NY, USA) and data were normalized on the 0% FBS samples. See Figure S.15.

RGD nanoemulsion synthesis

2.5% RGD functionalized nanoemulsions with no shielding (0% PEG-shielded), as well as with uncleavable (10% mPEG3000-DSPE) and cleavable shielding (10% mPEG-MMP2p-DSPE) were synthesized using the method previously described but replacing the biotin-PEG1000-DSPE with the RGD-PEG1000-DSPE and including 0.5% of rhodamine lipid.

Cell culture

MDA MB 231 breast cancer cells and J774A1 murine macrophage were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVEC) were maintained in EBM-2 medium supplemented with bullet kit. The cells were incubated in a 5% CO_2, water saturated atmosphere at 37 °C.

Flow cytometry experiments

1x10^5 MDA MB 231, J774A1 and HUVEC cells were seeded in 6 well plates. After 24 hours, the cells were incubated for 3 hours at 37 °C with 0% PEG-shielded 2.5% RGD, 10% PEG-shielded 2.5% RGD and 10% mPEG-MMP2-shielded 2.5% RGD nanoemulsions. After incubation cells were repeatedly washed with PBS and collect for flow cytometry analysis. Percentage of positive cells was measured using a BD FACSCalibur flow cytometer.

Transmission electron microscopy (TEM) and relaxometry

The instrument used was a Hitachi H7650 instrument linked to a SIA (Scientific Instruments and Applications) digital camera controlled by Maxim CCD software. TEM was performed on nanoemulsions diluted on ammonium acetate buffer and using 2% sodium phosphotungstate (pH=7) solution as a negative staining. Iron and gold concentration were measured by ICP-MS (Cantest, Canada). In order to determine the longitudinal (r_1) and transverse relaxivities (r_2) of the particles, T_1 and T_2 measurements of solutions were performed on a 60 MHz Bruker Minispec (Bruker Medical GmbH, Ettingen) operating at 40 °C. r_1 and r_2 were then obtained from the linear equation of $1/T_1$ and $1/T_2$ plotted against iron concentration obtained from ICP-MS. See Figure S.20.

Drug inclusion quantification by Nuclear Magnetic Resonance (NMR) spectroscopy

The simvastatin concentration in gold containing nanoemulsion was determined by ^{1}H-NMR spectroscopy using Bruker (MA, USA) 600 MHz spectrometer. A 100 μl of a nanoemulsion sample was dried under vacuum and resolubilized in 500 μl of dimethyl sulfoxide-d_6 containing 0.1% vol/vol tetramethylsilane as an internal standard. See Figure S.21.

Magnetic resonance imaging

In order to visualize the hypointense signal generated from iron oxide loaded nanoemulsions, 1:5, 1:10, 1:20, 1:100 dilutions of the samples in water were imaged on a 3T whole body MR scanner (Philips Achieva, X Series, Eindhoven, The Netherlands) using a conventional knee coil. Scout images were obtained to locate the phantom and a multi echo GRE sequence with the following imaging parameters was performed: field of view of 172 mm x 165 mm; resolution 1 mm x 1 mm (172x165 pixels); 1 slice of 5 mm thickness; repetition time (TR) of 150 ms, echo time (TE) of 3.2 ms, 15 echoes, with minimum echo time (TE1) of 0.9 ms and echo spacing (ΔTE) of 1.1 ms. See Figure S.20.

Computed tomography (CT) measurements

CT imaging was performed using Philips Brilliance iCT 256-slice clinical scanner on three dilutions of a nanoemulsion in water: 1:4, 1:2, and no dilution. Samples were scanned using 140 keV X-ray tube voltage, 250 mA tube current, 0.67 mm slice thickness, 0.3 mm increment, 20x0.625 collimation, field of view of 200 by 200 mm, 768x768 matrix, 0.252 pitch, 0.75s rotation time and reconstructed with the Smooth protocol. Images were analyzed using Osirix v.3.9.4 32-bit (Geneva, Switzerland; www.osirix-viewer.com). See Figure S.20.

Statistical analysis

For statistical analysis of avidin induce aggregation and binding experiments t-test was applied. For analysis of MMP2 dose dependence, MMP2 incubation time dependence and nanoparticles stability in presence of FBS one-way ANOVA test was used.

Supporting Figures and Tables

	10% PEG-shielded 1% biotin	10% PEG-shielded 2.5% biotin	20% PEG-shielded 1% biotin	20% PEG-shielded 2.5% biotin	0% PEG-shielded 1% biotin	0% PEG-shielded 2.5% biotin
mPEG 3000-DSPE	**10**	**10**	**20**	**20**	0	0
Biotin-PEG1000-DSPE	**1**	**2.5**	**1**	**2.5**	1	2.5
mPEG 350-DSPE	89	87.5	79	77.5	99	99
Rhod-lipid	0.2	0.2	0.2	0.2	0.2	0.2
Cholesterol	20	20	20	20	20	20

Table S.1 Lipid mole percentages for synthesis of nanoemulsions in preliminary experiments

	10% PEG-MMP2p-shielded 2.5% biotin	10% PEG-shielded 2.5% biotin	0% PEG-shielded 2.5% biotin
mPEG 3000-DSPE	0	10	0
mPEG-MMP2p-DSPE	10	0	0
Biotin-PEG1000-DSPE	2.5	2.5	2.5
mPEG 350-DSPE	87.5	87.5	97.5
Rhod-lipid	0.4	0.4	0.4
Cholesterol	20	20	20

Table S.2 Lipid mole percentages for synthesis of nanoemulsions used in the main experiments

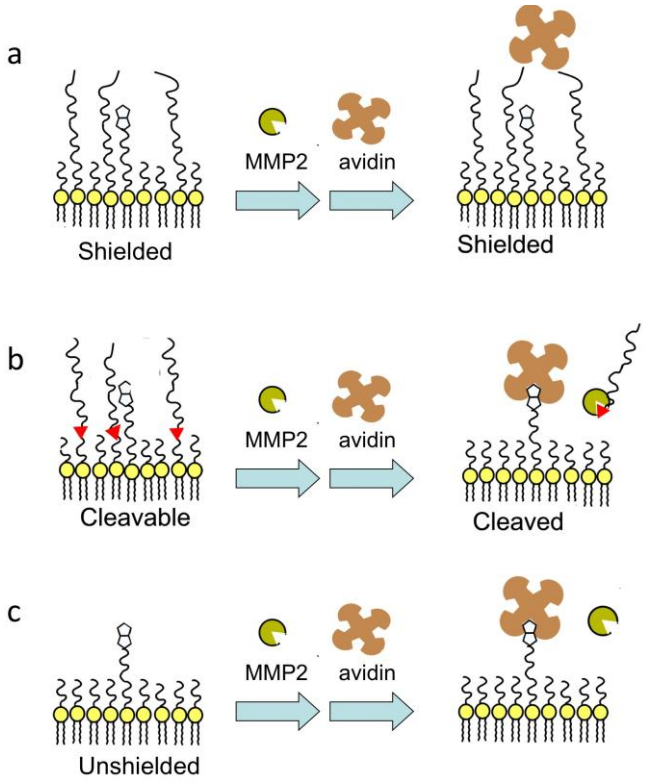


Figure S.1 Schematic showing (a) non-cleavable shielding, (b) cleavable shielding and (c) unshielding control surfaces before and after MMP2 and avidin treatment.

