Electronic Supplementary Information

Surface optimization of gold nanoparticle mass tags for the sensitive detection of protein biomarkers via immuno-capture LI-MS

Toby Siu-Chung Tam,‡[a] Yu-Hong Cheng,‡[a] Chun-Nam Lok,[a] Ho Yu Au-Yeung,[a] Wen-Xiu Ni,[d] Xiao-Long Wei,[e] Kwan-Ming Ng*[a,b,c]

[a] *Department of Chemistry and State Key Laboratory of Synthetic Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong S.A.R., P.R. China*

[b] *Department of Chemistry and Key Laboratory for Preparation and Application of Ordered Structural Materials of Guangdong Province, Shantou University, Guangdong, 515063, P. R. China*

[c] *Chemistry and Chemical Engineering Guangdong Laboratory, Shantou, Guangdong, 515063, P.R. China.*

[d] *Shantou University Medical College, Guangdong, 515041, P.R. China.*

e Department of Pathology, The Cancer Hospital of Shantou University Medical College, Guangdong, 515041, P. R. China

* To whom correspondence should be addressed: kwanming@stu.edu.cn (KMN)

‡ These authors contributed equally to this work

Table of Contents

I. Chemical and Materials

For the synthesis of 20nm AuNPs, potassium gold(III) chloride ($KAuCl_4$, 99.99% Au) was purchased from Strem Chemicals (Newburyport, MA, USA). Sodium citrate tribasic dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$, ≥99.0%), L-ascorbic acid ($C_6H_8O_6$, 99%), and sodium borohydride ($NaBH_4$, 98.0%) were purchased from Sigma Aldrich (St. Louis, MO, USA).

For PEGylation of synthesized AuNPs, bifunctional PEG linker ortho-pyridyl disulfide-poly(ethylene glycol)-N-hydroxysuccinimide, 5kDa (OPSS-PEG-NHS, 5kDa); and carboxylate-capped PEG (cPEG) stabilizer which is thiol-poly(ethylene glycol)-carboxyl 5kDa, (HS-PEG-COOH, 5kDa) were purchased from Creative PEGWorks (Chapel Hill, NC, USA).

To fabricate the immuno-capture substrate, glass coverslips of size (75mm x 25mm, 0.13-0.16mm thick) were purchased from Bellco Glass Inc. (Vineland, NJ, USA). (3-Glycidyloxypropyl)trimethoxysilane (GOPTS, 98.0%) used for the epoxy-silanization was purchased from Sigma Aldrich. ImmEdge Hydrophobic Barrier PAP Pen used for demarcating individual sample wells on the coverslips was purchased from Vector Laboratories (Burlingame, CA, USA).

Human papillomavirus type 18 E7 (HPV18E7) recombinant protein was purchased from ProteinX Labs (San Diego, CA, USA) and resuspended in a buffer of 1x PBS containing 0.05% Tween-20 and 125mM imidazole according to manufacturer's instruction to give a stock solution of HPV18E7 with a concentration of 0.5mg/mL or 36uM.

A pair of anti-HPV18E7 mouse monoclonal antibodies were purchased and used to capture and detect the recombinant protein in the immuno-capture assay. Anti-HPV18E7 (clone 718-15) was purchased from Novus Biologicals (Littleton, CO, USA) and were immobilized on the immuno-capture glass substrate and used as the capturing antibody. Anti-HPV18E7 (clone M1862HP7) was purchased from Fitzgerald Industries International (Acton, MA, USA) and used as the detection antibody via conjugation with either AuNPs or fluorescein.

Anti-βhCG mouse monoclonal antibody (clone M1709HCGb2) was purchased Fitzgerald Industries International and used as control antibodies for the preparation of non-specific antibody-AuNP conjugates.

Fluorescein-conjugated anti-HPV18E7 for fluorescence imaging experiments were prepared using NHS-fluorescein purchased from Thermo-Scientific Fisher (Waltham, MA, USA). Fluorescence conjugates were prepared according to manufacturer's instruction described further in section IV. A micro-dialysis kit (MWCO 1kDa) was purchased from G-Biosciences (St. Louis, MO, USA) for the dialysis of antibody reagents.

