# Supplementary Information

## Tunable gold nanorod/NAO conjugates for selective drug delivery in mitochondria-targeted cancer therapy

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**Keywords:** nonyl-acridine-orange NAO, Cetuximab, gold nanoparticles, mitochondria-targeted therapy, epidermal growth factor receptor EGFR

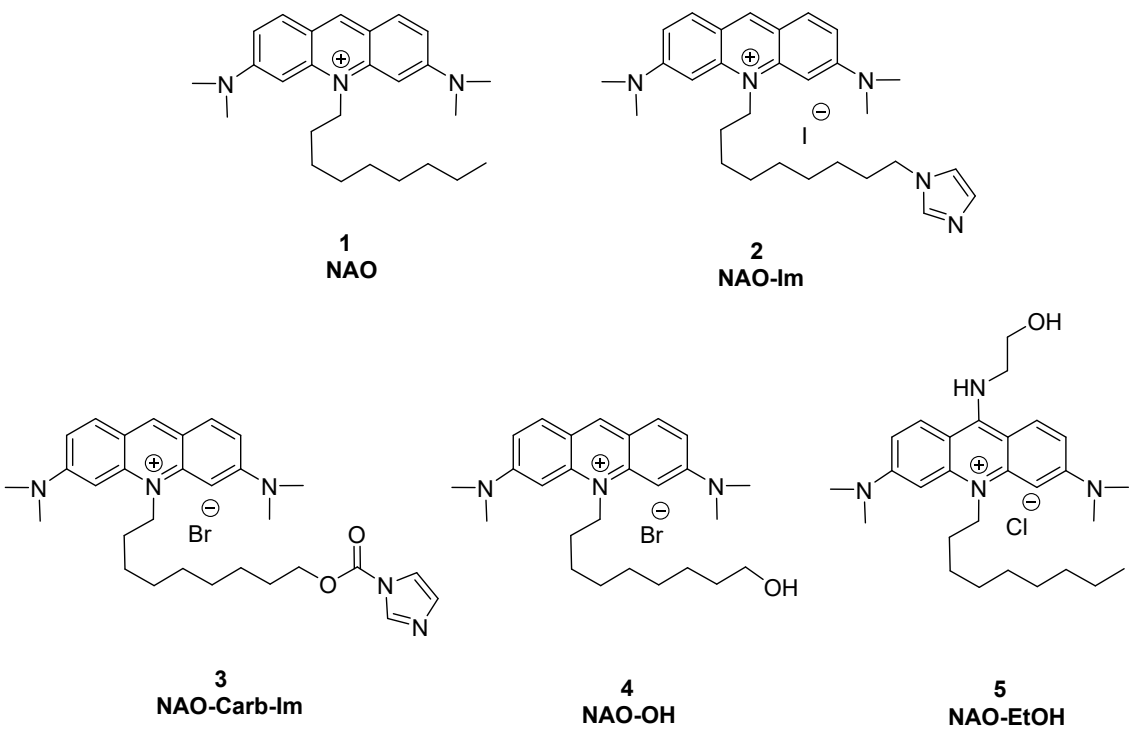
**Abbreviations:** AuNRs, gold nanorods; ATP, adenosine triphosphate; CLSM, Confocal Laser Scanning Microscopy; Cmab, Cetuximab; CTAB, Cetyl Trimethyl Ammonium Bromide; DLC, delocalized lipophilic cations; DMEM, Dulbecco Modified Eagle Medium; EGFR, epidermal growth factor receptor; FLIM, fluorescence lifetime imaging microscopy; HER2, epidermal growth factor receptor 2; IMM, inner mitochondrial membranes; LSPR, localized surface plasmon resonance; MEFs, mouse embryonic fibroblasts; MPPs, mitochondrial penetrating peptides; MTT, methylthiazoletetrazolium; NAO, 10-nonyl-acridine orange; NAO-Im, compound 2; NAO-Carb-Im, compound 3; NAO-OH, compound 4; NAO-EtOH, compound 5; NTA, nitrilotriacetic; PDX, patient-derived xenografts; PPTT, plasmonic photothermal therapy; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; SS, Szeto-

Schiller; TCA, tricarboxylic acid; TPP, triphenyl phosphonium; UV-VIS, ultraviolet – visible.

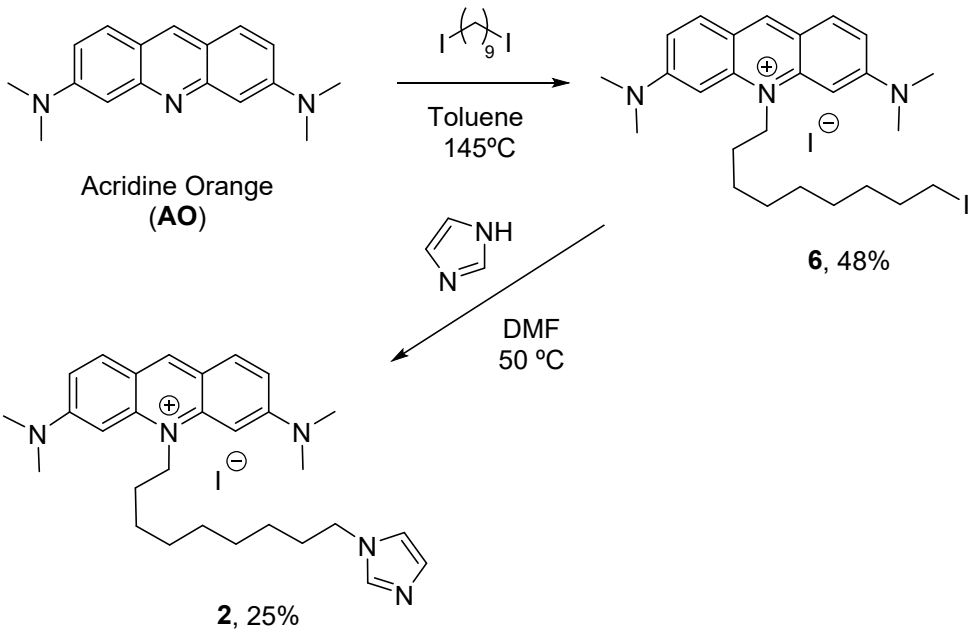
# 1. Synthesis of NAO and NAO derivatives