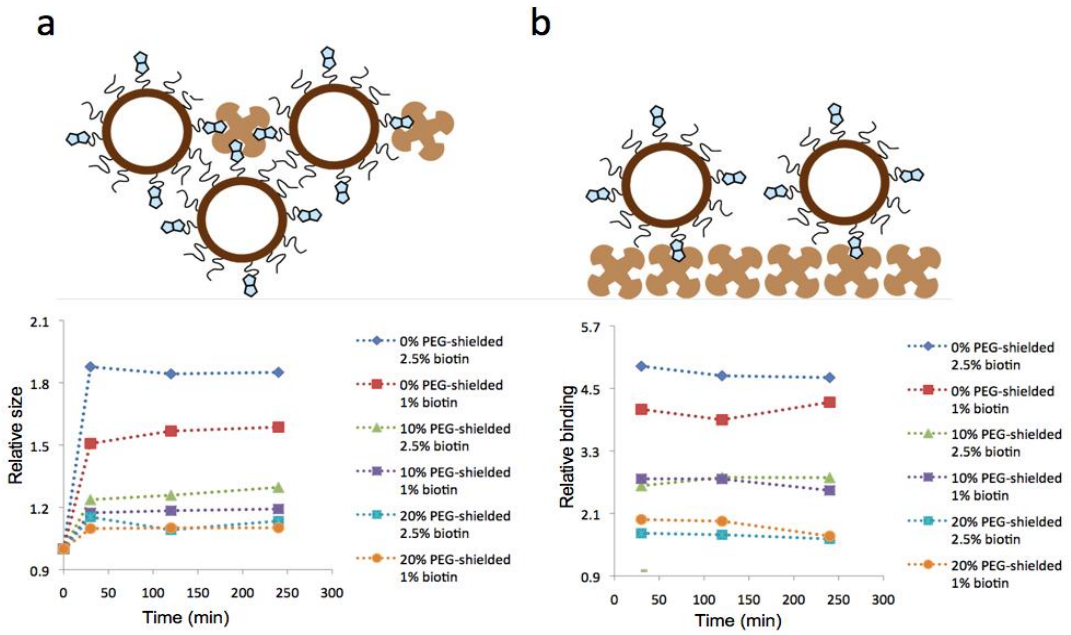
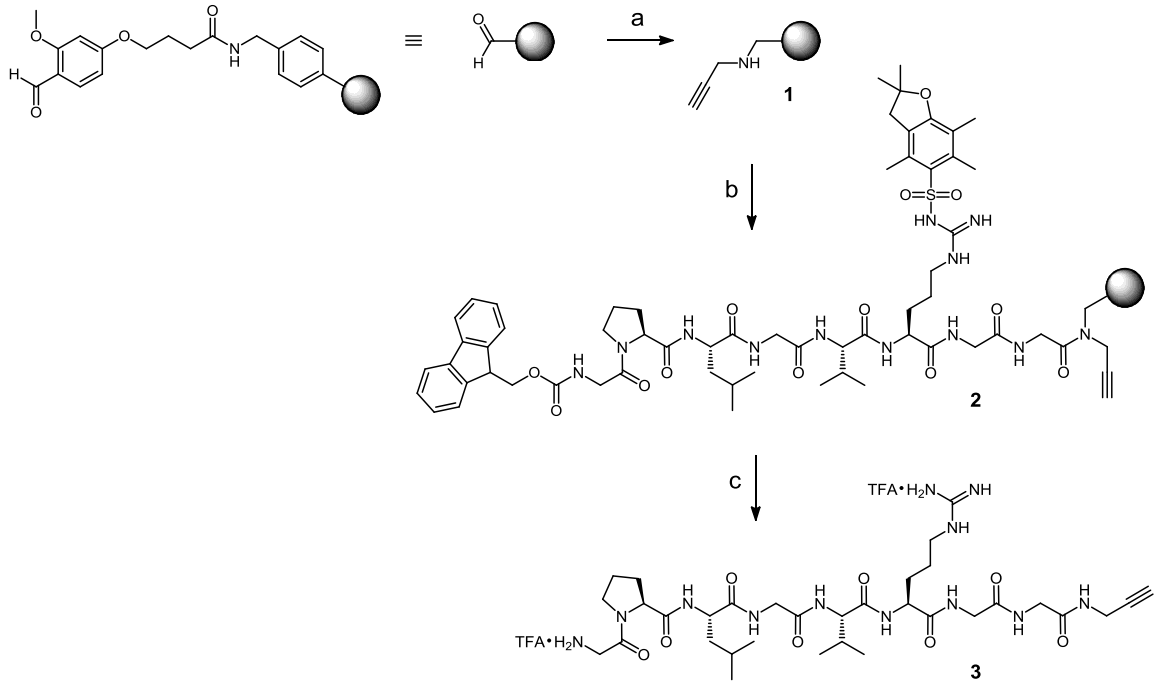
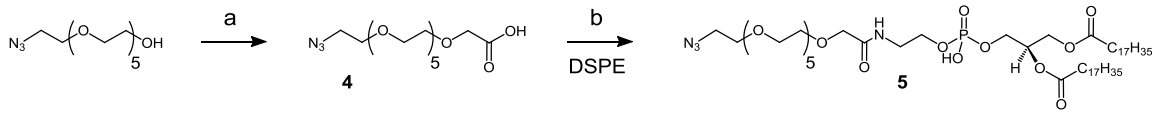


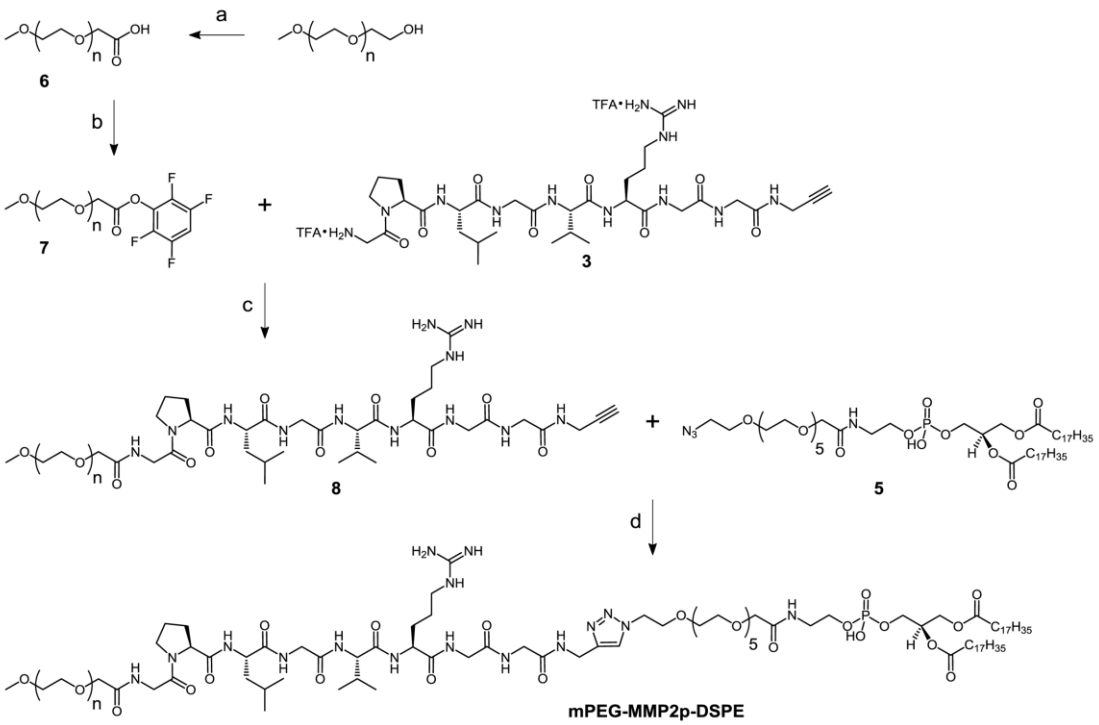
Figure S.2 (a) Avidin-induced aggregation of biotin functionalized shielded and unshielded nanoemulsions. (b) Relative binding of biotin functionalized shielded and unshielded nanoemulsions incubated on avidin coated plate



Scheme S.1 Synthesis of C-terminally alkynylated H_2N-GPLGVRGG-≡ **3**: (a) propargylamine, AcOH, $NaCNBH_3$, MeOH / DMF, 80 °C, 3 h (b) fmoc-SPPS (c) (i) piperidine / CH_2Cl_2 1:4, r.t., 2 h (ii) 5% H_2O in TFA, r.t., 3 h.



Scheme S.2 Synthesis of N_3-HEG-DSPE **5**: (a) (i) NaOH, bromoacetic acid, DMSO, r.t., 22 h (ii) HCl, 62% (b) DIPEA, HBTU, DSPE, DMF / $CHCl_3$ 1:3, 50 °C, 1½ h, 74%.



Scheme S.3 Synthesis of mPEG-MMP2p-DSPE: (a) NaOCl, TEMPO, NaBr, 0.25 M Na_2CO_3 (pH = 11), r.t., 1 h, 99% (b) TFP, PPTS, EDCI·HCl, CH_2Cl_2, r.t., 15 h, 98% (c) DIPEA, DMF, r.t., 2 h, 50% (d) Na-ascorbate, $CuSO_4 \cdot 5H_2O$, CH_2Cl_2 / H_2O, r.t., 18 h, 86%.

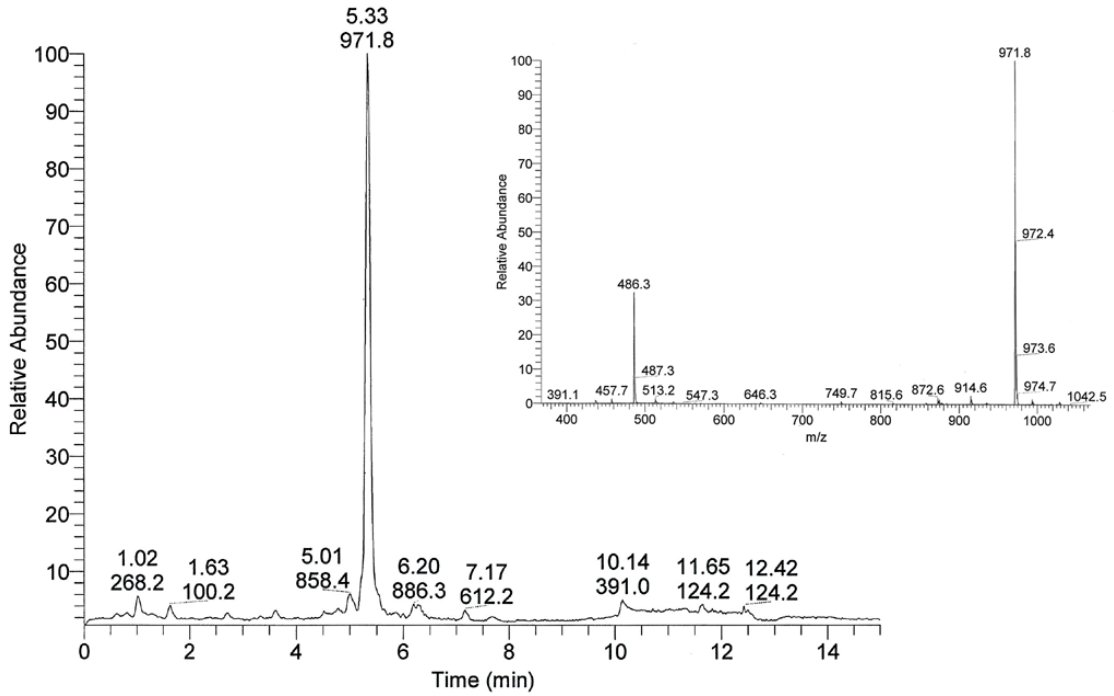


Figure S.3 LC-MS characterization (TIC, positive mode) for non-precipitated **2** after cleavage from the resin. The inset shows the mass spectrum of the main peak, showing the $[M+2H]^{2+}$ and $[M+H]^{+}$ ions, respectively.