Other reagents and solvent, including absolute ethanol, (ACS, Reag. Ph Eur. Grade), acetone (HPLC grade, >99.5%) and 30% hydrogen peroxide (Ph Eur. Grade) were purchased from VWR Chemicals (Radnor, PA, USA). Sulfuric acid (A.R. grade, 98%) was purchased from RCI Labscan (Bangkok, Thailand). HPLC grade water was purchased from Scharlau (Barcelona, Spain). Phosphate buffered saline (1x PBS) buffer solution was prepared using the Ultrapure, ACS Reagent grade Na_2HPO_4, KH_2PO_4, NaCl, KCl purchased from Affymetrix (Santa Clara, CA, USA). Tween-20 (Affymetrix) mixed with PBS was used for the preparation of 1x PBST buffer solution. Bovine serum albumin (BSA) (≥ 98%) was purchased from Sigma Aldrich for the preparation of antibody-AuNP conjugate storage buffer. Nonfat-Dried Milk

Bovine (NFDM) (Sigma Aldrich) was used to prepare the blocking buffer for immunoassays. Tris-Acetate-EDTA (1x TAE) buffer was prepared using Tris (Affymetrix), EDTA (Sigma Aldrich) and glacial acetic acid (Sigma Aldrich). Roswell Park Memorial Institute (RPMI) medium, fetal bovine serum (FBS) and penicillin-streptomycin solution that were used for the cell culture were purchased from Gibco (Waltham, MA, USA).

II. Synthesis and Characterization of 20nm Citrate-Capped AuNPs

Synthesis of 5nm AuNP Seeds

15mL of ~5nm AuNP seeds were prepared in the following manner. 5mL solutions of 0.75mM $KAuCl_4$, 3mM $NaBH_4$ and 3.75mM sodium citrate were prepared using HPLC grade H_2O in separate glass bottles. The three solutions were placed in an ice bath for 15 minutes to cool. Then, 5mL of 0.75mM $KAuCl_4$ and 3.75mM sodium citrate were transferred to a 25mL beaker and mixed under magnetic stirring. 5mL of 3mM $NaBH_4$ solution was then added to the $KAuCl_4$ - sodium citrate mixture solution dropwisely with a 200uL autopipette, at a rate of 1 drop per second. A colour change from clear to faint purple to light red (~1mL added) was observed. Once the addition was completed, the solution was stirred for 30 minutes.

Synthesis of 20nm AuNPs via Serial Reduction

Seed-mediated growth was performed to increase the size of AuNP seeds from 5nm to 10nm first, then from 10 to 20nm. To synthesize 10nm AuNPs, 73.6uL of 0.1M sodium citrate solution was added to 800uL of 5nm AuNP seeds. This solution was then diluted to 4mL using H_2O. Serial reduction was carried out twice. In each instance, 1mL of 1.41mM $KAuCl_4$ solution and 1mL of 4.24mM ascorbic acid (pH=7, neutralized using 1M NaOH) was added to the diluted 5nm seeds in an alternating dropwise fashion using two autopipettes. The solution was stirred for 30 minutes between each instance. The growth from 10nm to 20nm was carried out in a similar fashion, where 4.8uL of 0.1M sodium citrate solution was added to 4mL 10nm AuNPs solution. Serial reduction was again performed twice. In each instance, 1mL of 5.62mM $KAuCl_4$ and 1mL of 16.9mM ascorbic acid was added to the above solution in an alternating dropwise fashion using two autopipettes. The solution was then stirred for 30 minutes. Once seed-mediated growth was completed, 0.1M citrate solution was added to the 20nm AuNPs to adjust the final concentration of citrate to 5.68mM for a longer stabilization of AuNPs at 4^oC.

TEM Characterization and Size Measurement of 20nm AuNPs

TEM images were taken to determine the size of synthesized AuNPs, using a Philips CM100 Transmission Electron Microscope (FEI, Hillsboro, OR, USA). AuNPs were applied and dried on a discharged copper grid (Formvar/Carbon 400 mesh, Copper, approx. grid hole size: 42µm; TED Pella, Inc., Redding, CA, USA) for the TEM measurement. Images were captured at different positions on the TEM grid, and individual NP diameters were determined using the software ImageJ (version 1.51, National Institutes of Health, USA). The average diameter of observed NPs was determined to be 20.9 ± 1.8 nm ($N = 150$), based on a total of three different images.