### Materials and methods

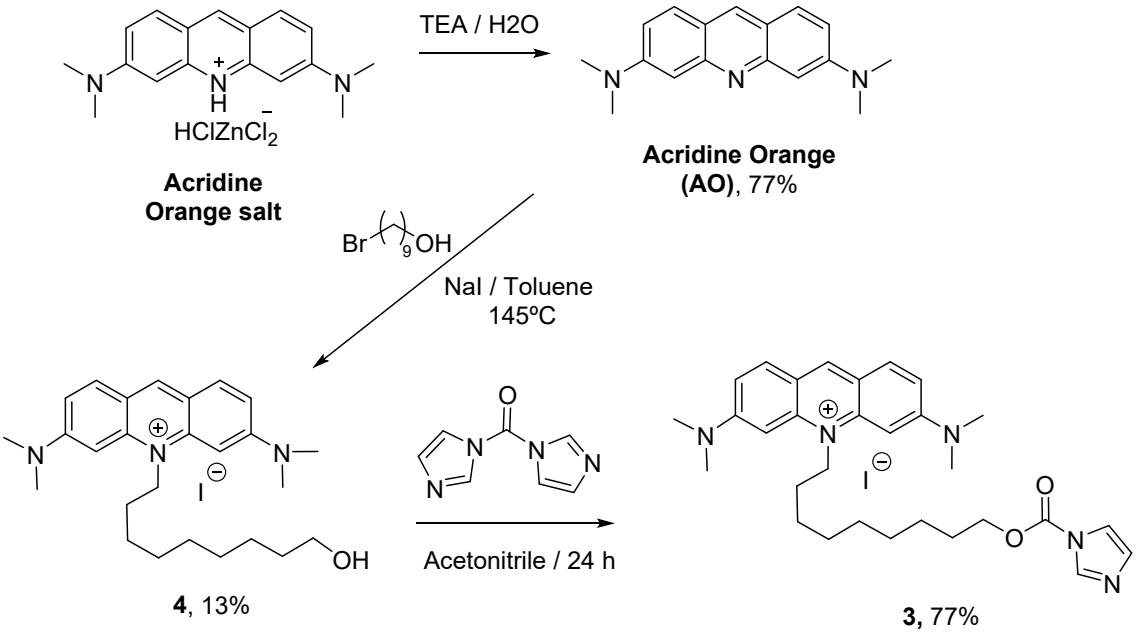
Commercially available compounds were purchased from commercial suppliers and used without further purification. All experiments were carried out under argon atmosphere using standard Schlenk techniques. Anhydrous solvents were distilled under argon following standard procedures. Flash chromatography was performed over silica gel 60 (230–400 mesh) or neutral alumina (70-290 mesh). NMR spectra were recorded at room temperature on a Bruker DPX 300 MHz BAC S-60 spectrometer instrument. The chemical shifts (δ) are given in ppm (TMS as internal standard). Coupling constants (*J*) are given in hertz (Hz). High resolution mass spectra were recorded on a MALDI-TOF/TOF Bruker ULTRAFAX mass spectrometer.



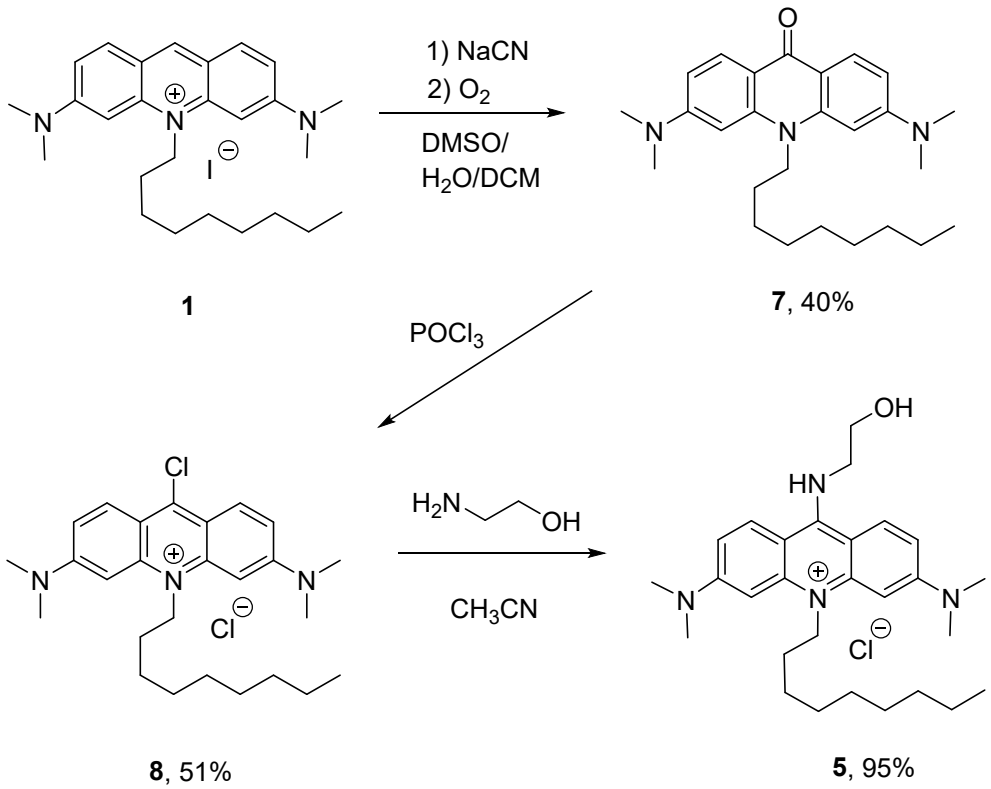

**Scheme 1.** The synthesized nonyl-acridine derivatives.




**Scheme 2.** Synthesis of **NAO-Im** (**2**).

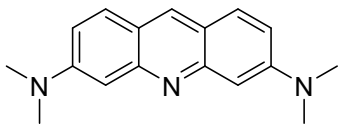



**Scheme 3.** Synthesis of **NAO-OH** (**4**) and **NAO-Carb-Im** (**3**).




**Scheme 4.** Synthesis of **NAO-EtOH** (**5**).

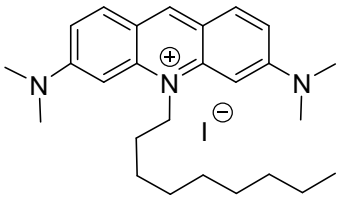
**Preparation of acridine orange (AO)**



Acridine Orange
(**AO**)

Acridine orange (**AO**) hemi-(zinc chloride) salt (4.3 g, 11.6 mmol) was dissolved in 30 mL of deionized water and stirred until a clear solution was obtained. Next, 6 mL of 25 wt% ammonia solution was added dropwise. The suspension was stirred for 30 min, extracted with DCM (3×20 mL) and the combined organic layers were washed with distilled water (3×30 mL) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure to afford **AO** (2.4 g, 77 %) as a red solid. $^1$H NMR (300 MHz, $CDCl_3$) δ 8.32 (s, 1H), 7.70 (d, $J$ = 9.2 Hz, 2H), 7.10 (d, $J$ = 9.2 Hz, 2H), 7.08 (s, 2H), 3.14 (s, 12H) ppm.

