Supporting Information for:

Multifunctional Cytotoxic Stealth Nanoparticles – A Model Approach with Potential for Cancer Therapy

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Materials and Methods.

Materials: 1-Aminopropan-2-ol, methacryloyl chloride, glycylglycine, glycyl-L-phenylalanine, L-leucylglycine, 4-nitrophenol, 4,5-dihydrothiazole-2-thiol, 4-(dimethylamino)pyridine (DMAP), triethylamine (TEA), *N*,*N*-dimethylformamide (DMF), *N*,*N*′-dicyclohexylcarbodiimide (DCCI), 2,2'-azobisisobutyronitrile (AIBN), doxorubicin hydrochloride (Dox.HCl), Cathepsin B, ethylenediaminetetraacetic acid (EDTA), H_3BO_3, $Na_2B_4O_7$ $10H_20$, NaCl, KCl, KH_2PO_4, Na_2HPO_4 $2H_2O$, KCN and dimethyl sulfoxide (DMSO) were from Fluka AG, Buchs (Switzerland). Poly(allyl amine hydrochloride) Mw=15000 g/mol (PAH), tetrachloroauric acid (99.9%), trisodium citrate dihydrate, N_α -benzoyl-L-arginine 4-nitroanilide (Bz-Arg-Nap) and reduced glutathione were purchased by Sigma-Aldrich, hyperbranched poly(ethylene imine) LUPASOL, Mw=25 000 g/mol was gratefully given by BASF, poly(styrene sulfonate) (PSS-pss-13k) was purchased from Polymer Standard Service (PSS). THP-1 cells (ATCC, TIB-202), RPMI 1640 w/o HEPES (Invitrogen, Ref 21875-034), fetal calf serum (Eurobio), HEPES (Invitrogen, Ref 15630056), Glucose (Sigma, Ref G-7021), Sodium Pyruvate (Serva, Ref 15220), Gentamicine (Kalys, Ref G0124-25), β-mercaptoethanol (Sigma, Ref 63690) and phorbol ester 12-O tetra decanoyl phorbol 13 acetate (TPA, Sigma).

Synthesis of monomers: 1) N-(2-hydroxypropyl)methacrylamide (HPMA): the monomer was synthesized as described earlier.[1] 2) Ma-Gly-DL-PheLeuGly-Doxorubicin: i) The Schotten-Baumann acylation with methacryloyl chloride in an aqueous alkaline medium was employed for methacryloylation of the oligopeptide. The *N*-methacryloylglycyl-DL-phenylalanylleucylglycine (Ma-Gly-DL-PheLeuGly-OH) was synthesized as described earlier.[2] ii) *N*-methacryloylglycyl-DL-phenylalanylleucylglycine 4-nitrophenyl ester (Ma-Gly-DL-PheLeuGly-ONp) was prepared by the reaction of Ma-Gly-DL-PheLeuGly-OH with 4-

nitrophenol in the presence of DCCI in THF as described earlier.[2] iii) *N*-Methacryloyl-glycyl-DL-phenylalanyl-leucyl-glycyl doxorubicin (Ma–Gly–DL-PheLeuGly–Dox) was prepared by the reaction of 100 mg (0.172 mmol) Ma–Gly–DL-PheLeuGly–ONp with 100.5 mg (0.173 mmol) of doxorubicin hydrochloride in 2 mL of DMF. Triethylamine (26.2 μL, 0.173 mmol, 4×5.2 μL and 5.4 μL) was slowly added every ten minutes under stirring. The reaction mixture was stirred in the dark for 4 h at room temperature and left in a refrigerator overnight. DMF was evaporated and 10 mL of diethyl ether was added. Crystals were filtered off and washed with 20 mL of diethyl ether. The powder was then dissolved in methanol (2 w%) and purified on a Sephadex LH-20 column in methanol. 3) Ma-GlyGly-TT: *N*-methacryloylated amino acids bearing thiazolidine-2-thione reactive groups are synthesized by the reaction of *N*-methacryloylated amino acids with 4,5-dihydrothiazole-2-thiol in the presence of DCCI. Addition of catalytic amount of DMAP increased the reaction rate. For the synthesis of *N*-methacryloyl-glycyl-glycyl thiazolidine-2-thione (Ma-GlyGly-TT) , *N*-methacryloyl-glycylglycine (Ma-GlyGly-OH) (2.00 g, 10 mmol) and 4,5-dihydrothiazole-2-thiol (1.25 g, 10.5 mmol) were dissolved in 30 mL of freshly distilled DMF and the solution kept at –15 °C for 1 h. DCCI (2.5 g, 12 mmol) was dissolved in 10 mL of DMF and the solution was cooled down to –15 °C for 1 h. Afterwards, both solutions were mixed, DMAP (~50 mg) was added and the mixture was kept at 0-5 °C for 24 h. The mixture was stirred 1 h at room temperature, acetic acid (50 μL) was added followed by stirring for another 1 h. *N*,*N*′-dicyclohexylurea (DCU) was filtered off, and DMF was removed on vacuum evaporator to dryness. The crude product was dissolved in 40 mL of dry acetone and stirred at room temperature for 1 h. The residue of DCU was filtered off. Dry diethyl ether (40 mL) was added to the filtrate and the resulting solution was kept at 0-5 °C for crystallization. Ma-GlyGly-TT was obtained as yellow crystals after recrystallization from ethyl acetate. Yield: 1.8 g (60 %). ^{1}H NMR [$(CD_3)_2SO$]: δ 1.87 s, 3H (CH_3); 3.44 t, 2H (CH_2S); 3.78 d, 2H (Gly); 4.50 t, 2H (CH_2N); 4.65