UV-Vis Characterization of Synthesized 20nm AuNPs

Synthesized AuNPs were diluted 12x prior to the UV-Vis measurements. UV-Vis spectra were taken using Cary 60 UV-vis Spectrometer to estimate the concentration of nanoparticles.

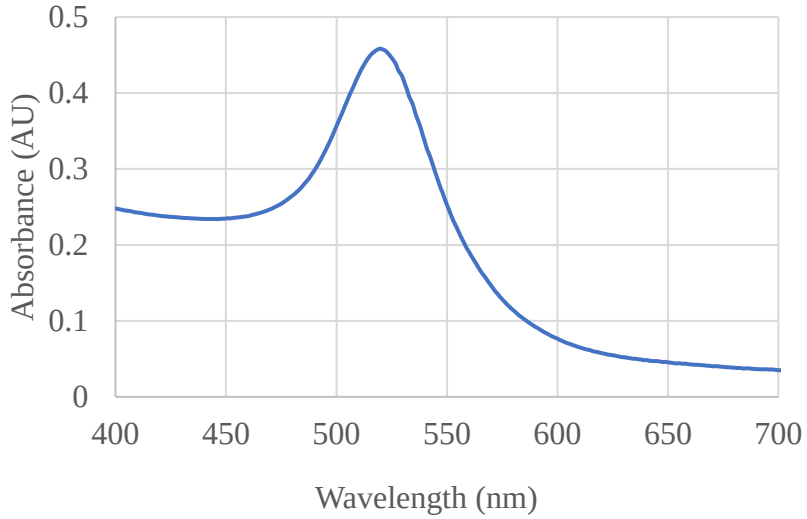

Fig. S1 UV-Vis spectrum of synthesized 20nm AuNPs. The λ_{LSPR} was measured to be 520nm.

Determination of Nanoparticle Concentration (number of particles/mL)

Calculations for nanoparticle concentration of 20nm of citrate-stabilized AuNPs were performed based on UV-Vis measurements according to Haiss et al.[1].

$$C = \frac{A_{450}}{\varepsilon_{450}}$$

where, at a path length of 1 cm:

C = Concentration (mol L^{-1})

A_{450} = Absorbance at 450nm

ε_{450} = Molar extinction coefficient at 450nm (L mol^{-1})

At a dilution factor of 12, the measured absorbance at 450nm was 0.235. According to Haiss et al.[1], $\varepsilon_{450} = 6.31 \times 10^8$ for ~21nm AuNPs, therefore giving a particle concentration of 2.69×10^{12} NPs/mL.

III. PEGylation and Characterization of Antibody-AuNP Conjugates

To synthesize anti-HPV18E7-AuNP conjugates, 20nm citrate-stabilized AuNPs were PEGylated using a mixture of PEG solutions containing Ab-PEG and cPEG.

Mixtures were prepared from the following three PEG solutions:

1. Antibody-capped PEG / Ab-PEG solution (Sol. 1)
2. Stock Carboxylate-PEG / cPEG solution (Sol. 2)
3. Diluted Carboxylate-PEG / cPEG solution (Sol. 3)

Similar to other reported studies,[2-5] Ab-PEG solution (Solution 1) was prepared in the following manner. First, 1mg/mL anti-HPV18E7 (clone M1862HP7) was dialyzed against 1x PBS prior to conjugation to remove interferants. To conjugate, OPSS-PEG-NHS (MW 5kDa) was dissolved in 1x PBS at a concentration of 6.7mg/mL (1.34mM). Immediately, 1uL of the OPSS-PEG-NHS solution was added to 99uL of the antibody solution (2:1 NHS:antibody mol ratio). The conjugation reaction then proceeded at room temperature for 4 hours. Afterwards, the Ab-PEG solution was aliquoted and stored at -20^{o}c until use.

The stock cPEG solution (Solution 2) was prepared by dissolving HS-PEG-COOH (5kDa) in 1x PBS at a concentration of 33.5mg/mL.

