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Supplementary Information

Polymer control of ligand display on gold nanoparticles for multimodal switchable cell targeting

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Materials and Instrumentation.

Poly-N-isopropylacrylamide-co-acrylamide-COOH (pNIPAm-co-Am-COOH, 8 kDa, PDI 1.35, 9:1 isopropylacrylamide/acrylamide molar ratio), pNIPAm-co-Am-SH and folate-SH were prepared according to previously published methods[1, 2, 3]. Biotin-N-hydroxysuccinimide, 4-nitrophenyl disodium salt (Sigma 104®), foetal bovine serum, folate-free Dulbecco's modified eagle's medium (FFMEM) low glucose, L-glutamine solution, trypsin solution, penicillin-streptomycin-amphotericin B solution, sodium bicarbonate solution, D-(+)-glucose solution, were purchased from Sigma (St. Louis MO, USA). Cysteamine, cysteine, dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), 2,4,6-trinitrobenzensulfonic acid (TNBS), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), p-dimethylaminocinnamaldehyde (p-DACA), silica TLC plates (20 x 20 cm), Trityl-chloride poly(styrene) (1% divinyl benzene) resin, nitric acid and hydrochloric acid TraceSELECT® were furnished by Fluka Chemika (Buchs, Switzerland). Avidin and streptavidin-alkaline phosphatase-conjugated were obtained from Biospa (Milan, Italy). Sephadex G25 superfine resin was purchased from GE healthcare (Chalfont St Giles, United Kingdom). Vectashield® mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA, USA). N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (fluorescein-DHPE) was purchased from Invitrogen (Carlsbad, CA, USA). DB Falcon™ 4 wells chamber slides were acquired by Becton, Dickinson and Company (NJ, USA).

Synthesis of AuNP.

AuNP in water were obtained by laser ablation synthesis in solution (LASiS) according to a previously reported protocol.[1, 4] Briefly, laser ablation was obtained with 1064 nm (9 ns) laser pulses of a Quantel YG981E Nd:YAG laser focused on a 99.99% pure gold plate placed at the bottom of a cell containing bidistilled water. AuNP were characterized by UV-Vis spectroscopy

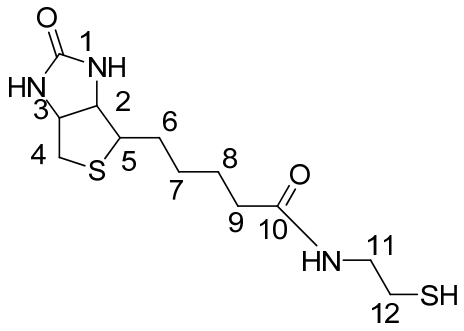
using a Varian Cary 5 spectrometer in 2 mm optical path quartz cells, and by transmission electron microscopy (TEM) collecting images at 300 kV with a JEOL JEM 3010 microscope equipped with a Gatan Multiscan CCD Camera model 794TEM (Fig. S1). The samples for TEM analysis were prepared by evaporating AuNP suspension on a copper grid covered with an amorphous carbon holey film. AuNP concentration was estimated by the Mie-Gans model fitting of UV-Vis spectra, as previously reported.[1, 4, 5]

Synthesis and characterization of biotin-cysteamine (biotin-SH).

The biotin-SH synthesis was carried out as described in Fig. S2A. Cysteamine (0.45 mg, 5.8 μmol) was dissolved in 100 μL of anhydrous DMF and added of biotin-NHS (1.0 mg, 2.9 μmol). After 30 minutes reaction at room temperature, the mixture was spotted on a precoated silica gel TLC plate and the plate was run with $CHCl_3$/MeOH 8/2 v/v mixture and biotin was detected by colorimetric test using p-DACA 0.2% (p/V), 2% H_2SO_4 (v/v) in EtOH as reported in literature[6] while the thiol groups were detected by the Ellman's test.[7] Biotin-SH showed a Rf value of 0.63. The reaction mixture was purified using preparative silica TLC plates run with $CHCl_3$/MeOH 8/2 v/v mixture and the conjugate was characterized by RP-HPLC, ESI-TOF mass spectrometry and ^{13}C NMR.

RP-HPLC analysis: ten microliters of the reaction mixture was added of 10 μL of 0.2% (w/V) p-DACA in EtOH, 10 μL of 2% (V/V) H_2SO_4 in EtOH and 70 μL of EtOH. After 1 hour incubation the sample was analysed by RP-HPLC using a Phenomenex Luna C18 column (Torrance, CA, USA) eluted with water/0.05% TFA (eluent A) and acetonitrile/0.05% TFA (eluent B) in a gradient mode from 20% to 50% eluent B in 20 minutes. The UV-Vis detector was set at 533 nm.

ESI-TOF mass spectrometry [m/z signal of 304.11 (M+H), calculated for $C_{12}H_{21}N_3O_2S_2$: 303.11, Fig. S2B] was carried out using a MARINER Biospectrometry Workstation (PerSeptive Biosystems, Stafford, TX, USA).



^{13}C NMR (300 MHz, DMSO-*d6*): δ 24.37 (C-6), 25.13 (C-8), 28.6 (C-12), 28.94 (C-7), 34.38 (C-9), 62.55 (C-5), 65.41 (C-2), 69.21 (C-4), 162.62 (C-1), 172.88 (C-10).