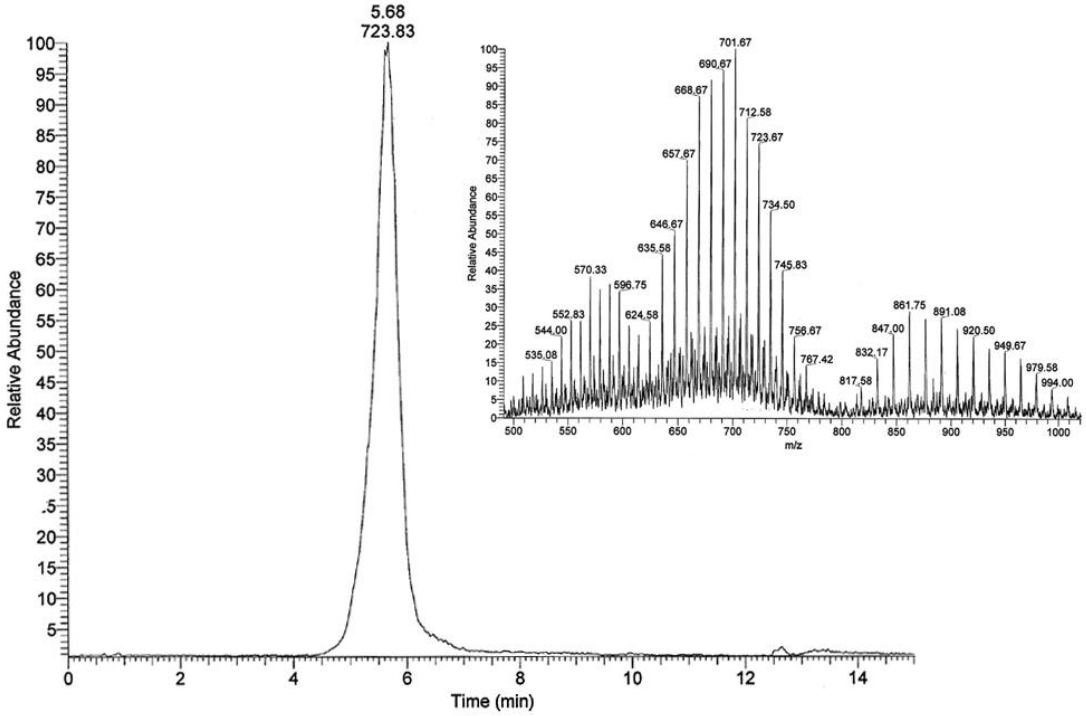


Figure S.4 LC-MS characterization (TIC, positive mode) for **8**, as purified with RP-HPLC. The inset shows the mass spectrum of the main peak from which the respective

$\Delta m/z$ = 44/z mass envelopes for the $[M+5H]^{5+}$, $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions can clearly be observed.

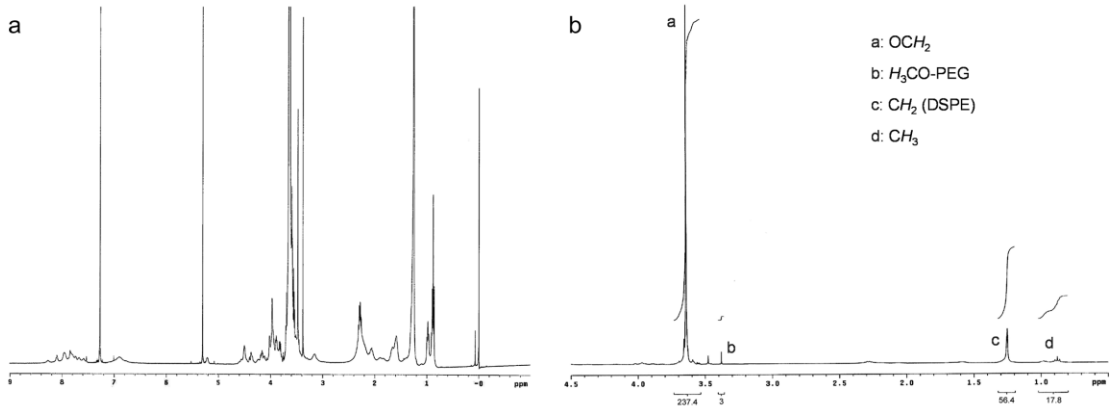


Figure S.5 (a) ^{1}H-NMR spectrum for mPEG-MMP2p-DSPE in $CDCl_3$. Residual solvent signals are visible at 5.30 ppm (CH_2Cl_2) and 3.48 ppm (MeOH) (b) Partially integrated (zoomed-out) spectrum; the integral of group b (OCH_3 at the end of the mPEG chain) is fixed at 3 (this resonance is free from spectral overlap). The expected integrals of groups c and d are 56 and 18, respectively, and these are indeed observed. The expected integral of group a is less clear due to spectral overlap.

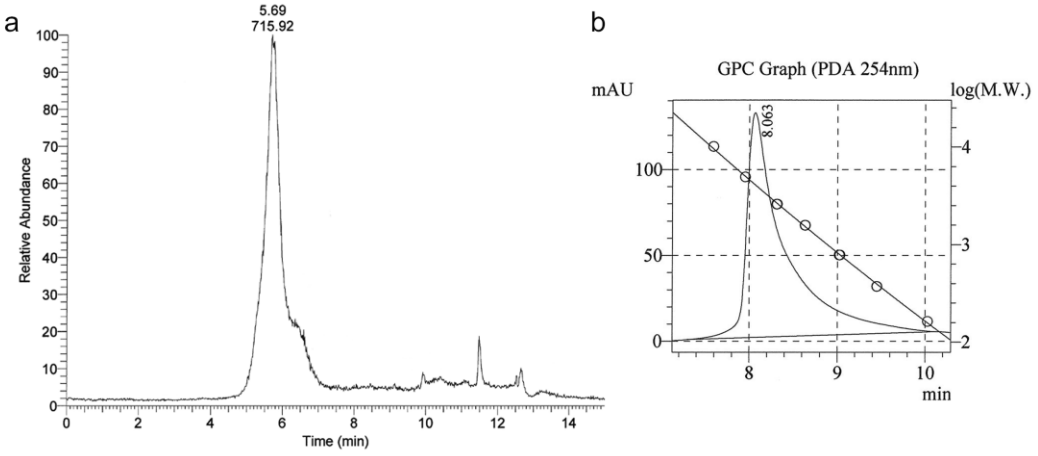


Figure S.6 (a) LC-MS trace (TIC, positive mode) for mPEG-MMP2p-DSPE in CH_3CN / H_2O. It has been found difficult to obtain an acceptable chromatogram and mass spectrum, presumably due to the incompatibility of DSPE with the acetonitrile eluent. The peaks around t_R = 10 min and higher are background signals (b) GPC trace for mPEG-MMP2p-DSPE in $CHCl_3$ against polystyrene calibration.