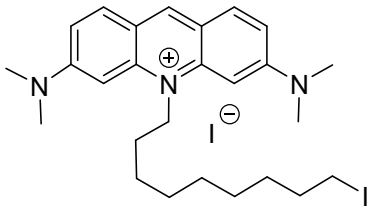
**Synthesis of compound 1 (NAO)**



**1**

**AO** (1.02 g , 3.85 mmol) was dissolved in 40 mL of toluene at 110 ºC. Next, a solution of of 1-iodononane (5.6 g, 23.1 mmol) in 10 mL of toluene and sodium bicarbonate (1.94 g. 23.1 mmol) were added. The reaction mixture was stirred at 145 ºC for 12 h, cooled and the solid was filtered, washed with hot toluene and recrystallized twice from DCM/$Et_2O$ to afford **1** (1.5 g, 99 %) as an orange solid. $^1$H NMR (300 MHz, $CDCl_3$) δ 8.76 (s, 1H), 7.94 (d, *J* = 9.3 Hz, 2H), 7.07 (dd, *J* = 9.3, 2.1 Hz, 2H), 6.61 (d, *J* = 2.1 Hz, 2H), 4.81(t, *J* = 7.7 Hz, 2H), 3.32 (s, 12H), 1.98 (quintet, *J* = 7.5 Hz, 2H), 1.66 (quintet, 7.7 Hz, 2H), 1.64-1.53 (m, 2H), 1.50-1.37 (m, 2H), 1.38-1.19 (m, 8H), 0.86 (t, *J* = 6.7 Hz, 3H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$) δ 155.4, 143.0, 142.4, 133.3, 117.0, 114.1, 92.5, 48.4, 41.2, 31.8, 29.5, 29.4, 29.2, 27.2, 26.1, 22.6, 14.1 ppm.

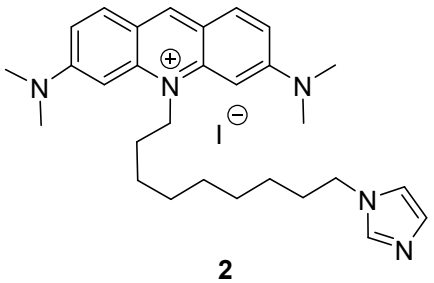
**Synthesis of compound 6**



**6**

**AO** (430 mg, 1.62 mmol) was dissolved in 30 mL of toluene at 110 ºC. Then, a solution of 1,9-diiodononane (10 g, 26.3 mmol) in 10 mL of toluene and sodium bicarbonate (1.94 g, 23.08 mmol) were added. The reaction mixture was stirred at 150 ºC for 12 h, cooled, filtered and the solid was washed with hot toluene and purified by flash chromatography (neutral alumina, DCM/MeOH 9:1) to afford **6** (398 mg, 48%) as an orange solid. $^1$H NMR (300 MHz, $CDCl_3$) δ 8.73 (s, 1H), 7.89 (d, *J* = 9.3 Hz, 2H), 7.06 (dd, *J* = 9.3, 2.1 Hz, 2H), 6.51 (d, *J* = 2.1 Hz, 2H), 4.77 (t, 8.1 Hz, 2H), 3.32 (s, 12H), 3.17 (t, *J* = 7 Hz, 2H), 1.94 (quintet, *J* = 7.2 Hz, 2H), 1.79 (quintet, *J* = 7.1 Hz, 2H), 1.66 (quintet, *J* = 7.6 Hz, 2H), 1.46-1.27 (m, 8H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$) δ 155.5, 143.1, 142.6, 133.4, 117.1, 114.1, 92.8, 48.4, 41.3, 33.4, 30.4, 29.5, 29.3, 28.5, 27.3, 26.3, 7.7 ppm. HRMS (MALDI-TOF, DCTB) *m/z*: calcd. for $C_{26}H_{37}IN_3^+$ 518.2032, found 518.2044.

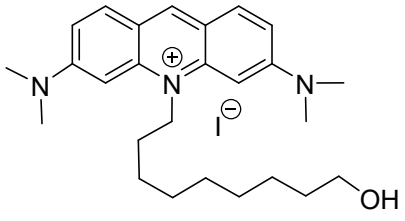
**Synthesis of compound 2 (NAO-Im)**



**2**

Imidazole (38 mg, 2 eq., 0.55 mmol) was dissolved in 2 mL of anhydrous DMF. Next, a solution of **6** (143 mg, 0,22 mmol) in 2 mL of DMF was added. The solution was stirred for 12 h at 50 ºC and purified by flash chromatography (neutral alumina, EtOAc to remove DMF and DCM/MeOH 9:1). Then, the red eluent solution containing **2** was washed with distilled (2×20 mL) to remove the excess of imidazole and dried over $MgSO_4$. The solvent was evaporated under reduced pressure to afford **2** (32 mg, 25%) as a red solid. $^1$H NMR (300 MHz, $CDCl_3$) δ 8.70 (s, 1H), 7.89 (d, *J* = 9.3 Hz, 2H), 7.45 (t, 1.3 Hz, 1H), 7.04 (dd, *J* = 9.3, 2.0 Hz, 2H), 7.02 (s, 1H), 6.91 (t, *J* = 1.3 Hz, 1H), 6.62 (d, *J* = 1.5 Hz, 2H), 4.85 (t, *J* = 7.8 Hz, 2H), 3.93 (t, *J* = 7.0 Hz, 2H), 3.32 (s, 12H), 1.95 (quintet, *J* = 8.7 Hz, 2H), 1.81-1.61 (m, 4H), 1.45-1.18 (m, 8H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$) δ 155.6, 143.0, 142.7, 137.1, 133.3, 129.3, 118.9, 117.2, 114.1, 93.0, 48.4, 47.0, 41.2, 31.0, 29.4, 29.3, 29.0, 27.2, 26.4, 26.3 ppm. HRMS (MALDI-TOF, DCTB) *m/z*: calcd. for $C_{29}H_{40}N_5^+$ 458.3283, found 458.3272.