The diluted cPEG solution (Solution 3) was prepared by diluting the stock solution by 500 times with 1x PBS to a concentration of 0.067mg/mL.

To prepare the mixture of cPEG and Ab-PEG in different ratios for the PEGylation of 20nm AuNPs, the above three PEG solutions were mixed in a total volume of 11uL according to **Table S1**. Then, the 11uL of the mixed PEG solutions in different ratio of cPEG:Ab-PEG were added to each 90uL of 20nm AuNPs for the PEGylation and mixed thoroughly using an autopipette. The PEGylation occurred overnight at 4^{o}C (at pH ~7) to ensure complete ligand exchange. Afterwards, the anti-HPV18E7-AuNP conjugates were centrifuged at 3500g for 20 minutes. The supernatant was removed, and the remaining pellet was resuspended in 1% BSA-PBST.

AuNP controls using βhCG antibodies (as the non-specific antibodies) to produce anti-βhCG-AuNPs conjugates were prepared in the same manner as described above.

Dynamic Light Scattering Characterization

For DLS, antibody-AuNP conjugates and AuNP-cPEG control samples were prepared with a storage buffer containing no stabilizing protein i.e. BSA. These AuNPs were diluted 10 times using 1x PBS for the measurements. For citrate-capped AuNPs, the sample was diluted with H_2O instead. 2mL of diluted sample was transferred to a quartz cuvette for the analysis.

The DLS size measurements were taken with a Malvern Instruments Zetasizer 3000HSa (Malvern Panalytical, Worcestershire, UK), at an angle of 90^{o}. The raw data was processed using monomodal analysis to obtain the size distribution by intensity.

Agarose Gel Electrophoresis Characterization

For agarose gel electrophoresis, a 2% agarose gel was prepared by dissolving 500mg of agarose into 25mL of hot 1x TAE buffer using an appropriate gel caster. Once the gel is prepared, it was placed into the electrophoresis tank containing sufficient 1x TAE buffer to completely immerse the gel. 1uL of glycerol was mixed with 9uL of antibody-AuNP conjugates and AuNP-cPEG control prior to loading into sample wells. Electrophoresis was performed at 80V for 1 hour.

Zeta Potential Characterization

Zeta potential measurements were performed with a ZetaView PMX-120 BASIC NTA (Particle-Metrix, Meerbusch, Germany). Samples were diluted 2000 times using distilled water prior to measuring their Zeta potential. Measurements were taken at 11 different positions for each analysis. For every sample, 5-10 replicate analysis were performed.

TEM Characterization of Antibody-AuNP Conjugates

Procedures for the TEM imaging of the antibody-AuNP conjugates and AuNP-cPEG control were performed as described in section II.

The measured sizes of the antibody-AuNP conjugates and AuNP-cPEG control were found to be 21.3 ± 1.8 nm (N = 120) and 21.2 ± 1.8 nm (N = 150) respectively, indicating no significant change in nanoparticle size or aggregation during PEGylation.

Table S1. Table depicting the preparation of the mixture of cPEG and Ab-PEG in different ratios for the PEGylation of 20nm AuNPs. Various volumes of antibody-capped PEG (**Sol. 1**) and diluted carboxylate-PEG (**Sol. 3**) was mixed at a fixed volume of 10uL. 1uL of stock carboxylate-PEG (**Sol. 2**) was then added into the 10uL mixture of Sol. 1 & 3. This 11uL PEG solution was then pipetted and mixed with 90uL of citrate-capped AuNPs for PEGylation.

Vol. of Sol. 1 (0.067 mg/mL PEG) (uL)	Vol. of Sol. 3 (0.067 mg/mL PEG) (uL)	Vol. of Sol 1 & 3 Mixture (uL)	Amount of Ab-PEG in mixture (ug)*	Amount of cPEG in mixture (ug)	Vol. of Sol. 2 added (33.5mg/mL PEG) (uL)	Amount of cPEG (ug)	Total Volume (uL)	Total amount of PEG (ug)	Ratio of cPEG:Ab-PEG[a,b]
10.00	0.00	10	0.335	0.335	1	33.5	11	34.17	100:1
7.50	2.50	10	0.251	0.419	1	33.5	11	34.17	134:1
5.00	5.00	10	0.168	0.503	1	33.5	11	34.17	202:1
2.50	7.50	10	0.084	0.586	1	33.5	11	34.17	406:1
1.00	9.00	10	0.034	0.637	1	33.5	11	34.17	1018:1
0.50	9.50	10	0.017	0.653	1	33.5	11	34.17	2038:1
0.25	9.75	10	0.008	0.662	1	33.5	11	34.17	4078:1