Synthesis and characterization of Folate-cysteamine (folate-SH).

The folate-cysteamine synthesis described in Fig. S3A was carried out according to the literature protocol[8], and characterised by RP-HPLC, ESI-TOF mass spectrometry and ^{1}H NMR.

RP-HPLC analyses were performed using a Phenomenex Luna C18 column eluted with water/0.05% TFA (eluent A) and acetonitrile/0.05% TFA (eluent B) in a gradient mode from 10% to 20% eluent B in 20 minutes. UV-Vis detection was recorded at 363 nm.

Folate-SH was characterized by ESI-TOF mass spectrometry [m/z signal of 499.06 (M-H), calculated for $C_{21}H_{24}N_8O_5S_1$: 500.16 – Fig. S3B] and by ^{1}H NMR. Peaks and assignments agreed with literature values.

^{1}H NMR (300 MHz, DMSO-*d6*): δ 11.4 (s, 1H, -C=N-C(O*H*)=C), 8.65 (s, 1H, -C-C(*H*)=N-), 8.160 (d, 1H, J=7.4 Hz, Ar-CO-N*H*-), 8.0 (t, 1H, J=6.5 Hz, SH-CH_2-CH_2-CO-N*H*-), 7.66 (d, 2H, J=8.4 Hz, aromatic protons C_{13}-H/C_{15}-H), 6.93 (bs, 1H, Ar-CH_2-N*H*-Ar), 6.62 (d, 2H, J =8.4 Hz,

aromatic protons C_{12}-H/C_{16}-H), 4.48 (d, 2H, J=6.0 Hz , Ar-C*H*$_2$-NH-Ar), 4.29 (m, 1H, -CO-NH-C*H(C)*-COOH), 3.50 (m, 2H, -NH-C*H*$_2$-CH_2-SH), 2.54 (m, 2H, -NH-CH_2-C*H*$_2$-SH), 1.8-2.2 (m, 4 H, -CH-C*H*$_2$-C*H*$_2$-CONH-).

Conjugation study of pNIPAm-co-Am-SH to AuNP.

Increasing amounts of pNIPAm-co-Am-SH (from 17.6 to 140.7 μg) were added to 4 mL of 1.57 nM AuNP in order to yield 350:1, 700:1, 1400:1 and 2800:1 polymer/AuNP molar ratio. The mixtures were maintained overnight under mild stirring at room temperature. The colloidal mixtures were centrifuged at 14000 rpm for 5 minutes and the supernatants were lyophilized and re-dissolved in 1 mL of water. The polymer amount in the supernatant was assessed by an iodine assay.[9] All conjugation runs yielded ~ 100% pNIPAm-co-Am-SH conjugation (Table S1).

The amount of pNIPAm-co-Am on the gold nanoparticles surface was also determined by thermal gravimetric analysis (TGA) using a TA Instruments TGA SDT Q600 (TA instruments, New Castle, DE, USA) under flowing nitrogen atmosphere. The temperature was increased from 20 to 800 °C at a rate of 10 °C/min.[10] The data obtained from the TGA analysis confirmed the results from iodine assay.

Conjugation study of biotin-SH to AuNP.

Aqueous volumes (2.7, 54.1, 108.3, 216.5 μL) of 0.29 mM biotin-SH were added to 10 mL of 1.57 nM AuNP to yield 50:1, 1000:1, 2000:1 and 4000:1 biotin-SH/AuNP molar ratios. After 6 hours of mild stirring at room temperature, the nanoparticles were removed by centrifugation at 14000 rpm for 5 minutes and the supernatants were lyophilized. The dry material was re-dissolved in 50 μL of anhydrous DMF and added to 50 μL of 0.2% (w/V) p-DACA in ethanol and 50 μL of 2% (V/V) H_2SO_4 in ethanol. After 1 hour incubation at room temperature, 350 μL of EtOH was

added and the resulting solution was analyzed by RP-HPLC with a Phenomenex Luna C18 column eluted with water/0.05% TFA (eluent A) and acetonitrile/0.05% TFA (eluent B) in a gradient mode from 20% to 50% eluent B in 20 minutes. UV-Vis detection was recorded at 533 nm. The biotin conjugation degree increased as the biotin/AuNP molar ratio increased. The reaction yielded 100% biotin coupling with 50:1, 1000:1 and 2000:1 biotin/AuNP molar ratio while maximal biotin coupling (3320:1 biotin/AuNP) was obtained using 4000:1 biotin/AuNP molar ratio.

Syntesis of biotin/pNIPAm-co-Am decorated AuNP.

Water solutions (50 or 100 µL) of 18 µM biotin-SH were added to 15 mL of 1.2 nM water AuNP dispersion to yield respectively a 50:1 and 100:1 biotin-SH/AuNP molar ratio. The mixtures were incubated for 6 hours at room temperature, and then 10 mL of the biotin-SH/AuNP mixtures were added of 4.2, 8.4, 16.8 or 33.6 µL of 1.0 mM pNIPAm-co-Am-SH solution to achieve a 50:350:1 50:700:1, 100:700:1, 100:1400:1 and 100:2800:1 biotin-SH/polymer/AuNP molar ratios. Untargeted particles were obtained by incubating naked nanoparticles with the same pNIPAm-co-Am-SH/AuNP ratios while biotinylated AuNP were produced with the same biotin-SH/AuNP molar ratios without pNIPAm-co-Am-SH addition. The colloidal mixtures were stirred overnight at room temperature. The degree of biotin and polymer conjugation was determined by chromatographic and spectrometric analysis as reported above.