**Synthesis of compound 4 (NAO-OH)**

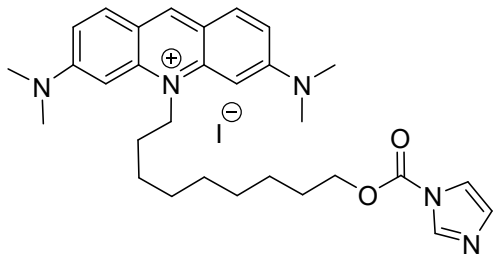


**4**

**AO** (150 mg, 0.56 mmol) was dissolved in 10 mL of toluene at 110 ºC. Then, a solution of 9-bromo-1-nonanol (760 mg, 3.39 mmol) in 5 mL of toluene was added. Finally, sodium iodine (508 mg, 3.39 mmol) was added to the solution and the mixture was stirred for 12 h at 150 ºC. The reaction mixture was filtered and the solid washed with toluene and purified by flash chromatography (neutral alumina, DCM/MeOH 99:1) to afford **4** (38 mg, 13%) as an orange solid. $^1$H NMR (300 MHz, $CDCl_3$) δ 8.69 (s, 1H), 7.87 (d, *J* = 9.3 Hz, 2H), 7.00 (dd, *J* = 9.3, 1.7 Hz, 2H), 6.50 (d, *J* = 1.6 Hz, 2H), 4.72 (t, *J* = 8.0 Hz, 2H), 3.60 (t, *J* = 7 Hz, 2H), 3.28 (s, 12H), 2.03 (bs, 1H), 1.90 (quintet, *J* = 7 Hz, 2H), 1.63 (quintet, *J* = 7 Hz, 2H), 1.53-1.15 (m, 10H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$) δ 155.4, 143.2, 142.9, 133.4, 117.1, 114.1, 92.7, 62.6, 48.4, 41.3, 32.7, 29.8, 29.6, 29.3, 27.1, 26.2,

25.7 ppm. HRMS (MALDI-TOF, DCTB) *m/z*: calcd. for $C_{26}H_{38}N_3O^+$ 408.3015, found 408.2995.

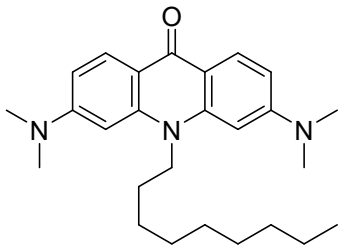
**Synthesis of compound 3 (NAO-Carb-Im)**



**3**

Compound **4** (89 mg, 0.17 mmol) was dissolved in 5 mL of chloroform. Then, a solution of CDI (40 mg, 0.25 mmol) in 8 mL of acetonitrile was added. The resulting solution was refluxed for 24 h (the progress of the reaction was followed by TLC, DCM/MeOH 9:1). The reaction mixture was poured over distilled water (100 mL) and extracted with DCM (3×25 mL). The combined organic layers were dried over $MgSO_4$ and the solvent evaporated under reduced pressure to afford a red solid, which was purified by precipitation from DCM/$Et_2O$ yielding **3** (82 mg, 77%). $^1$H NMR (300 MHz, $CDCl_3$) δ 8.71 (s, 1H), 8.08 (s, 1H), 7.89 (d, *J* = 9.2 Hz, 2H), 7.38 (s, 1H), 7.02 (s, 1H), 7.00 (dd, 2H), 6.54 (s, 2H), 4.76 (t, *J* = 8 Hz, 2H), 4.36 (t, *J* = 6.6 Hz, 2H), 3.28 (s, 12H), 1.92 (quintet, *J* = 7 Hz, 2H), 1.75 (quintet, *J* = 7 Hz, 2H), 1.64 (quintet, *J* = 7 Hz, 2H), 1.45-1.20 (m, 8H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$) δ 155.6, 148.8, 143.2, 142.6, 137.1, 133.4, 130.6, 117.2, 114.2, 92.7, 68.5, 48.3, 41.2, 29.6, 29.4, 29.1, 28.5, 27.3, 26.3, 25.7 ppm. HRMS (MALDI-TOF, DCTB) *m/z*: calcd. for $C_{30}H_{40}N_5O_2^+$ 502.3182, found 502.3203.

**Synthesis of compound 7**

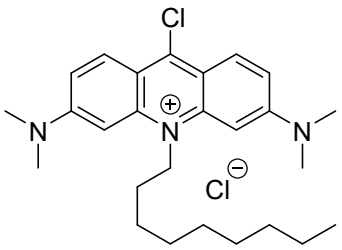


**7**

NAO (1.5 g, 2.88 mmol) and sodium cyanide (170 mg, 3.47 mmol) were dissolved in a mixture of DMSO/$H_2O$/DCM 90:5:5 (30 mL). The reaction mixture was stirred at room temperature for 3 h and then exposed to air for 24 h. The crude reaction mixture was poured over 20 mL of distilled water, extracted with ethyl acetate (3×25 mL) and dried

over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue purified by flash chromatography (neutral alumina, hexane/ethyl acetate/MeOH 5:10:1) yielding **7** (470 mg, 40%) as a pale yellow solid. $^1$H NMR (300 MHz, $CDCl_3$) δ 8.36 (d, *J* = 9.0 Hz, 2H), 6.66 (dd, *J* = 9.0, 2.2 Hz, 2H), 6.29 (d, *J* = 2.2 Hz, 2H), 4.14-4.04 (m, 2H), 3.08 (s, 12H), 1.90 (quintet, *J* = 8 Hz, 2H), 1.58-1.37 (m, 4H), 1.37-1.25 (m, 8H), 0.91-0.87 (m, 3H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$) δ 175.7, 153.7, 143.9, 129.0, 113.7, 107.5, 94.0, 46.1, 40.3, 31.9, 29.6, 29.4, 29.3, 27.2, 26.7, 22.8, 14.2 ppm. HRMS (MALDI-TOF, DCTB) *m/z*: calcd. for $C_{26}H_{37}N_3O^+$ 407.2937, found 407.2940.