[a] At a pH of 7.4, the estimated half-life of NHS esters is 2-3 hours.[6] At a 2:1 NHS:antibody ratio, it is assumed that half of the NHS esters will conjugate to an antibody, whilst the remaining half will hydrolyze to form carboxylic acid.[4] For the calculation of ratios, half of the Ab-PEG mass is assumed to be bound to an antibody, whilst the other half of the Ab-PEG mass is unconjugated as cPEG.

[b] According to Fig. 3(a) of the manuscript, the optimized ratio of cPEG:Ab-PEG was determined to be 202:1. With reference to a similar experiment conducted by Chattopdhayay et al. using 30nm AuNPs and 5kDa mPEG and Ab-PEG at a ratio of 150:1, around 10 Ab-PEG was bound on each AuNP.[7] Since our optimized ratio between cPEG and Ab-PEG is 202:1 and the AuNPs used were 20nm, it is estimated that the number of Ab-PEG on the 20nm AuNPs was 3, and thus the estimated number of cPEG is 606.

IV. Preparation of Fluorescein-Conjugated Anti-HPV18E7 Antibodies

Fluorescein-conjugated anti-HPV18E7 were prepared according to manufacturer's protocol. A solution of NHS-fluorescein was freshly prepared by dissolving 1mg NHS-fluorescein in 100μL DMSO. To 1mL of 1mg/mL antibody in 1xPBS, 4.7μL of the NHS-fluorescein solution was added and mixed thoroughly with a pipette, giving a 15x molar excess of fluorescein:antibody. The conjugation reaction proceeded for 1 hour in the dark whilst shaking at 400rpm on a thermo-mixer. The solution was then dialyzed overnight using 1x PBS, with buffers exchanged at least twice to remove the excess unreacted NHS-fluorescein. The fluorescein-conjugated antibody solution was kept in the dark at 4^{o}Cuntil use.

V. Preparation of HeLa and NB4 Cell Lysate

Preparation of HeLa Cell Lysate

HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. Cell cultures were maintained at 37°C in humidified atmosphere with 5% CO_2. Confluent cells were trypsinised, washed with ice-cold PBS and counted using a haemocytometer. ~ 1 x 10^7 cells were then pelleted by centrifugation at 400 g for 5 min and lysed in 1 mL of RIPA lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 150 mM sodium chloride, 50 mM Tris, pH 8.0 supplemented with protease inhibitor cocktail) for 30 min on ice with constant agitation. The cell lysates were then centrifuged at 12,000 *g* for 20 min at 4°C. The supernatants were collected and stored at -20°C until use.

Preparation of NB4 Cell Lysate

Acute promyelocytic leukemia cell line NB4 was used as control in this study. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in humidified atmosphere with 5% CO_2. Upon reaching confluency, the cells were collected by centrifugation at 400 g for 5 min and then washed with ice-cold PBS. The number of cells was counted using a haemocytometer. ~ 1 x 10^7 cells were pelleted by centrifugation at 400 g for 5 min and lysed in 1 mL of RIPA lysis buffer (1 % Triton X-100, 0.5% sodium deoxycholate, 150 mM sodium chloride, 50 mM Tris, pH 8.0 supplemented with protease inhibitor cocktail) for 30 min on ice with constant agitation. The cell lysates were then centrifuged at 12,000 g for 20 minutes at 4°C. The supernatants were collected and stored at -20^oc until use.