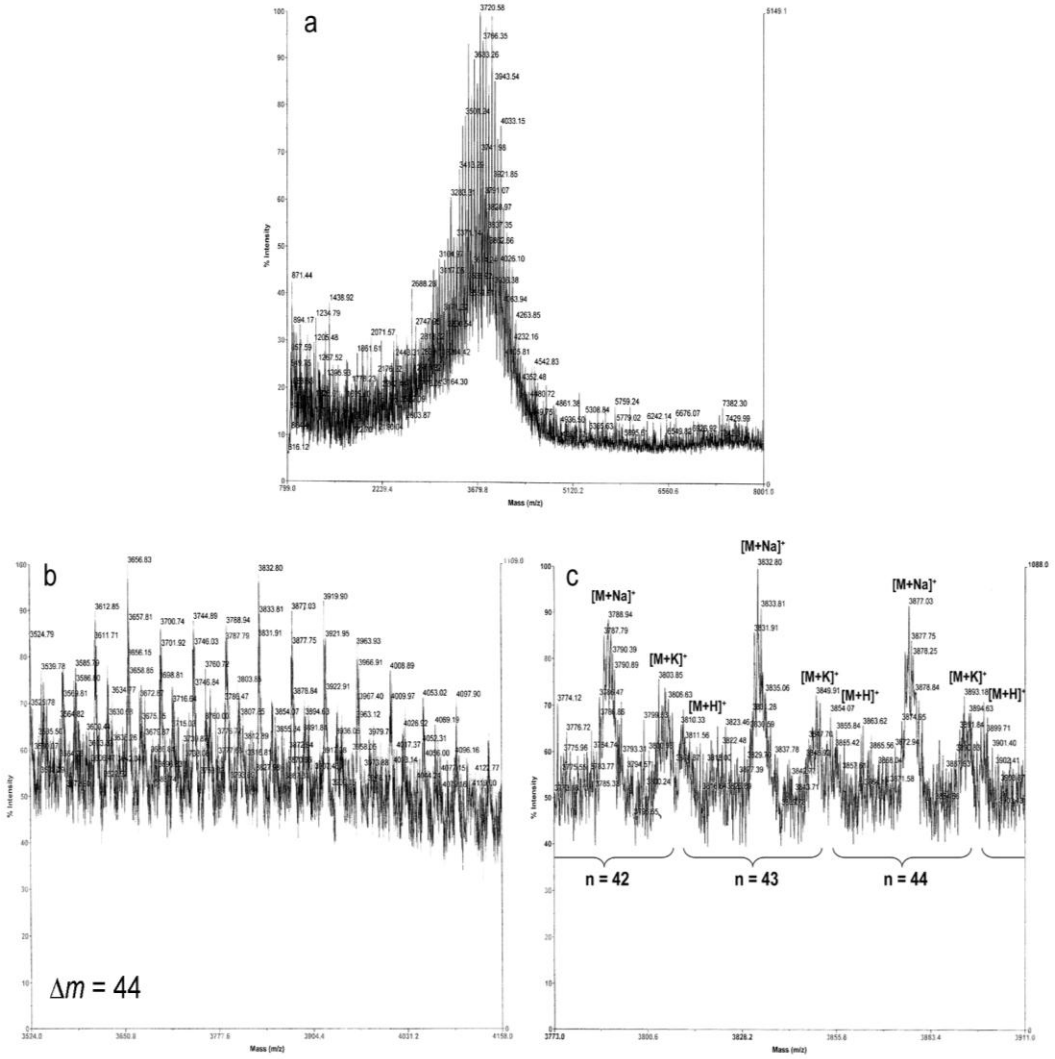


Figure S.7 MALDI-TOF-MS spectra of mPEG-MMP2p-DSPE; (a) linear mode, (b) reflector mode; the Δm = 44 signature of the PEG repeating unit is clearly visible, (c) expansion of (b) showing individual ion signals.

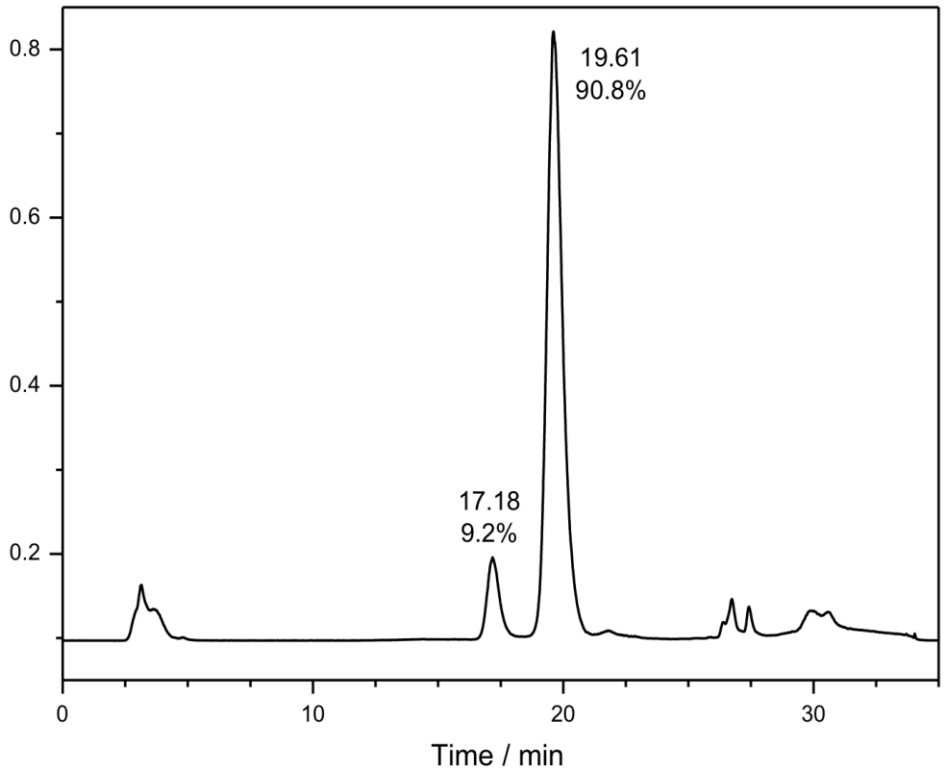


Figure S.8 HPLC trace for mPEG-MMP2p-DSPE in THF/H_2O with 0.1 % TFA (ELSD detection). On the basis of this spectrum, the purity of mPEG-MMP2p-DSPE is estimated to be 91%, where the 9% material is believed to have one aliphatic C18-tail missing from the DSPE group. Background signals at t_R 2-3 and at > 25 min are not integrated.

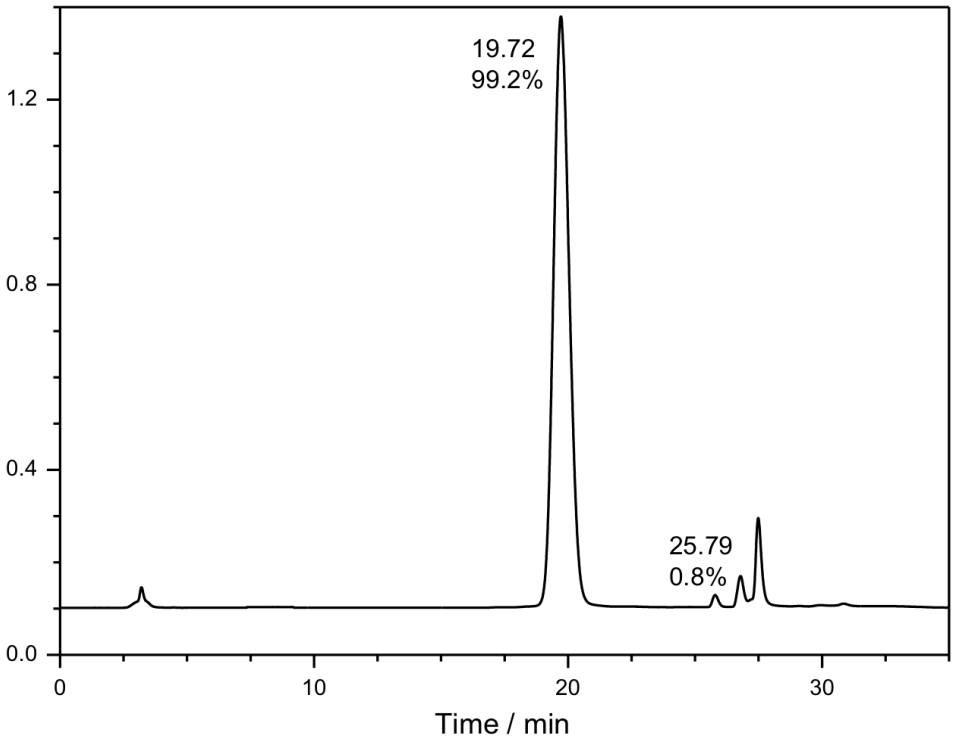


Figure S.9 HPLC trace for mPEG-MMP2p-DSPE (after a full synthetic repeat) in THF/H_2O with 0.1 % TFA (ELSD detection). On the basis of this spectrum, the purity of mPEG-MMP2p-DSPE is estimated to be 99%. Background signals at t_R 2-3 and at > 26 min are not integrated.

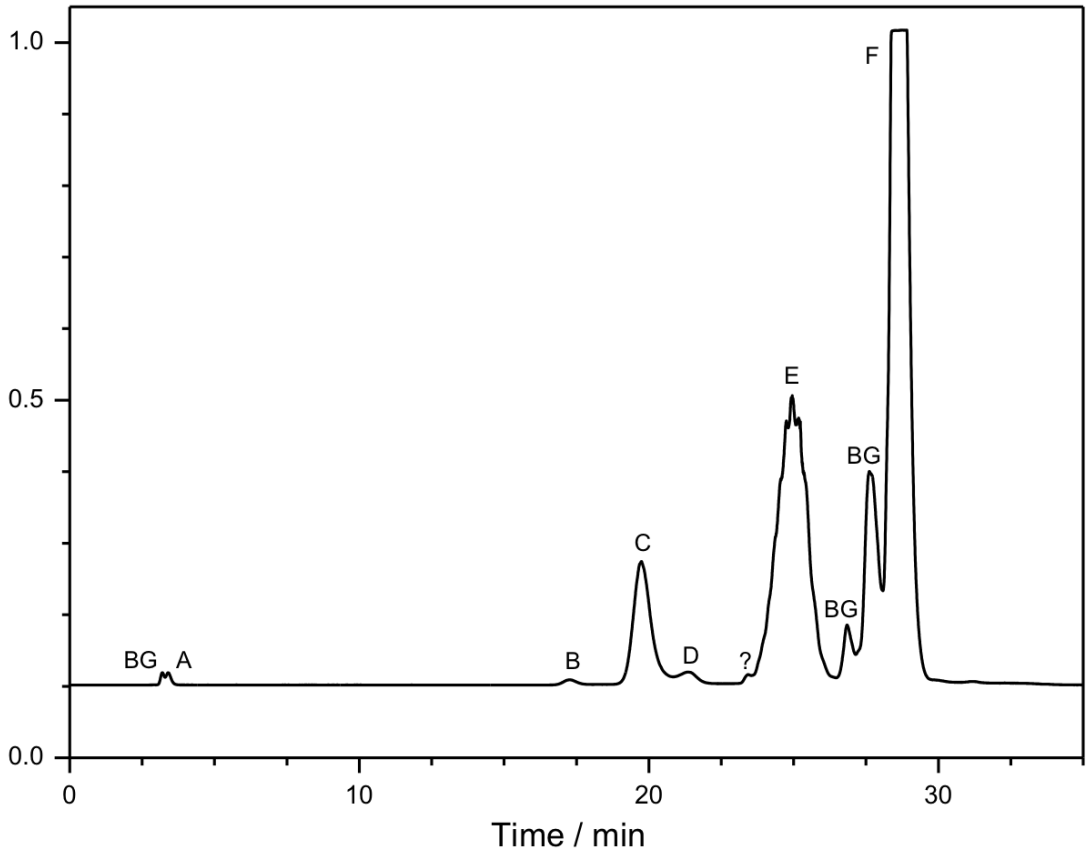


Figure S.10 HPLC trace for nanoemulsion sample #4 (see Table S.3) in THF/H_2O with 0.1 % TFA (ELSD detection). BG = background signal, A = buffer and salts, B = minor impurity in mPEG-MMP2p-DSPE, C = mPEG-MMP2p-DSPE, D = biotin-PEG1000-DSPE, E = mPEG350-DSPE and F = soybean oil.