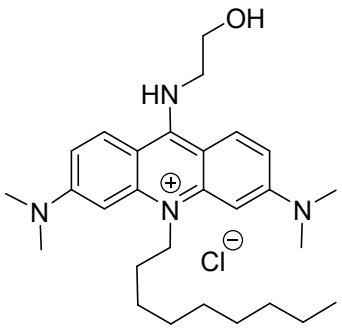
**Synthesis of compound 8**




**8**

Compound **7** (44 mg, 0.11 mmol) was dissolved in 5 mL of $POCl_3$ and refluxed for 12 h at 125 ºC. The excess of $POCl_3$ was removed under reduced pressure and the resulting solid was purified by flash chromatography (neutral alumina, DCM/MeOH 9:1) to afford **8** (26 mg, 51%) as a red solid. $^1$H NMR (300 MHz, $CDCl_3$) δ 8.19 (d, *J* = 9.6 Hz, 2H), 7.20 (dd, *J* = 9.6, 2.0 Hz, 2H), 6.67 (d, *J* = 2.0 Hz, 2H), 4.87 (t, *J* = 8 Hz, 2H), 3.36 (s, 12H), 1.96 (quintet, *J* = 7.2 Hz, 2H), 1.64 (quintet, *J* = 7.5 Hz, 2H), 1.40 (quintet, *J* = 7.0 Hz, 2H), 1.35-1.15 (m, 8H), 0.86 (t, *J* = 6.6 Hz, 3H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$) δ 155.7, 147.4, 143.0, 129.6, 115.5, 114.9, 93.1, 48.7, 41.2, 31.9, 29.6, 29.5, 29.3, 27.2, 26.4, 22.7, 14.2 ppm. HRMS (MALDI-TOF, DCTB) *m/z*: calcd. for $C_{26}H_{37}ClN_3^+$ 426.2676, found 426.2660.

**Synthesis of compound 5 (NAO-EtOH)**




**5**

Compound **8** (21 mg, 45.4 mmol) was dissolved in 3 mL of acetonitrile. Then, ethanolamine (50 mg, 81.9 mmol) was added and the resulting solution was stirred for 24 h at 25 ºC. The reaction mixture was washed with distilled water (3×20 mL), extracted with DCM (3×20 mL) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure to afford **5** (21 mg, 95%) as a yellow solid that was used without further purification. $^1$H NMR (300 MHz, $CDCl_3$) δ 8.58 (d, *J* = 9.5 Hz, 2H), 6.86 (dd, *J* = 9.5, 2.2 Hz, 2H), 6.32 (d, *J* = 2.2 Hz, 2H), 4.24 (t, *J* = 7.4 Hz, 2H), 4.17-4.06 (m, 2H), 4.03-3.96 (m, 2H), 3.16 (s, 12H), 1.94 (quintet, *J* = 7.9 Hz, 2H), 1.57 (quintet, *J* = 7.6 Hz, 2H), 1.45 (quintet, *J* = 7.1 Hz, 2H), 1.37-1.16 (m, 8H), 0.87 (t, *J* = 7 Hz, 3H) ppm. $^{13}$C NMR (75 MHz, $CDCl_3$) δ 156.4, 154.0, 142.9, 129.2, 109.9, 105.7, 93.3, 60.3, 53.8, 48.1, 40.3, 31.9, 29.6, 29.4, 29.3, 27.2, 26.3, 22.7, 14.2 ppm. HRMS (MALDI-TOF, DCTB) *m/z*: calcd. for $C_{28}H_{43}N_4O^+$ 451.3437, found 451.3428.

## 2. Synthesis and characterization of AuNRs and conjugates

### Materials

All the starting materials were obtained from commercial suppliers and used without further purification. Hexadecyltrimethylammonium bromide (CTAB, ≥99%), hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot H_2O$, ≥99.9%), silver nitrate ($AgNO_3$, ≥99.0%), L-ascorbic acid (≥99%), sodium borohydride ($NaBH_4$, 99%), poly(ethylene glycol) methyl ether thiol (Peg-SH, Mw 6000), (±)-α-lipoic acid (0≥98.0%), *N*α,*N*α-bis(carboxymethyl)-L-lysine hydrate (NTA, ≥97.0%, TLC), cobalt($^{2+}$) chloride hexahydrate (ACS reagent, 98%), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, purum, ≥98.0% (AT)), and *N*-hydroxysuccinimide (NHS, 98%) were purchased from Aldrich. Nanopure water (resistivity 18.2 MΩ cm at 25 °C) was used in all experiments.

### Synthesis and characterization of AuNRs

The AuNRs were prepared using a seeded growth method with some modifications.

*Synthesis of 1–2 nm Au seeds.*

A 0.05 M $HAuCl_4$ solution (500 µL) and a 0.1M ascorbic acid solution (250 µL) were added under stirring (ca. 400-500 rpm) to 50 mL of a 50 mM CTAB and 13.5 mM *n*-decanol solution in a 50 mL glass beaker. After 2 min, 2000 µL of a freshly prepared 0.02 M $NaBH_4$ solution was injected to the former colorless solution under vigorous stirring (1500 rpm using a PTFE plain magnetic stirring bar: 30 × 6 mm), affording a brownish-yellow solution. The seed was aged for 1 h at 25–27 °C prior to use.

*Synthesis of small anisotropic seeds (21 nm in length and 7.5nm in width).*

In a typical synthesis, 500 mL of a 50 mM CTAB and 11 mM n-decanol solution was placed in a 1000 mL Erlenmeyer and 5000 µL of 0.05 M $HAuCl_4$, 4000 µL of 0.01 M $AgNO_3$, 35 mL of 1 M HCl, and 6500 µL of 0.1 M ascorbic acid were sequentially added. The temperature of the solution was maintained at 25 °C. Then, 30 mL of the 1–2 nm seed solution was added under stirring. The mixture was left undisturbed at 25 °C for at

least 4 h until the solution changed from colorless to dark brownish gray. The obtained small anisotropic seeds (longitudinal LSPR located at 721 nm) were centrifuged at 15000 rpm for 45 min. The precipitate was redispersed with 100 mL of 10 mM CTAB solution and centrifuged under the same conditions. The final Au concentration was fixed to 4.25 mM (Abs 400 nm: 1, optical path: 0.1 cm).