d, 2H (Gly); 5.37 s, 1H (CH_2=); 5.73 s, 1H (CH_2=); 8.14 t, 1H (NH); 8.20 t, 1H (NH). Elemental analysis: C: 43.79 (th:43.83), H: 5.21 (th:5.02), N: 13.87 (th:13.95), S: 21.12 (th:21.28), mp: 83 C , ε(305nm) in MeOH=10860 $Lmol^{-1}cm^{-1}$, R_F=0.221 (in ethyl acetate).[3]

Synthesis of the F-HPMA terpolymer (e.g. p(HPMA)-co-p(GlyGly-TT)-co-p(Gly-D,L-PheLeuGly-Dox) by radical copolymerisation: The mixture of HPMA (84 mol %, 200 mg), Ma-GlyGly-TT (12 mol %, 60.1 mg), Ma-Gly-DL-PheLeuGly-Dox (4 mol %, 65.6 mg)and 21.7 mg of AIBN (1 w%) was dissolved in 1.82 g of DMSO (15 w%). The solution was inserted into a polymerization ampoule, bubbled with nitrogen for 5 min and the ampoule was sealed. Polymerization was carried out at 60°C for 6 h. The solution was precipitated in a mixture 2:1 diethyl ether/acetone, filtered off, washed with acetone and diethyl ether. The solid was then dissolved in methanol (10 w%) and precipitated into a mixture acetone/diethyl ether (3:1), filtered off and dried in vacuum.

Characterization of monomers, F-HPMA and nanoparticles: Monomers were characterised by melting point, elemental analysis, NMR (Bruker, 300MHz), HPLC (LDC Analytical) and UV/VIS spectroscopy. Purity of all monomers was examined by HPLC (LDC Analytical, USA) using a reverse-phase column Tessek SGX C18 (150 x 4 mm) with UV detection at 270 nm or 305 nm. Water–methanol containing 0.1 vol. % of TFA with gradient 50–100 vol. % methanol was used as a mobile phase; flow-rate was 0.5 mL/min. The content of 4-nitrophenyl ester or thiazolidine-2-thione reactive groups in copolymers was determined by UV spectrophotometry. The weight-average molecular weight and polydispersity of copolymers (in the case of reactive copolymers after aminolysis of reactive groups with 1-aminopropan-2-ol) were determined by SEC on Äkta Explorer HPLC (Amersham Biosciences) with Superose 6™ column equipped with UV, differential refractometer (Shimadzu RID-10A, Japan) and multiangle light scattering detector DAWN® 8™ (Wyatt

Technology Corp., USA). 0.3M sodium acetate buffer (pH 6.5) containing 0.5 g/L sodium azide was used as the mobile phase. The flow rate was 0.5 mL/min.

Characterisation procedure reveals: M_w = 56 400 g/mol, M_w/M_n = 1.9, TT mol % = 8.7, Dox mol % = 1.9 (6.5 molecules of doxorubicin per copolymer chain). The Dox content was determined by VIS spectrophotometry at 484 nm (ε = 13 000 $Lmol^{-1}cm^{-1}$) in methanol. The content of TT reactive groups was calculated from absorbance obtained by subtraction of absorbance of copolymer aminolysed with 1-aminopropan-2-ol from absorbance of copolymer containing TT reactive groups at 305 nm.

Electron micrographs were obtained with a transmission electron microscope (TEM) (Philips CM12) operating at 120 kV.

References.