Synthesis of folate and folate/pNIPAm-co-Am decorated AuNP.

A water solution (7 mL) of 3.1 nM AuNP was added of 71 µL water containing 30.6 µM folate-cysteamine (1.1 µg, 2.2 nmol) to yield 100:1 folate-SH/AuNP molar ratio. Part of the mixture was characterized for folate content as reported below and used for UV/Vis and DLS analysis and cell uptake studies as positive control. The remaining part of the mixture was incubated for 6 hours at

room temperature, and then added of 15.2 µL of 1.0 mM pNIPAm-co-Am-SH solution (121.5 µg, 15.2 nmol) to achieve 700:1 polymer/AuNP molar ratio. The sample was stirred overnight at room temperature and diluted to 0.62 nM with 0.025 M phosphate buffer, 0.187 M NaCl, pH 7.4 to obtain a final physiological saline solution. In order to assess the degree of conjugation of the polymer and the folate, reaction mixture volumes were centrifuged at 14000 rpm for 5 minutes and the amount of unconjugated polymer and folate in the supernatant was assessed by spectrometric analysis and the folate by RP-HPLC as reported above. The same protocol without the folate-SH conjugation step was followed to obtain pNIPAm-co-Am coated AuNP with the same degree of polymer conjugation (control).

Dispersion/aggregation assays for AuNP.

Naked, folate coated, folate/pNIPAm-co-Am coated AuNP and pNIPAm-co-Am coated AuNP (control) were diluted to 0.62 nM in 0.025 M phosphate buffer, 0.187 M NaCl, pH 7.4 with or without 10% foetal bovine serum (FBS). The dispersions were allowed to equilibrate to the experimental temperature (34 or 40°C) for 15 minutes and analyzed by photo correlation spectroscopy (PCS) at 34 and 40°C using a NICOMP 380ZLS Particle Sizing System (Nicomp, Santa Barbara, CA, USA) (Table S2). The dispersions were also analyzed by UV-Vis spectroscopy (Fig. S4, S5 and S6A). Folate/pNIPAm-co-Am coated AuNP were also analyzed by UV-Vis spectroscopy (Fig. S6B) and DLS (Table S3) at 37°C to evaluate the stability at physiological temperature. The tests confirmed the physico-chemical stability of nanoparticles at 37°C.

ELISA studies on biotinylated AuNP.

The biotin/pNIPAm-co-Am coated AuNP binding to avidin was evaluated by non-competitive enzyme-linked immunosorbent assay (ELISA).[11] A 96-wells microtitre plate was coated at 4°C

overnight with 100 µL of a 1 µg/mL avidin solution in 0.1 M bicarbonate buffer, pH 9.5. The plate was washed three times with 220 µL/well of PBS, 0.3% Tween 20 (PBS/Tween buffer) and then was incubated at 37°C for 1 hour with 200 µL/well of a 50 µg/mL BSA solution in 0.05 M TRIS buffer, 2 mM EDTA, 0.3 M KCl pH 8. After incubation, the plate was washed three times with 220 µL/well of PBS/Tween buffer. The plates were incubated with 100 µL of increasing concentrations (from 0.05 to 1.17 nM) of biotinylated AuNP, biotinylated pNIPAm-co-Am coated AuNP and pNIPAm-co-Am coated AuNP at 34 and 40°C. Each sample was pre-warmed 15 minutes at the incubation temperature, and the plates were incubated for 3 hours. The mixtures were then discharged and plates were washed five times with 220 µL/well of PBS/Tween buffer, and then 100 µl of 0.4 µg/mL of pre-warmed streptavidin-alkaline phosphatase solution in PBS/Tween buffer were added to each well. The plates were incubated for 1.5 hours at 40°C and then washed five times with 200 µL/well of PBS/Tween buffer and treated with 200 µL/well of a 1 mg/mL solution of 4-nitrophenyl disodium salt in 1 M diethanolamine, 0.5 mM $MgCl_2$ pH 9.8 at room temperature. After 1 hour the enzymatic reaction was quenched by adding 50 µL/well of 3 M NaOH and the plates were analyzed by an EL311SK microplate autoreader (Bio-Tek Instruments, Winooski, VT-USA) set at 405 nm. The optical density was normalized using the maximum signal raised by 1.17 nM biotinylated AuNP at 34 and 40°C (reference) (Fig. 2 and S7).

Cell culture.

Human breast adenocarcinoma MCF7 and nasopharyngeal epidermal carcinoma KB cell lines were cultured as a monolayer in a 75 cm^2 tissue culture treated flask at 37°C under humidified atmosphere containing 5% CO_2 in folate-deficient Dulbecco's modified Eagle's medium with 4.5 g/L glucose (FFDMEM) supplemented with 10% (v/v) heat-inactivated fetal bovin serum (FBS), 2 mM glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL of amphotericin B.

Cells were routinely harvested by treatment with 500 µg/mL trypsin in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS).

Cell uptake of AuNP at 34 and 40°C.