*AuNRs with LSPR at 800 nm.*

In a typical synthesis, 25 mL of 0.01 M $AgNO_3$, 10 mL of 0.05 M $HAuCl_4$, 50 mL of 1 M HCl, and 8000 µL of a 0.1 M ascorbic acid solution were added under stirring to 1000 mL of a 50 mM CTAB and 11 mM *n*-decanol solution at 35 °C. Then, 5000 µL of the small anisotropic seed suspension was added under stirring. The mixture was left undisturbed for 4-6 h. The AuNRs were forced to settle as sediment (by centrifugation at 8000 rpm, 30 min) to remove the excess of surfactant and redispersed in 100 mL of a 10 mM CTAB solution (AuNR stock solution). This procedure was repeated twice to remove n-decanol traces. Finally, they were redispersed in 60 mL of a 2 mM CTAB solution. The resulting AuNRs presented an average length of 61 ± 4 nm and diameter of 18 ± 2 nm.

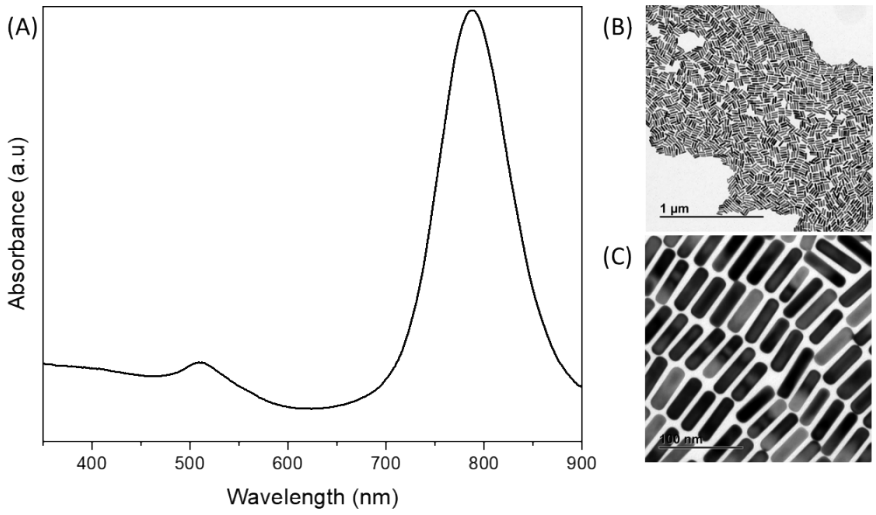



**Figure S1:** (A) Extinction spectra and representative TEM images at different magnifications of the synthesized AuNRs: (B) 5 k and (C) 40 k.

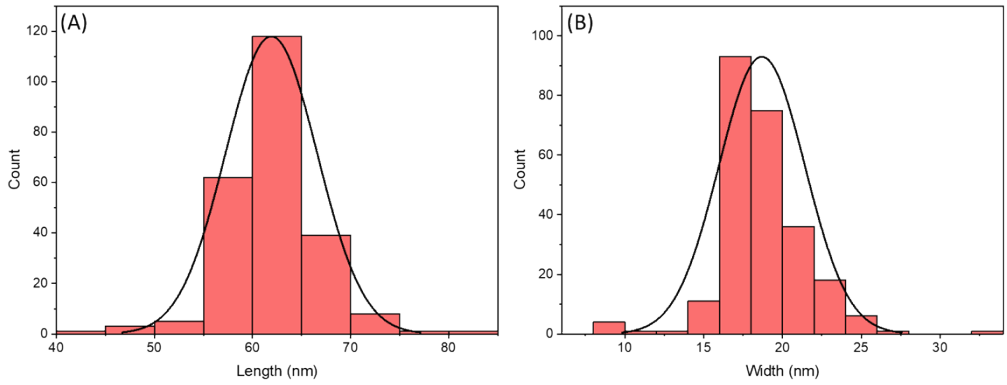



**Figure S2:** (A) Length and (B) width particle size distribution of the synthesized AuNRs.

**NTA-Cobalt AuNRs functionalization**

Typically, 15 mg Peg-SH was added under stirring to 5 mL of a freshly prepared aqueous suspension of AuNRs (2 mM of $Au^0$, 2 mM CTAB). After 1 h, the excess of free Peg-SH was removed by one centrifugation cycle (8000 rpm, 30 min). Then, the precipitate was redispersed in 2 mL of 10 mM lipoic acid in 200 mM Hepes (pH 8). After 4 h, the excess of free lipoic acid was removed by one centrifugation cycle (7000 rpm, 25 min). The precipitate was redispersed in 500 µL of 20 mM Hepes solution (pH 8). Activation of the carboxylic group was performed by addition of 500 µL of a 35 mM EDC and 500 µL of a 35 mM NHS solution (20 mM Hepes at pH 8). After 30 min, 500 µL of NTA-Cobalt complex (20 mM Hepes pH 8), which was previously prepared in water by mixing 250 µL mL of a 100 mM NTA solution and 250 µL mL of a 150 mM cobalt ($^{2+}$) chloride solution, were added to activate the lipoic acid-functionalized AuNRs. The mixture was left undisturbed at least for 1 h. After the reaction time, the AuNRs were centrifuged (6500 rpm, 20 min) and redispersed in 10 mL of Hepes 20 mM (pH 8). Two more centrifugation cycles were performed to obtain a final $Au^0$ concentration of 10 mM in buffer.

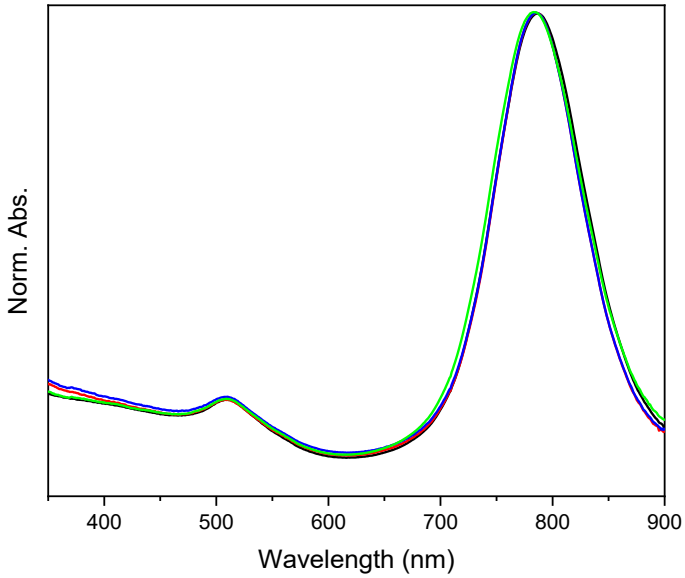



**Figure S3:** Extinction spectra of the longitudinal surface plasmon resonance of sequentially functionalized AuNRs with Peg-SH (black curve), with lipoic acid before and after activation of the carboxylic group (red and blue curves, respectively), and with the NTA-$Co^{2+}$ complex in buffer (green curve).