VI. Preparation of Immuno-Capture Array and Detection of HPV18E7

For the preparation of the immuno-capture substrates, glass coverslips were derivatized with GOPTS to functionalize the surface with epoxide groups for coupling with capture antibodies. Glass coverslips were first treated with piranha solution (2:1 H_2SO_4:H_2O_2) for 30 minutes to remove any organic contaminants. Afterwards, the coverslips were washed four times with distilled water whilst shaking on an orbital shaker at 120 rpm to wash away the remaining acid. The coverslips were then dried under a stream of argon gas and immediately silanized by immersion in 2% (v/v) GOPTS in toluene overnight (~21 hours). Afterwards, the coverslips were washed with toluene, followed by ethanol twice and then acetone twice, 2 minutes each time. The coverslips were then gently blown dry under a stream of argon gas.

Sample wells were demarcated on the epoxy-coverslips using a hydrophobic pen. For printing the capture antibodies, 0.3uL of 0.4mg/mL anti-HPV18E7 (clone 718-15) in 1x PBS was spotted onto each sample well of the coverslip. The coverslip was then stored in a humidity chamber (containing saturated NaCl solution, ~75% rH) at room temperature for overnight printing.

For the analysis of recombinant HPV18E7 in blocking buffer, the stock recombinant HPV18E7 protein solution was diluted to a concentration ranging from 14 pM to 1.8nM using 3% NFDM in 1x PBST. Sample wells were blocked with a solution of 3% NFDM in 1x PBST for 2 hours after printing with capture antibodies. The blocking solution was then removed and 2.5uL of the diluted recombinant HPV18E7 was added to each sample well. For the blank, 2.5uL of the 3% NFDM blocking solution was applied instead. The coverslip was then incubated at 4^{o}C for overnight to maximize the amount of antigen captured. Following overnight incubation, the antigen solution was removed, and sample wells were washed three times using 1x PBST for 5 minutes each, whilst shaking on an orbital shaker. To detect the immobilized antigen, 5uL of detection antibodies was applied onto sample wells and incubated for 1 hour. For mass spectrometric analysis, anti-HPV18E7-AuNPs was diluted 20x using 3% NFDM in 1x PBST. For fluorescence, 5ug/mL of anti-HPV18E7-fluorescein was prepared in 3% NFDM in 1x PBST. After incubation, the excess detection antibodies were removed by immersing the slide in 1x PBST, PBS, and then rinsed with water. The slide was finally dried under a stream of argon before mass spectrometric/ fluorescence measurements.

For the analysis of native HPV18E7 in HeLa cell lysate, the blocking, incubation and detection steps were performed in the same manner as above. After blocking, prepared HeLa cell lysate was diluted 2 times with 3% NFDM in 1x PBST, and 2.5µL was incubated in each sample well (corresponding to 12,500 cells/well). For the control, 2.5uL of diluted NB4 cell lysate was applied instead (12,500 cells/well).

Mass Spectrometric Measurement

Mass spectrometric measurements were performed according to the optimized conditions discussed in our previous work.[8]

All the mass spectrometric measurements were performed with UltrafleXtreme MALDI-TOF/TOF mass spectrometer equipped with a 355nm solid state smartbeam Nd:YAG laser (Bruker, Bremen, Germany). The glass coverslip was mounted onto the standard stainless steel MALDI plate using electrically conductive double-sided tape (9713 XYZ-Axis, 3M, St. Paul, MN, USA). The instrumental parameters were optimized to obtain high ion intensity with low background and stated as follows: ion source 1 at 25.0kV; ion source 2 at 22.1kV; lens voltage at 8.6kV; delay time at 10ns; detector gain at 2855V (20x); laser power was set in the range of 80 – 90 %; laser shot frequency at 2000Hz; laser shot number at 150,000 shots were used to raster each well of the array; acquired mass range at m/z 5 – 700; laser spot size at 100µm. The vacuum pressure was approximately 10^{-6} – 10^{-7} mbar in the source and 10^{-7} – 10^{-8} mbar in the analyzer. The instrument was controlled via the Bruker Daltonics flexControl 3.4 software (Bruker). Assisted by the AutoXecute function of flexControl software. The mass spectra recorded were analyzed using flexAnalysis 3.4 (Bruker). The ion intensities were reported as the total intensities of the major gold ion clusters formed, including Au^+ at m/z 197, Au_2^+ at m/z 394, and Au_3^+ at m/z 591.