MCF7 and KB cells were seeded in 6-well tissue culture treated plates at a density of 1.5 x 10^6 cells/well. After 24 hours, the culture medium was replaced with 1.5 mL of FFDMEM, 154 mM NaCl, containing 0.62 nM pNIPAm-co-Am or folate/pNIPAm-co-Am decorated AuNP preheated 15 minutes at 34 or 40°C. The cells were incubated for 2 hours at 34 or 40°C either in the presence or absence of 10% foetal bovine serum (FBS). Cells were incubated with folated AuNP, positive control, at 40°C only in the presence of FBS. The medium was removed, and the cells were washed with FFMEM (x 2) and detached by trypsin treatment. The samples were added to 1 mL of PBS and centrifuged at 1000 rpm for 5 minutes. The pellets were washed three times with PBS and lysated by addition of 600 µL of Triton® X-100 0.1% in water. The lysates (500 µL) were digested with aqua regia (HNO_3/HCl 1:3 v/v) at 100°C for 2 hours and the acid volume was brought to 5 mL with 4% HCl addition. Quantitative analysis of gold was performed by Atomic Absorption Spectrometry (AAS) using a Varian AA240 Zeeman instrument equipped with a GTA120 graphite furnace, Zeeman background corrector and an autosampler (Varian Inc., Palo Alto, CA-USA). 100 µL of the cell lysate in Triton® X-100 was analysed by BCA Protein Assay (Thermo Fisher Scientific Inc., Waltham, MA-USA), and the cells per mL were determined by referring to a calibration curve.

Cell uptake inhibition assay.

KB cells were seeded in 6-well tissue culture treated plates at a density of 1.5 x 10^6 cells/well. After 24 hours, the culture medium was replaced with 2 mL of FFDMEM, 154 mM NaCl, 200 µM folic acid. After 3 hours incubation, the medium was removed and replaced with 0.62 nM folate/pNIPAm-co-Am decorated AuNP, 200 µM folic acid medium, with or without 10% FBS, preheated at 40°C for 15 minutes. The cells were incubated for 2 hours at 40°C. After incubation, the samples were treated as described above for gold quantification (Fig. S8).

Confocal microscopy studies.

KB cells (10^5 cells in 0.5 mL) in folic FFDMEM supplemented with 10% FBS were seeded in DB Falcon™ 4 wells chamber slides. After 24 hours the medium was removed and the wells were washed three times with PBS. AuNP decorated with folic acid/pNIPAm-co-Am or pNIPAm-co-Am were dispersed in 1 mL FFMEM, 154 mM NaCl at a concentration of 0.62 nM and added to each wells. Chamber slides were incubated for 2 hours at 34°C or 40°C. The medium was then removed and the cells were washed three times with PBS. Fluorescein-DHPE dissolved in PBS was added to each well (1 mL, 20 µg/mL) and gently shaken in the dark for 10 minutes. The fluorescein-DHPE solution was removed, the chamber slide washed three times with PBS and samples were incubated with 500 µL of 1% paraformaldehyde in PBS for 20 minutes. The supernatant was removed, the sides of the chamber slides were pilled off and samples covered with coverslips using Vectashield® mounting media with DAPI. The samples were maintained in the dark at 4°C. Confocal microscopy was performed on a Leica TCS SP5 Leica Microsystems GmbH microscope (Wetzlar, Germany) using the Leica Application Suite advanced fluorescence 2.0.2 software for data acquisition. Images were acquired using a 63x oil immersion lens with argon laser set at 405, 488 and 561 nm to detect respectively DAPI, fluorescein-DHPE and gold nanoparticles. In the case of gold nanoparticles

scattered light was recovered between 550 and 580 nm on the base of investigations reported in the literature.[12]

FIGURES

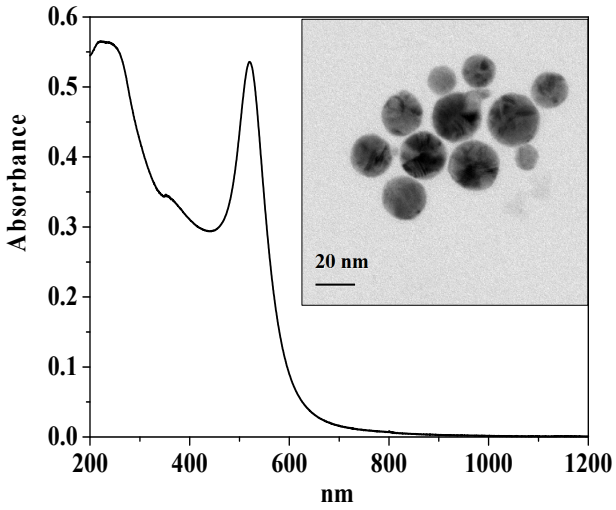


Fig. S1. The UV-visible spectrum of AuNP obtained by LASiS in the 0.4 mM NaCl aqueous solution and a representative TEM image of same nanoparticles.