Sample #	Type	mPEG3000-DSPE	mPEG-MMP2p-DSPE	mPEG350-DSPE	biotin-PEG1000-DSPE
1	mixture	22.7	-	75.9	1.4
2	emulsion	22.9	-	75.7	1.4
3	mixture	-	18.5	80.3	1.2
4	emulsion	-	20.1	78.9	1.0

Table S.3 Phospholipid composition (%area, disregarding BG and F, see Figure S.10) of mixtures and nanoemulsions, prior to and after nanoemulsion synthesis, respectively.

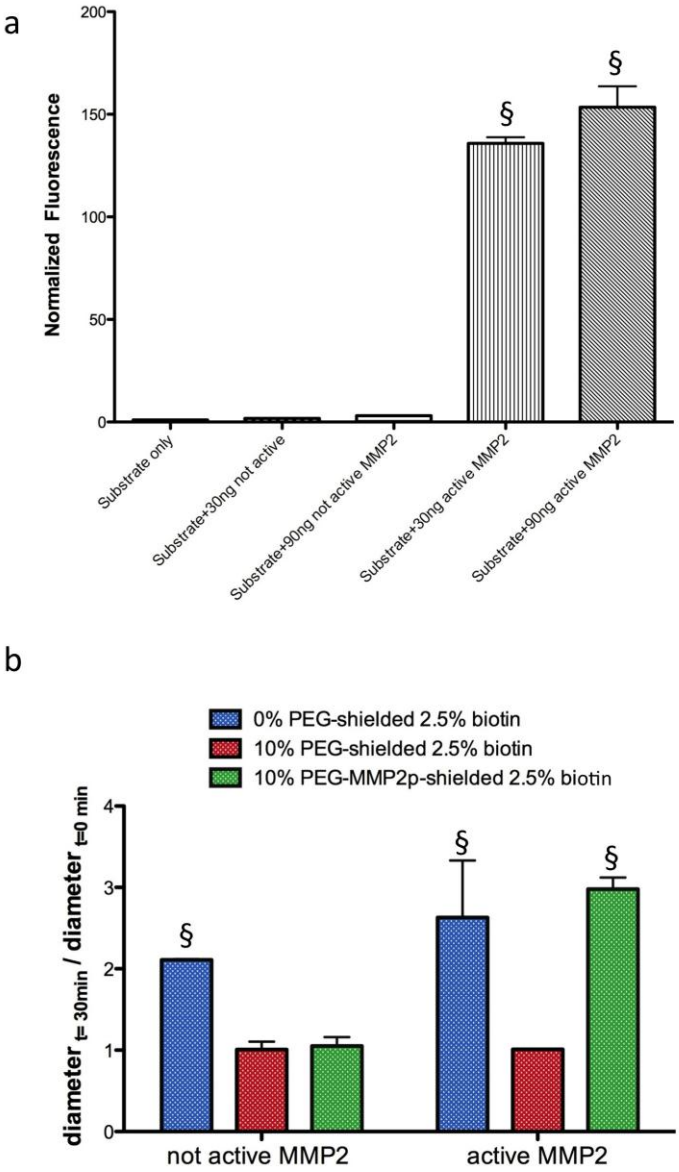


Figure S.11 (a) Normalized fluorescence emitted by MMP2-sensitive substrate in presence of not active and active MMP2 after 1 h of incubation at 37 °C (N=3). Bars represent mean ± SD (§ vs Substrate only, $P \leq 0.05$), (b) Avidin-induce aggregation of biotin functionalized shielded and unshielded nanoemulsions in presence of not active and active MMP2 measured by DLS (N=3). Bars represent mean ± SD (§ vs 10% PEG-shielded 2.5% biotin nanoparticles, $P \leq 0.05$).

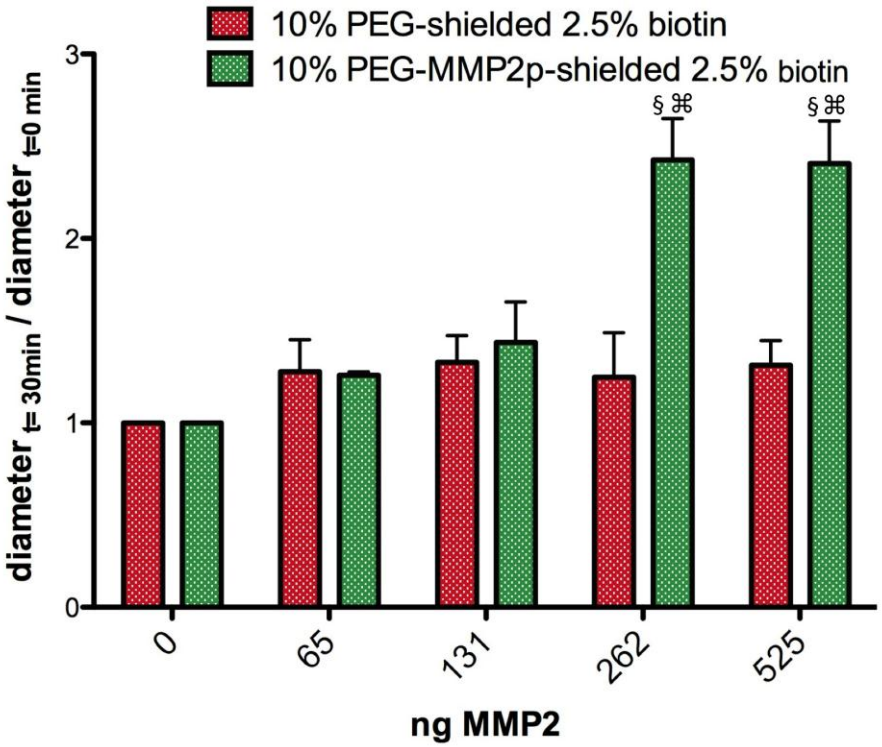


Figure S.12 (a) Avidin-induce aggregation of biotin functionalized nanoemulsions in presence of different amounts of active MMP2 measured by DLS (N=3). Mean ± SD (§ vs 0 ng MMP2, ⌘vs 65 ng MMP2).