**NAO-Im functionalization of AuNRs**

Excess of NAO-Im was mixed with the gold nanoparticles (final $Au^0$ concentration 10 mM) in a 2 mL Eppendorf tube and incubated for 2 h. Then, the AuNRs were centrifuged for 15 min and washed with buffer to remove the excess of unbound molecules. The functionalized AuNRs were resuspended again in the same buffer and stored at 4 °C for further use.

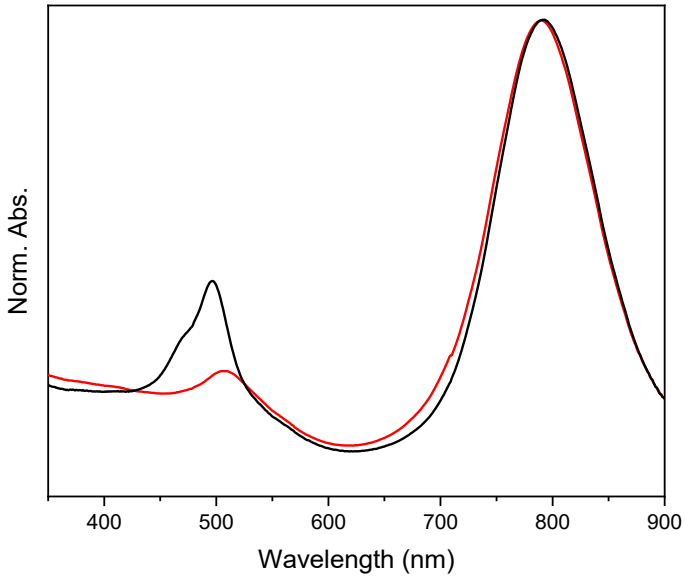



**Figure S4:** Extinction spectra of the longitudinal surface plasmon resonance of functionalized AuNRs with NAO-Im before and after washing with buffer (black and red curves, respectively).

**NAO-Carb-Im functionalization of AuNRs**

Excess of NAO-Carb-Im was mixed with the gold nanoparticles (final $Au^0$ concentration 10 mM) in a 2 mL Eppendorf tube and incubated for 2 h. Then, the AuNRs were centrifuged for 15 min and washed with buffer to remove the excess of unbound molecules. The functionalized AuNRs were resuspended again in the same buffer and stored at 4 °C for further use.

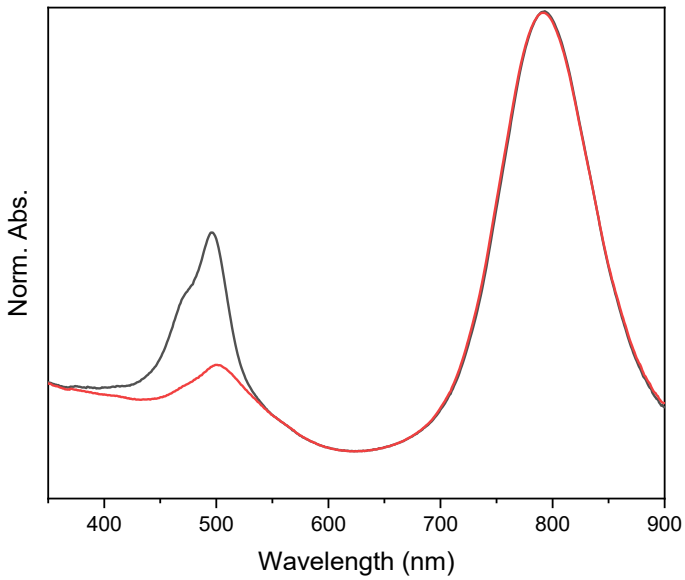



**Figure S5:** Extinction spectra of the longitudinal surface plasmon resonance of sequentially functionalized AuNRs with NAO-Carb-Im before and after washing with buffer (black and red curves, respectively).

**Estimation of NAO/AuNR coating ratio through UV-Vis spectroscopy**

To obtain the molar extinction coefficient of NAO-Carb-Im (3), the UV-vis absorption spectra of the NAO analogue were recorded at increasing concentrations in aqueous solution. As for NAO, compound **3** presents a broad band in the 390–540 nm region with a maximum at 495 nm, along with a shoulder at around 470 nm (**Figure S6A**) [1]. For NAO, the 495 nm band corresponds to monomer absorption in solution and the weaker shoulder at 470 nm corresponds to dimeric arrangements in solution. Here, the carbamate-imidazole group did not prevent the formation of dimeric arrangements of **3** compounds as confirmed by the presence of the 470 nm band with the increasing concentration of **3**. As AuNRs also display a small absorption band at 510 nm, we built the Lambert-Beer plot using the values from the low absorption band. From the linear fit (**Figure S6B**) we obtained the molar extinction coefficient of NAO-Carb-Im (**3**), $\varepsilon_0(\lambda = 470\ nm) \approx 28000\ M^{-1}cm^{-1}$. From the UV-vis spectra of different **3**-AuNRs preparations we estimated a NAO per AuNR coating ratio of $\approx 10^4$. Similar results were obtained with NAO-Im (**2**) (data not shown).

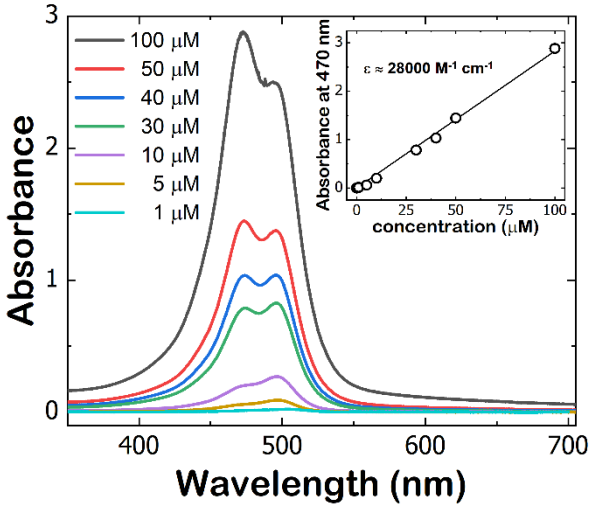



**Figure S6. A)** UV-vis absorption spectra of NAO- Carb-Im (**3**) at different concentration in aqueous solution. **Inset:** Lambert-Beer plot from the 470 nm absorptium peaks. The linear fit provided the value of the molar extinction coefficient of NAO-Carb-Im (**3**), $\varepsilon_0(\lambda = 470\ nm) \approx 28000\ M^{-1}cm^{-1}$.