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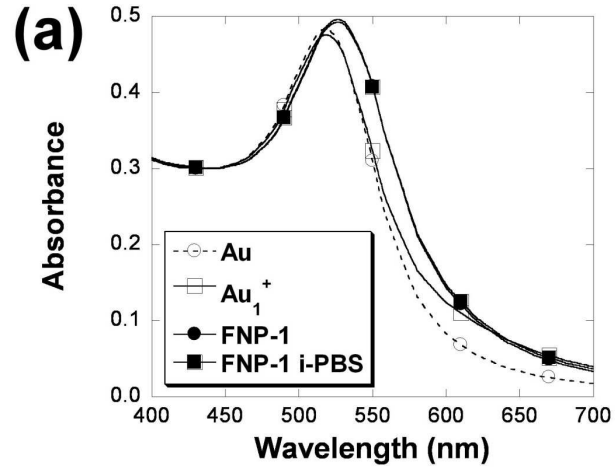
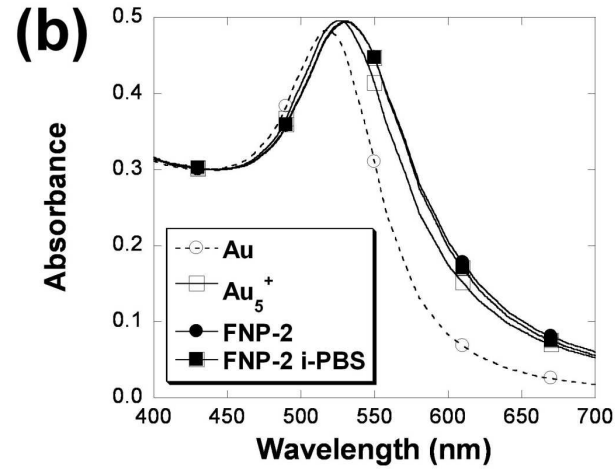
Figure S1. UV/Visible spectra during the nanoparticle functionalization for nanoparticles pre-coated with several layers of LBL-polymers and copolymer, dispersed in different buffers: a) Au/PAH (e.g. Au_1^+) in pure water, Au/PAH/F-HPMA (e.g. FNP-1) in pure water, FNP-1 in isotonic PBS (i-PBS); b) Au/$(PAH/PSS)_2$/PAH (e.g. Au_5^+) in pure water, Au/$(PAH/PSS)_2$/PAH/F-HPMA (e.g. FNP-2) in pure water, FNP-2 in i-PBS. Dotted lines correspond to native gold nanoparticles. All spectra were normalised in order to reach the same absorbance value at the wavelength of 440 nm. c) Statistical evaluation of the aggregation state derived from TEM images of 2 000 Au-cores.


(a)
Absorbance
Wavelength (nm)
Au
Au1+
FNP-1
FNP-1 i-PBS


(b)
Absorbance
Wavelength (nm)
Au
Au5+
FNP-2
FNP-2 i-PBS

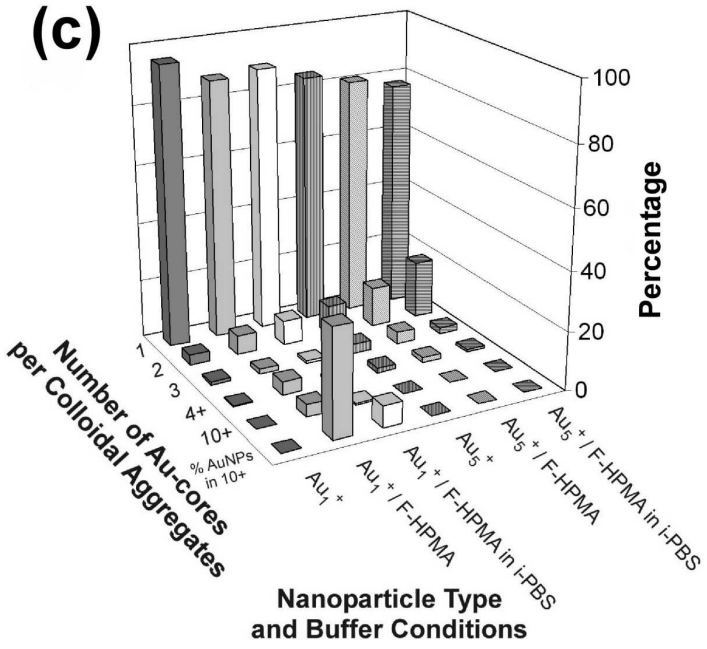

(c)
Number of Au-cores per Colloidal Aggregates
1
2
3
4+
10+
% AuNPs in 10+
Percentage
Au1+
Au1+ / F-HPMA
Au1+ / F-HPMA in i-PBS
Au5+
Au5+ / F-HPMA
Au5+ / F-HPMA in i-PBS
Nanoparticle Type and Buffer Conditions

FIGURE S1

Table S1. Position of plasmon bands and their displacement relative to pristine gold nanoparticles (in parenthesis) for nanoparticles bearing one or five PAH/PSS primer layers (in water), after their functionalization with F-HPMA (in water), and after their dispersion in i-PBS. Units used are nanometers.