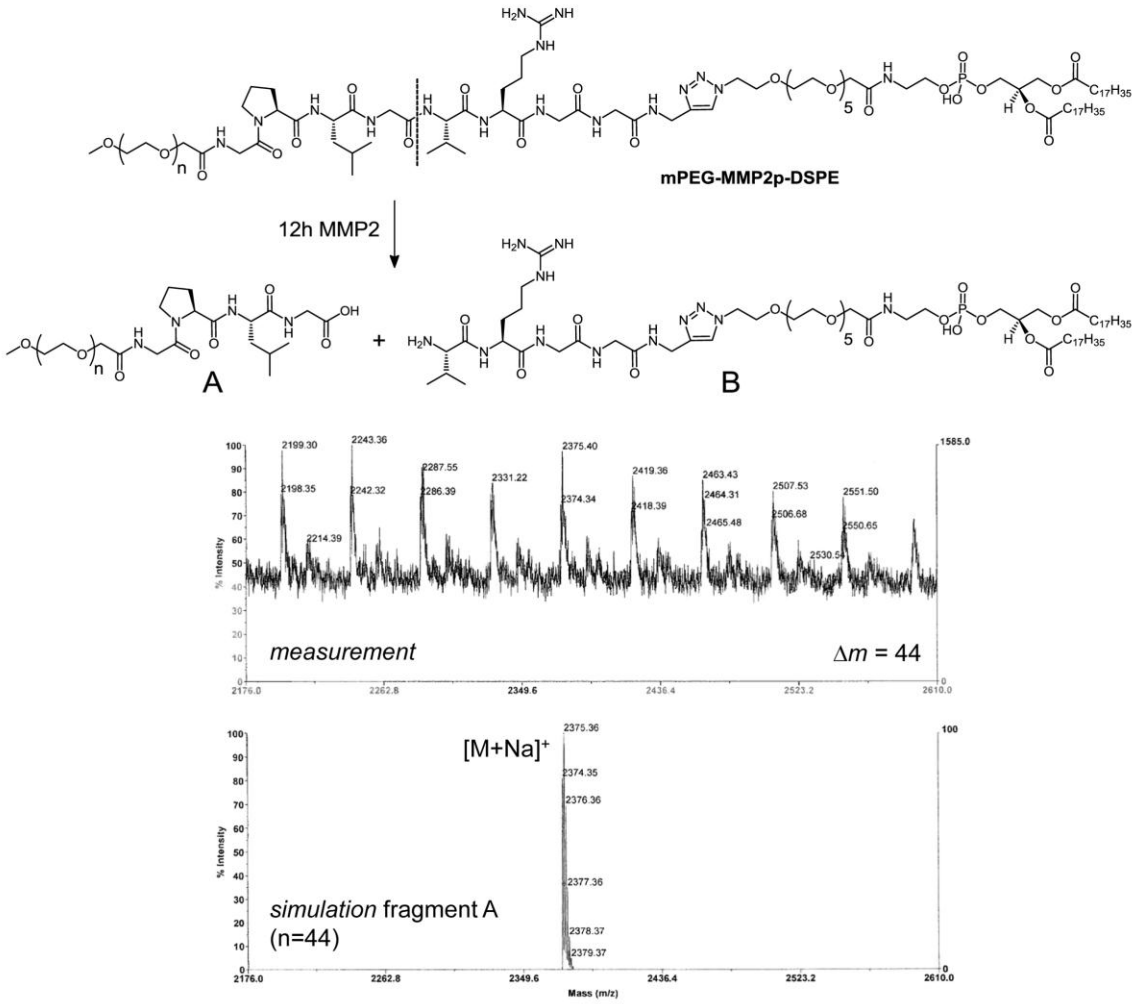


Figure S.13 MALDI-TOF-MS analysis of mPEG-MMP2p-DSPE after MMP2 incubation. The spectrum reveals the presence of one of the two expected cleavage fragments, *i.e.* mPEG-GPLG-COOH fragment A.

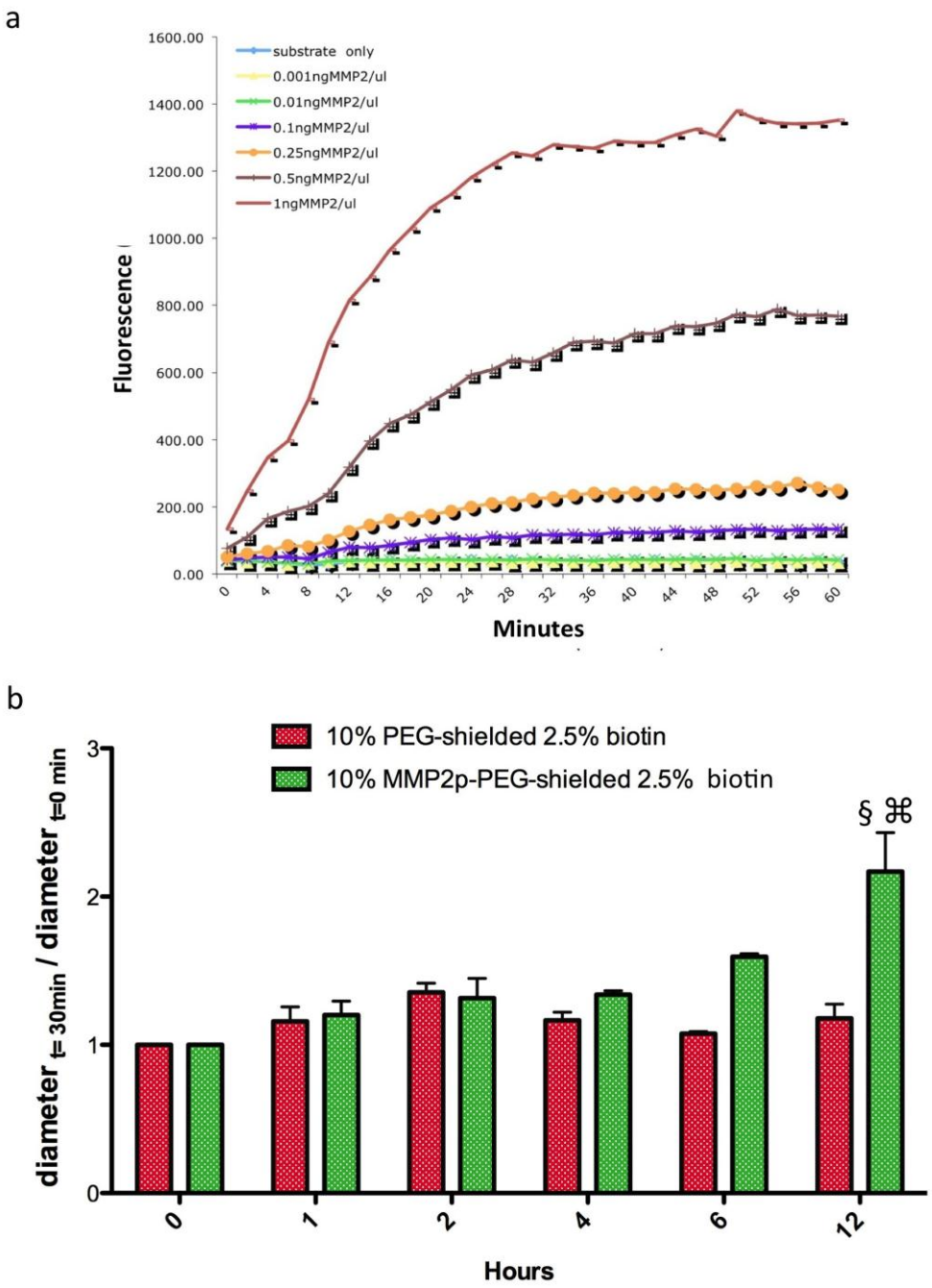


Figure S.14 (a) Fluorogenic MMP2 substrate cleavage kinetic by MMP2 at 37°C, (b) Nanoemulsion PEG cleavage kinetic by MMP2 shown through avidin-induced aggregation system. Mean ± SD, (§ vs sample 0 hr, ⌘vs sample 1 hr P≤ 0.05).

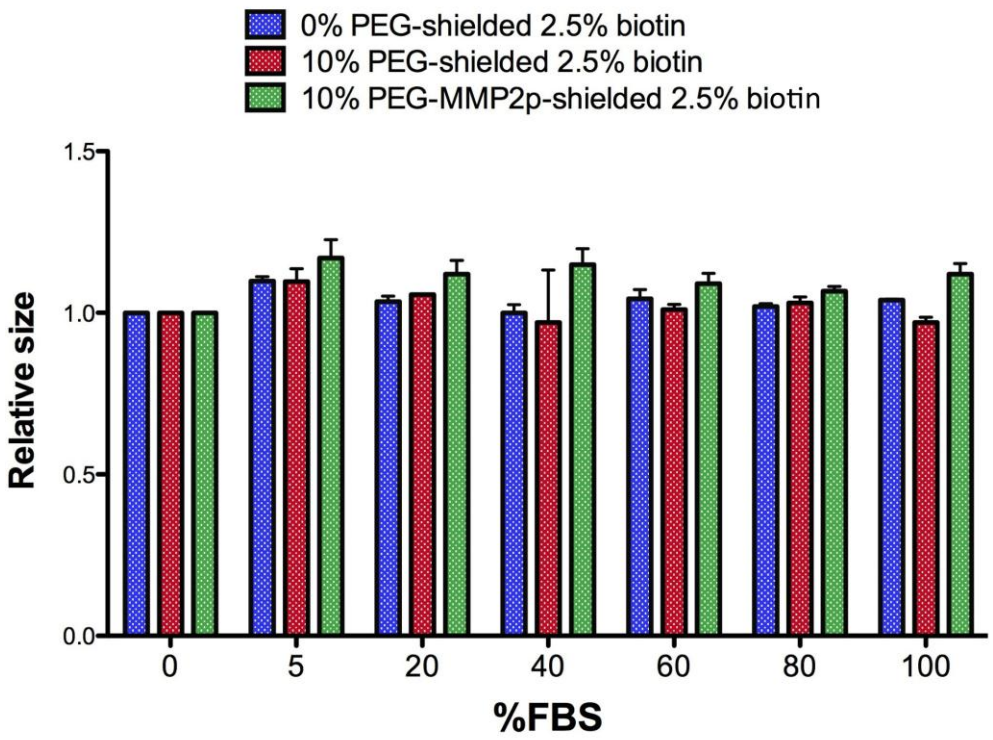
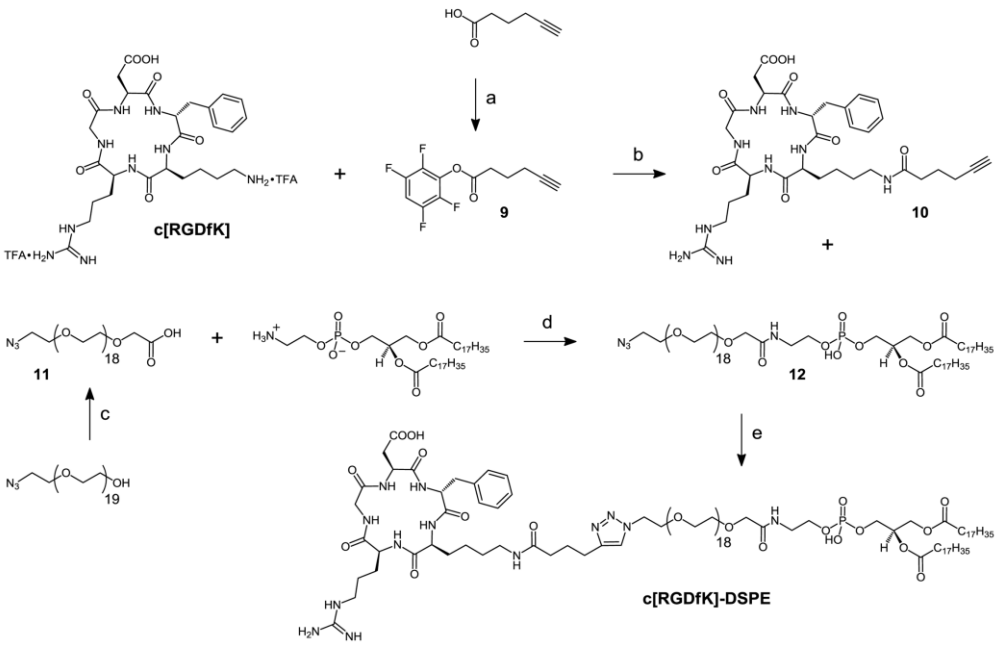