**Cetuximab functionalization of 3-AuNRs**

Excess of Cetuximab antibody was mixed with the NAO-Carb-Im functionalized gold nanoparticles and incubated for 1 h at 4 °C. Then, the AuNRs were centrifuged (15 min, 6500 rpm) at 4 °C and washed with PBS buffer to remove the excess of unbound antibody. The functionalized AuNRs were resuspended again in the same buffer and stored at 4 °C for further use.

[1] M. Septinus, W. Seiffert, H.W. Zimmermann. Über hydrophobe Acridinfarbstoffe zur Fluorochromierung von Mitochondrien in lebenden Zellen. Histochemistry, 79 (1983), pp. 443-456

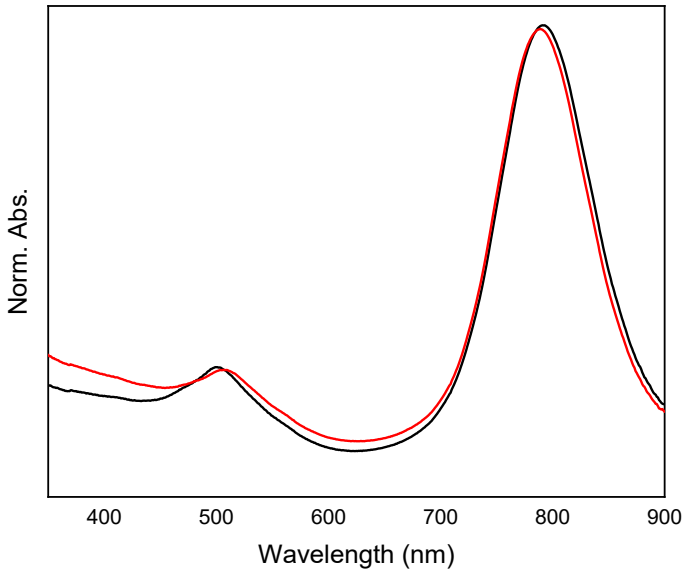



**Figure S7:** Extinction spectra of the longitudinal surface plasmon resonance of AuNRs with NAO-Carb-Im (black curve) and after 1 h incubation with Cetuximab antibody (red curve).

## Estimation of Cmab binding on 3-AuNRs by Coomassie blue staining gel electrophoresis

20 µL of Cmab-**3**-AuNRs (typically at 10-20 nM) or Cmab (1-20 µg typically) were heated at 100 ºC for 10 min in 5 µL NUPAGE LDS sample buffer (4×) supplemented with 5% of ditiotreitol. 20 µL of the mixture containing different concentration of Cmab-**3**-AuNRs or free Cmab were loaded per lane on a 7.5 % SDS-polyacrylamide gel. The gel was run and revealed following SDS-PAGE standard procedures. ImageJ was used for protein level quantification by densitometric analysis (**Figure S8**). From the standard curve of free Cmab we could estimate a binding of 0.1 µg of Cmab per nM of **3**-AuNR, i.e. ≥1 Cmab molecule per AuNR.

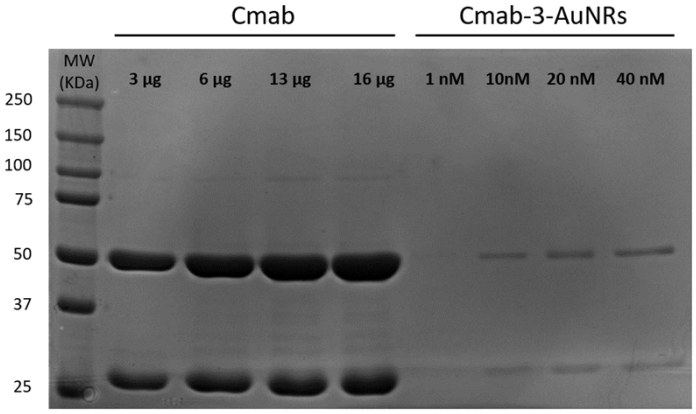



**Figure S8.** SDS-PAGE gel stained with Coomassie blue. Lanes 1-4 contain free Cetuximab in reducing conditions, where the bands at 50kDa and 25 kDa correspond to

the heavy and light chains, respectively. Lanes 5-8 contain Cmab-3-AuNRs and the bands correspond to the bound Cmab to **3**-AuNRs after incubation and washing.

## ζ–Potential

The most efficient way to determine correct functionalization is by ζ–potential measurements at each stage of the nanoparticle synthesis. The ζ–potential was evaluated using a Zeta PALS instrument (Brookhaven Instruments Corp., Holtsville, USA). The phase analysis light scattering technique was used to measure the electrophoretic mobility of the samples, from which the ζ–potential was obtained using a dispersant refractive index of 1.33 (water), viscosity of 0.9 cP, dispersant dielectric constant of 78.5, and temperature of 25 °C. In this study, ζ–potentials were calculated using the Smoluchowski equation, which assumes a spherical shape for particles. Thus, the value qualitatively indicates the sign and magnitude of the ζ–potential of the AuNRs. This is sufficient for the relative evaluation of the surface charge of AuNRs. The ζ–potential values for the gold nanoparticles at the different stages of functionalization are shown in Table S1.

| Sample | ζ-Potential (mV) |
|---|---|
| **CTAB-AuNRs in water** | 25.9±1.5 |
| **AuNRs-PEG-SH** | -3.1±1.2 |
| **AuNRs lipoic acid** | -11.5±0.9 |
| **AuNRs activated lipoic acid** | -12.7±0.9 |
| **AuNRs NTA-$Co^{2+}$ Complex** | -9.3±0.9 |
| **AuNRs NTA-$Co^{2+}$ + Cetuximab antibody** | -14.6±0.7 |
| **AuNRs NTA-$Co^{2+}$ + NAO-Carb-Im** | -14.6±0.5 |
| **AuNRs NTA-$Co^{2+}$ + NAO-Carb-Im + Cetuximab antibody** | -14.9±0.8 |
| **AuNRs NTA-$Co^{2+}$ + NAO-Im** | -8,4±0.5 |

**Table S1:** ζ–potentials at each stage of the AuNR conjugation procedure in buffer solution.