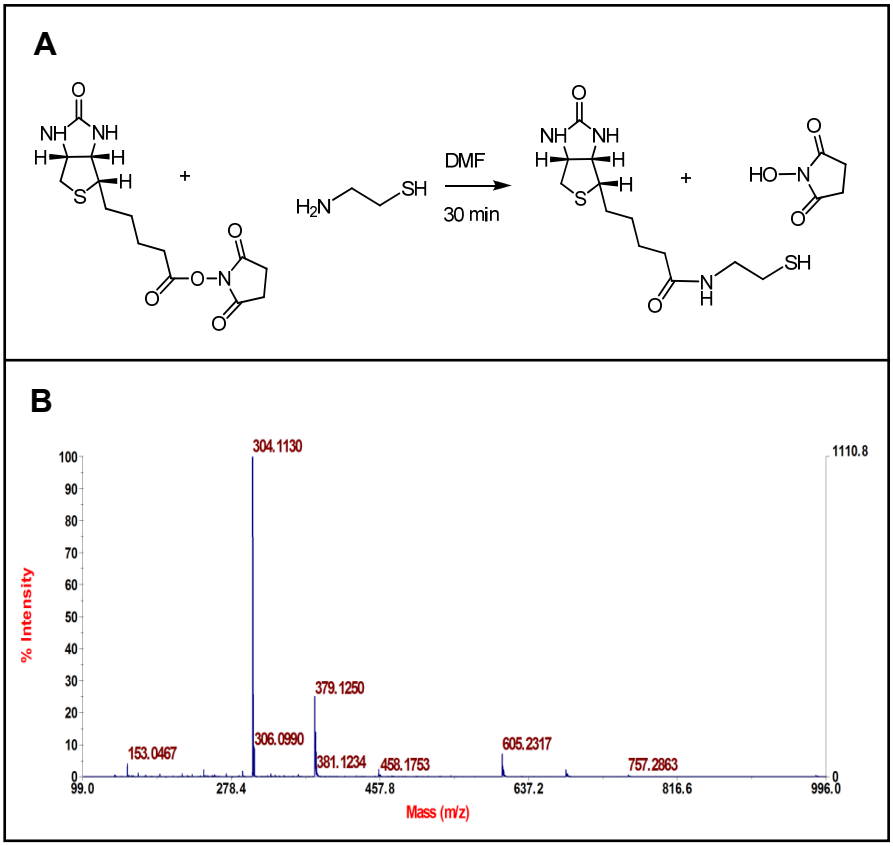


Fig. S2. Panel A: coupling reaction of biotin-NHS with cysteamine. Panel B: mass spectrometry analysis of biotin-cysteamine.

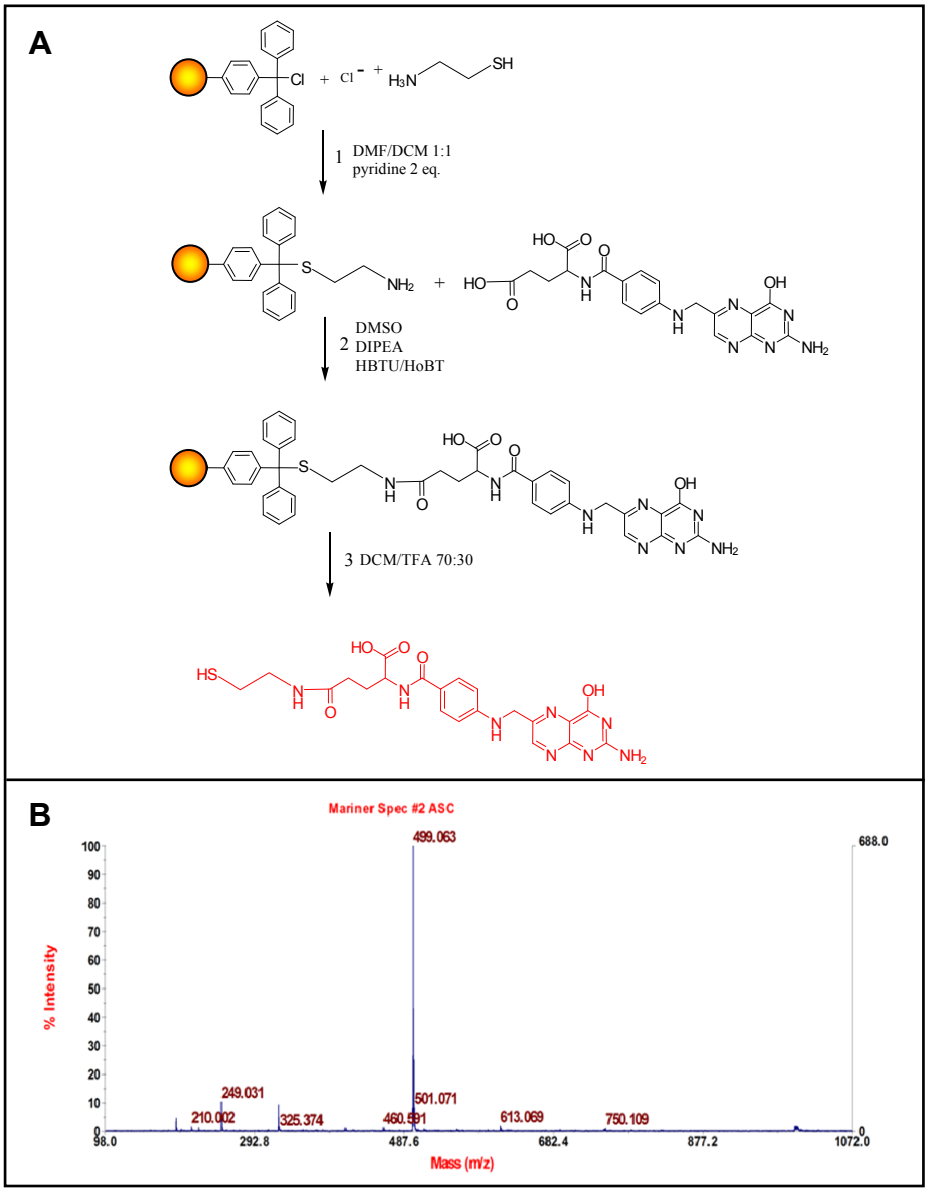


Fig. S3. Panel A: synthesis of folate-cysteamine conjugate. Cysteamine conjugation to the resin through the thiol group (1); folate γ–carboxyl group conjugation to amino group of cysteamine (2); cleavage of folate-cysteamine from the resin (3). Panel B: mass spectrometry analysis of folate-cysteamine.