Figure S.15 Nanoparticle effective diameter relative to serum-free condition for nanoemulsions in presence of FBS for 12 hrs at 37°C measured by DLS. Samples were normalized versus 0% FBS samples (N=3). Bars represent mean ± SD. Variations between samples are not statistically significant.



Scheme S.4 Synthesis of c[RGDfK]-DSPE: (a) TFP, PPTS, EDCI·HCl, CH_2Cl_2, r.t., 16 h, 100% (b) DIPEA, DMF, r.t., 2 h, 56% (c) NaOCl, TEMPO, NaBr, 0.25 M Na_2CO_3 (pH = 11), r.t., 3 h, 91% (d) DIPEA, HBTU, DMF / $CHCl_3$ 1:3, 50 °C, 2 h, 77% (e) Na-ascorbate, $CuSO_4 \cdot 5H_2O$, THF / H_2O, r.t., 2 h, 75%.

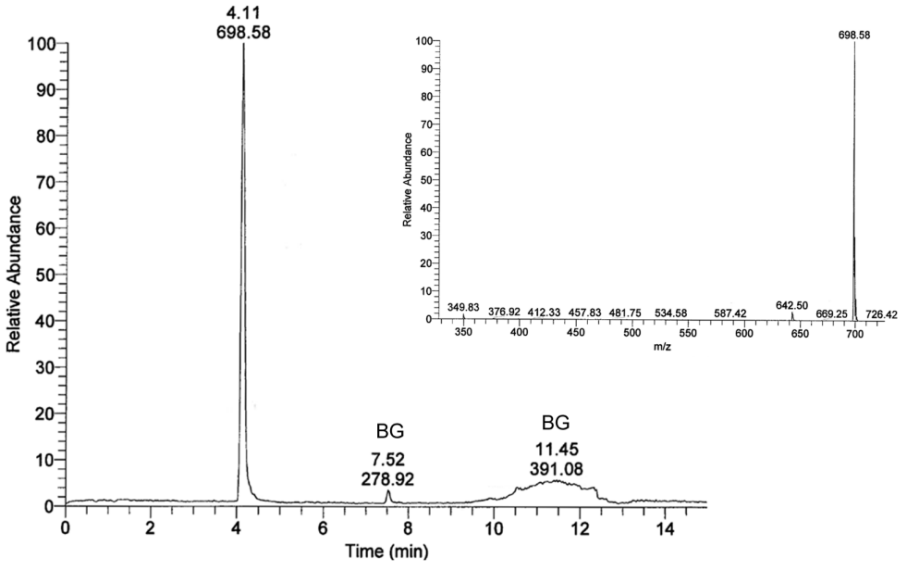


Figure S.16 LC-MS characterization (TIC, positive mode) of **10**, the peaks at t_R = 7.5 min and around t_R = 11.5 min are background signals (BG, dibutylphtalate and dioctylphtalate, respectively; the latter are plasticizers used in HPLC-tubings). The inset shows the mass spectrum of the main peak, showing predominantly the $[M+H]^+$ ion.

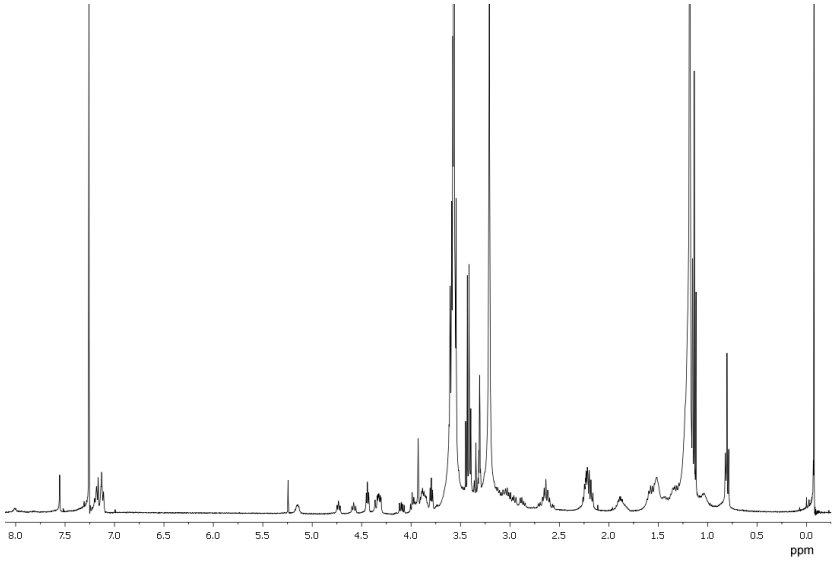


Figure S.17 ^{1}H-NMR spectrum of c[RGDfK]-DSPE in $CDCl_3$ / MeOD-d_4 95:5. Residual solvent signals are visible at 1.21 and 3.52 ppm (diethyl ether).

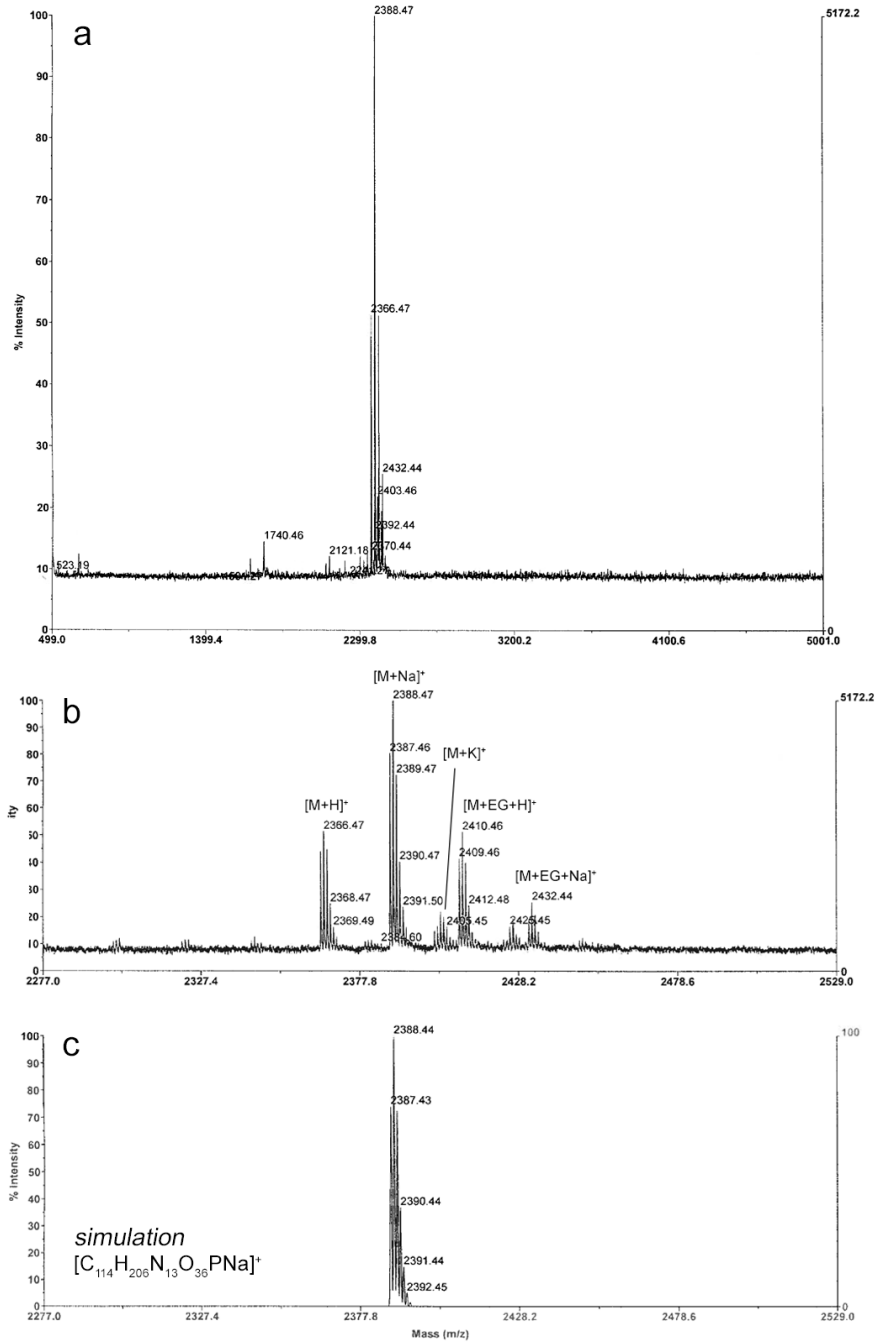


Figure S.18 (a) MALDI-TOF-MS spectrum of c[RGDfK]-DSPE in reflector mode (b) expansion of (a) showing isotope distributions of individual ions. The presence of a second oligomer (Δm = 44 due to an additional EG unit) is clearly discernable* (c) simulation of the expected isotope distribution for the $[M+Na]^+$ ion, showing an excellent match between theory and experiment.