	$(PAH/PSS)_n$ / PAH	F-HPMA	i-PBS
Au_1^+ (n=0)	519.3 (1.6)	527.0 (9.3)	527.0 (9.3)
Au_5^+ (n=2)	526.0 (8.3)	531.0 (13.3)	531.0 (13.3)

For one primer layer (FNP-1) the attachment of the F-HPMA corona layer causes a much larger bathochromic shift (8 nm) than in the case of five primer layers (4 nm, FNP-2, Figure S1a-b): the attachment of F-HPMA occurs much closer to the AuNPs core in FNP-1 compared to FNP-2.

This analysis revealed that the attachment of the corona layer in the case of a single primer layer (FNP-1) leads to the formation of significant amounts of aggregates composed of 10 or more Au-cores. The size of such aggregates can reach 300 nm or more (a TEM picture of such aggregates is available in Figure S2).

Figure S2. TEM micrographs of Au_1^+ (e.g. Au/(PAH/PSS)$_1$/PAH) and FNP-1 (e.g. Au/(PAH/PSS)$_1$/PAH/F-HPMA) in different dispersion media. a) From left to right: Au_1^+ dispersed in pure water, Au_1^+/ F-HPMA (FNP-1) dispersed in pure water, FNP-1 dispersed in i-PBS, FNP-1 dispersed in i-PBS in the presence of 0.5mg/mL of HSA; b) Overview image of FNP-1 dispersed in pure water. The inset is a close-up of the sub-micrometer aggregate identified by the black arrow.

In Figure S2a (from top left to top right) we present TEM micrographs of isolated gold nanoparticles with a single layer of PAH and with an additional corona layer of F-HPMA, dispersed in i-PBS (e.g. *isotonic* phosphate buffer saline) and in i-PBS containing 0.5mg/mL HSA (e.g. human serum albumin). After an incubation time of two days, a majority of isolated particles but also submicrometer aggregates form (Figure S2b); These submicrometer aggregates are totally absent when the gold cores are ensheathed by five polyelectrolyte primer layers.

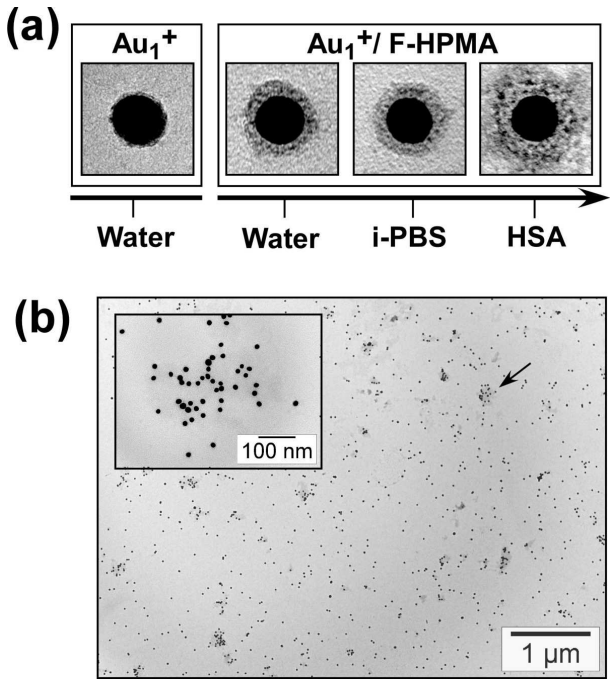


FIGURE S2

Figure S3. a) Top left: FNP-2 before removing the gold core by reaction with KCN. Bottom left: FNP-2 after removing the gold core by etching with KCN. The right TEM micrograph corresponds to FNP-2 after the dissolution of the core and shows four empty nanospheres. b) and c) UV/Visible spectra before (open circles) and after (open squares) core dissolution of FNP-1 (b) and FNP-2 (c). The left axis shows the absorbance of AuNP dispersions, the right axis refers to the absorbance of AuNP after dissolution of the gold core.