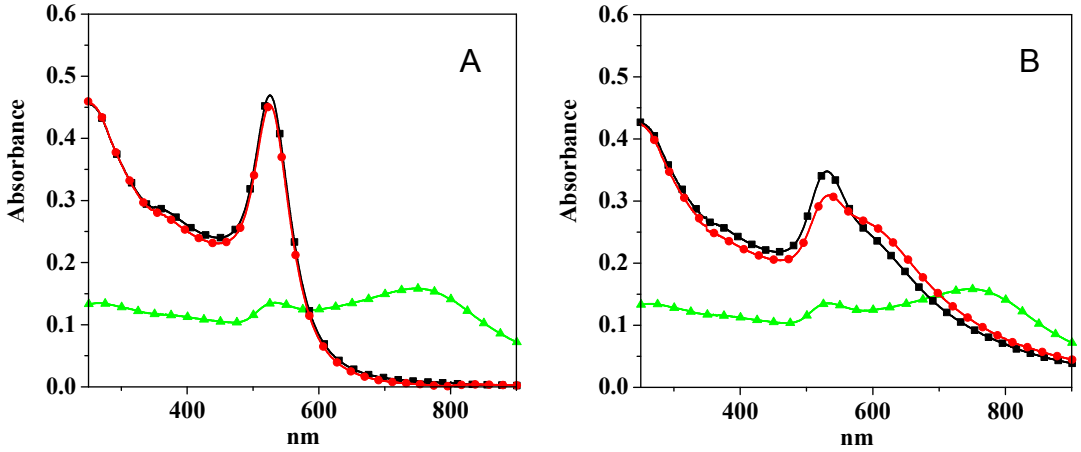


Fig. S4. UV-Vis spectra of naked AuNP (▲) and AuNP decorated with folate/pNIPAm-co-Am (■) and pNIPAm-co-Am (●) in 0.02 M phosphate buffer, 0.150 M NaCl, at 34°C (Panel A) or 40°C (Panel B). The analyses were carried out with 100:700:1 folate/polymer/AuNP molar ratio decorated nanoparticles.

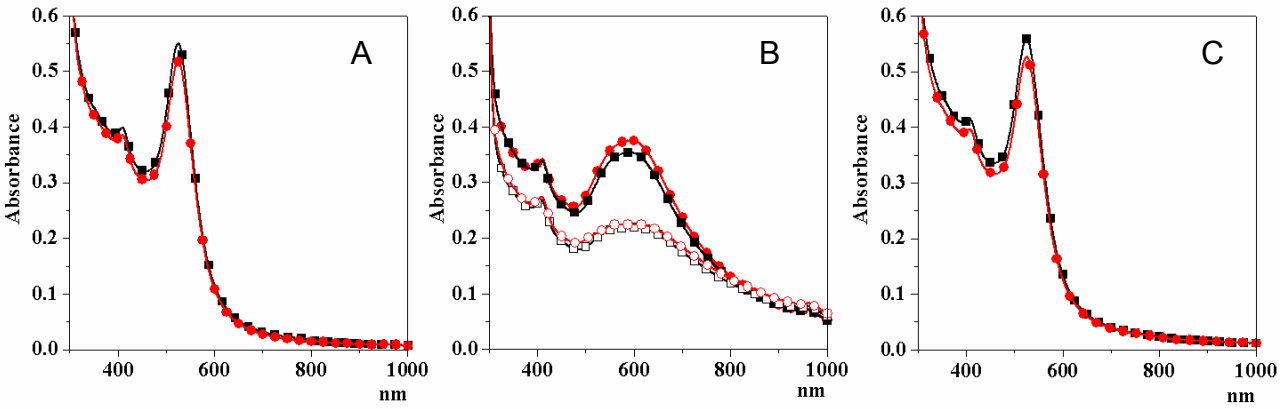


Fig. S5. UV-Vis spectra to show difference in aggregation of AuNP decorated with folate/pNPAm-co-Am (■) and pNPAm-coAm (●) in 10% foetal bovine serum (FBS)/PBS after: 120 minutes incubation at 34°C (Panel A); 10 minutes (full symbols) and 120 minutes (empty symbols) incubation at 40 °C (Panel B); incubation for 120 minutes at 40°C followed by 10 minutes incubation 34°C (Panel C). The analyses were carried out with 100:700:1 folate/polymer/AuNp molar ratio decorated nanoparticles.