* the supplier reports the oligomer purity of the starting *O*-(2-azidoethyl)nonadecaethylene glycol as 95% so this is rather unexpected

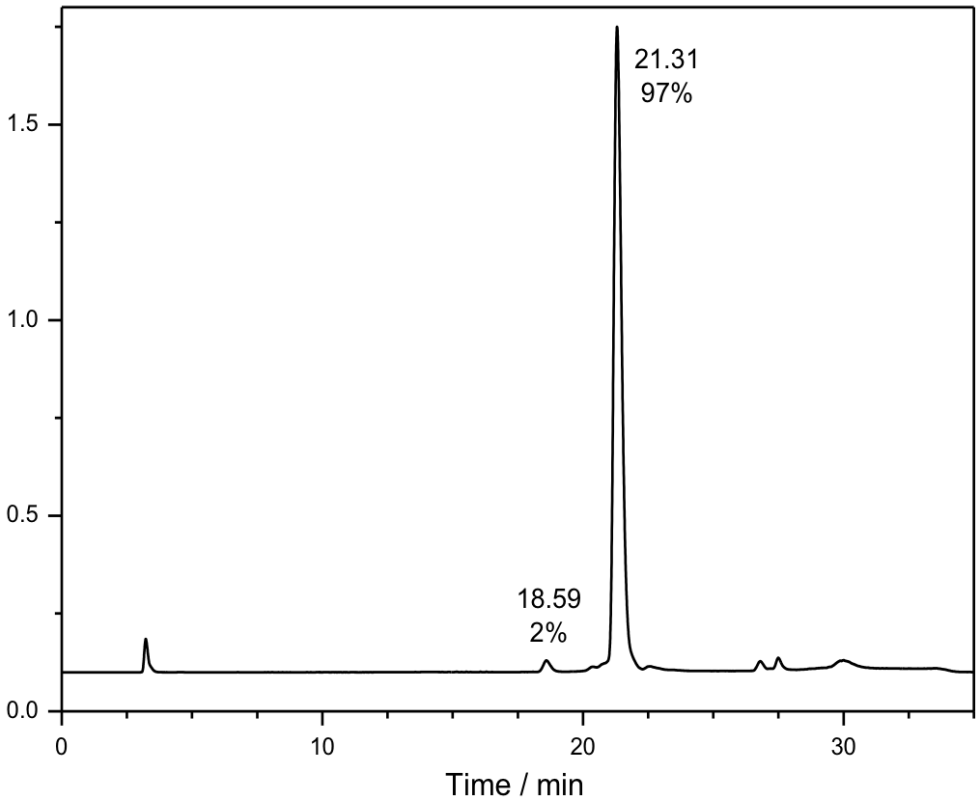


Figure S.19 HPLC trace of c[RGDfK]-DSPE in THF/H_2O with 0.1 % TFA (ELSD detection). On the basis of this spectrum, the purity of c[RGDfK]-DSPE is estimated to be 97%. Background signals (BG) have not been integrated. Ca. 2% of the compound is believed to have one aliphatic C18 tail missing from the DSPE unit.

a

	Au-MMP2p nano	IO-MMP2p nano
DLS (nm)	74	70.4
Polydispersity	0.108	0.169
r1 ($mM^{-1}s^{-1}$)	n.a.	1.41
r2 ($mM^{-1}s^{-1}$)	n.a.	56.3
HU mM-1	3.11	n.a.

b

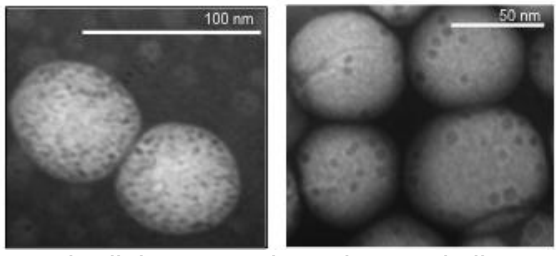


Figure S.20 (a) Dynamic light scattering size, polydispersity, relaxivities and CT attenuation measurements of theranostic nanoemulsions loaded with Au and IO nanocrystals. (b) TEM images of Au theranostic nanoemulsions (left) and IO theranostic nanoemulsions (right).

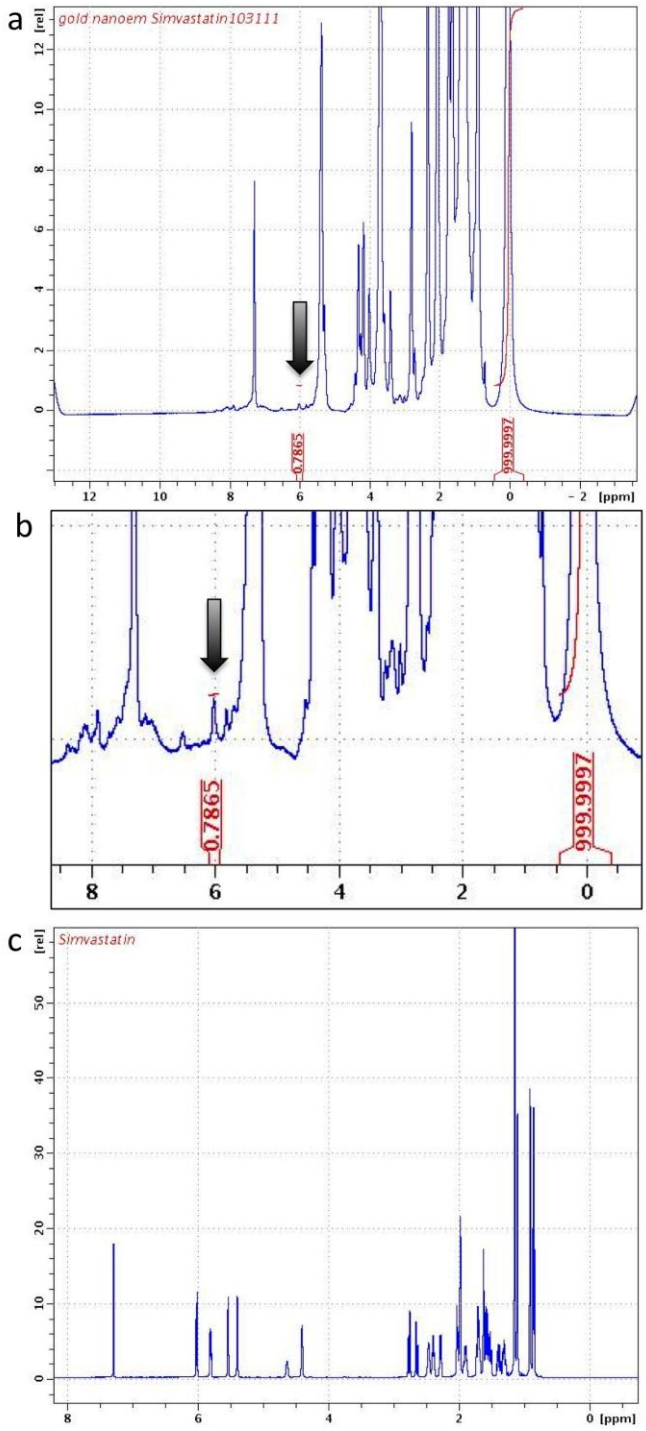


Figure S.21 ^{1}H-NMR spectrum. (a) Au-nanoparticles loaded with Simvastatin, (b) Au-nanoparticles loaded with Simastatin detail, (c) Simastatin. The arrow shows the presence the H atom specific from Simvastatin that has been used for drug inclusion.

References

(1) H. T. ten Brink, J. T. Meijer, R. V. Geel, M. Damen, D. W. P. M. Löwik, and J. C. M. van Hest, *J. Pept. Sci.* **2006,** *12*, 686–692.
(2) A. Dif, F. Boulmedais, M. Pinot, V. Roullier, M. Baudy-Floc'h, F. M. Coquelle, S. Clarke, P. Neveu, F. Vignaux, R. Le Borgne, M. Dahan, Z. Gueroui and V. Marchi-Artzner, *J. Am. Chem. Soc.* **2009**, *131*, 14738–14746.
(3) X. Dai, Z. Su, J.O. Liu, *Tetrahedron Lett.* **2000,** *41* 6295-6298.
(4) G. B. Fields and R. L. Noble, *Int. J. Pept. Prot. Res.***1990**, *35*, 161–214.
(5) M. Brust, M. Walker, D. Bethell, D.J. Schiffrin, R. Whyman, *Chem.Commun*. **1994**, 801-802.