Fluorescence Imaging and Data Processing

Fluorescence imaging was performed on a Bio-Rad Chemidoc MP System. For fluorescein, the excitation source is a blue epi-illuminator, with a 532/28 emission filter. The exposure time was set to 30 seconds. Size of images were taken at the preset standard of small (95mm x 76mm, pixel size: 34.5µm x 34.5µm). The raw data obtained was processed using the accompanying software Image Lab (Ver 6.0.0) to obtain the fluorescence intensities of each sample spot.

VII. Other Supplementary Data

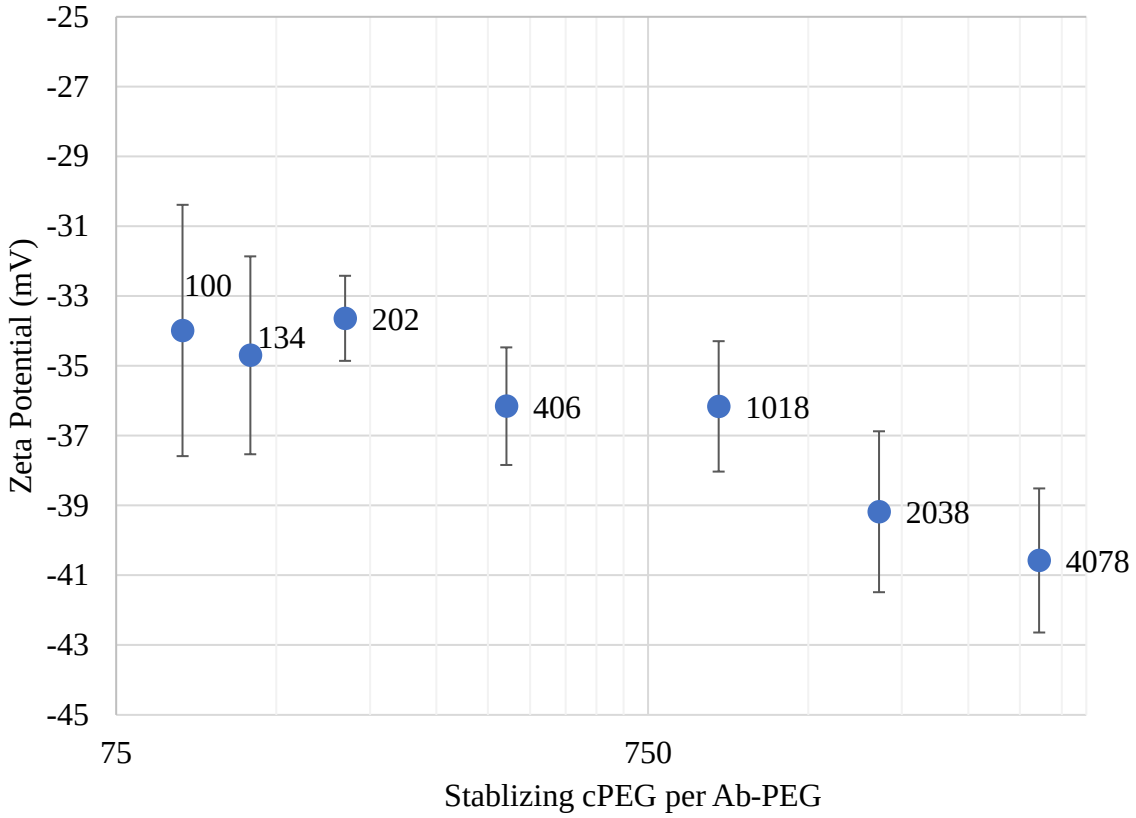

Fig. S2 Graph depicting the measured average Zeta potential of anti-HPV18E7-AuNP conjugates prepared with different ratios of cPEG per Ab-PEG, ranging from 100:1 to 4078:1. 5-10 replicate measurements were performed at each ratio.