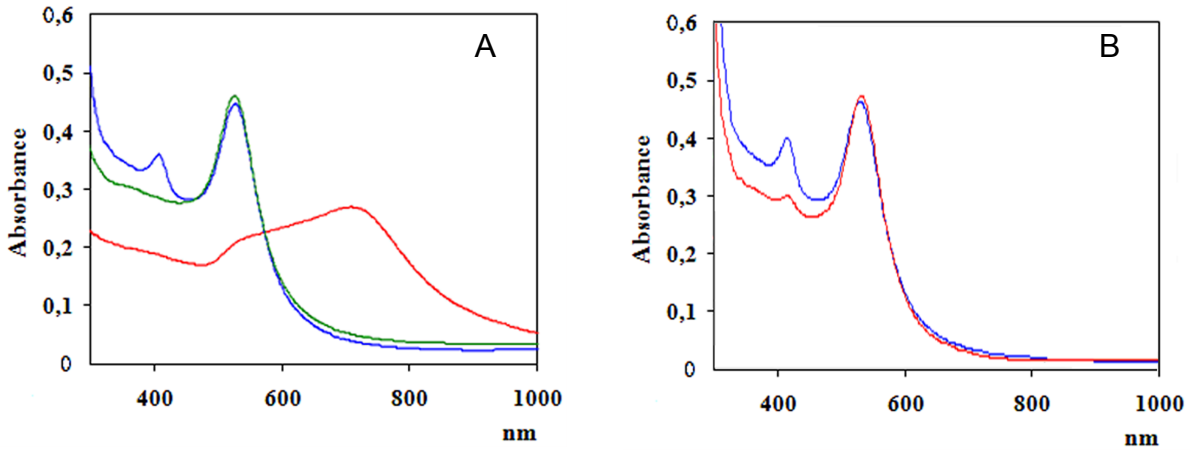


Fig S6. Panel A: UV-Vis spectra of: naked AuNP in water (green); folated AuNP in 10% foetal bovine serum (PBS/FBS) (blue); folated AuNP in PBS (red). Overlapped UV-Vis spectra were obtained at 34 and 40°C. **Panel B:** UV-Vis spectra of: folate/pNPAm-co-Am decorated AuNP in PBS (red) and in PBS with 10% foetal bovine serum (PBS/FBS) (blue) at 37°C.

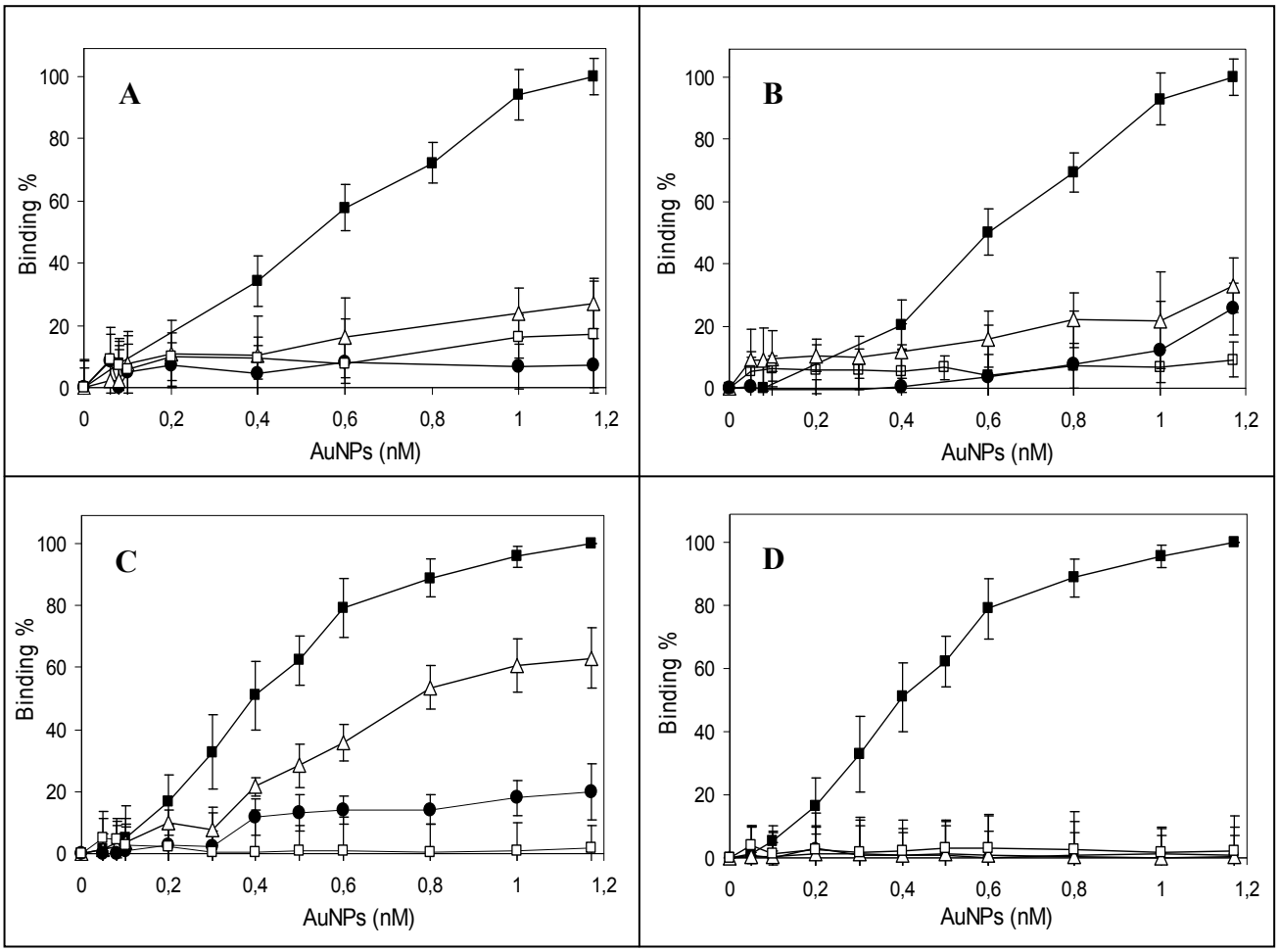


Fig. S7. Binding profile to avidin coated ELISA plates of gold nanoparticles: biotinylated AuNP incubated at 34° and 40°C (overlapped) (■); biotinylated pNIPAm-co-Am decorated AuNP incubated at 40°C (Δ); biotinylated pNIPAm-co-Am decorated AuNP incubated at 34°C (●); pNIPAm-co-Am decorated AuNP incubated at 40°C (□).

Panel A: 50:350:1 biotin-SH/polymer/AuNP molar ratio (Δ, ●); 50:1 biotin-SH/AuNP molar ratio (■); 350:1 polymer/AuNP molar ratio (□). Panel B: 50:700:1 biotin-SH/polymer/AuNP molar ratio (Δ, ●); 50:1 biotin-SH/AuNP molar ratio (■); 700:1 polymer/AuNP molar ratio (□). Panel C: 100:1400:1 biotin-SH/polymer/AuNP molar ratio (Δ, ●); 100:1 biotin-SH/AuNP molar ratio (■); 1400:1 polymer/AuNP molar ratio (□). Panel D: 100:2680:1 biotin-SH/polymer/AuNP molar ratio (Δ, ●); 100:1 biotin-SH/AuNP molar ratio (■); 2680:1 polymer/AuNP molar ratio (□).

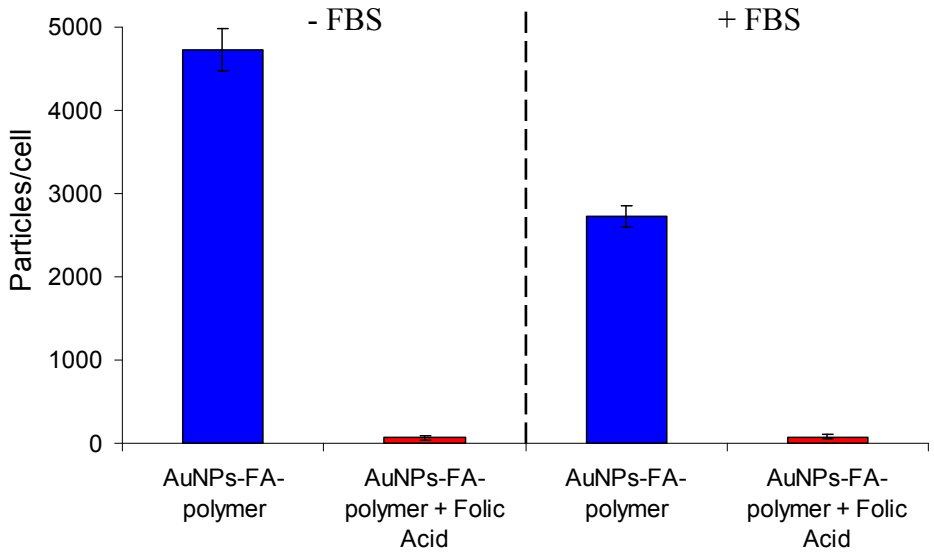


Fig. S8. Cell uptake of folate/pNIPAm-co-Am decorated AuNP by KB cells at 40°C in FFDMEM (left) and in FFDMEM supplemented with foetal bovine serum (right) in folic acid free medium (blue) and with medium supplemented with 200 μM folic acid (red).

TABLES

Table S1. Polymer conjugation efficiency with gold nanoparticles.

AuNP/polymer feed	AuNP/bound polymer ratio
1/350	1/350
1/700	1/700
1/1400	1/1400
1/2800	1/2680

Table S2. Particle sizes (DLS) of naked, folate decorated and pNIPAm-co-Am decorated AuNP with or without folate.

	34°C (nm)	40°C (nm)
Naked AuNP (water)	14.9 ±6.9	14.9 ±6.9
Naked AuNP (PBS)	638.1 ±280.1	551.9 ±378.6
Folate AuNP (PBS)	425.3 ±64.4	468.8 ±87.6
Folate AuNP (PBS/serum 10%)	28.0 ±4.8	31.5 ±4.9
pNIPAm-co-Am AuNP (PBS)	36.5 ±13.0	50.5 ±19.5
pNIPAm-co-Am AuNP (PBS/10% serum)	21.1 ±9.2	36.2 ±15.8
Folate/pNIPAm-co-Am decorated AuNP (PBS)	36.3 ±13.0	57.5 ±21.2
Folate/pNIPAm-co-Am decorated AuNP (PBS/10% serum)	19.1 ±8.7	59.2 ±28.3

Table S3. Particle sizes (DLS) of folate/pNIPAm-co-Am decorated AuNP.

	37°C (nm)
Folate/pNIPAm-co-Am decorated AuNP (PBS)	34.3 ±5.2
Folate/pNIPAm-co-Am decorated AuNP (PBS/10% serum)	26.9 ±4.0

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