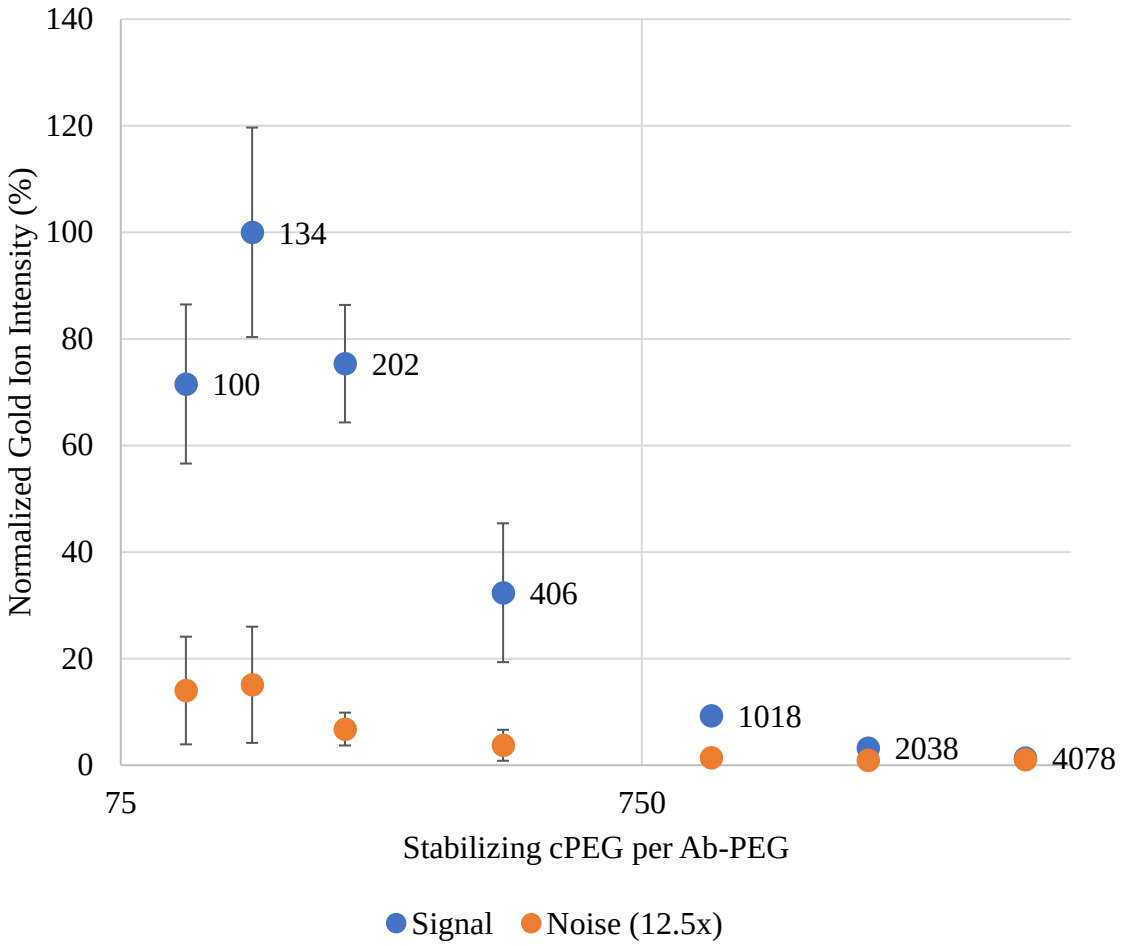

Fig. S3 Supplementary data for Fig. 3(a), showing the effect of varying the ratio of cPEG per Ab-PEG on the measured Signal (blue) and Noise (orange) for the detection of 4.5 fmol of recombinant HPV18E7 using 20nm AuNP mass tags with a fixed PEG loading amount of 34ug. The relative total gold ion intensity for the measured signal (N=6) and noise (N=3) across triplicated independent assays are depicted. The intensity of the noise has been multiplied by 12.5 times for better visual clarity. (Remark: The Au ion intensity changes of noise (i.e., in the absence of HPV18E7) were relatively small across different ratios of cPEG/Ab-PEG. Yet, the changes of signal (i.e., in the presence of HPV18E7) were more significant across the ratios of cPEG/Ab-PEG. This suggested that the presence of antigen was the key factor for the signal increase but not the change of cPEG/Ab-PEG ratio.)

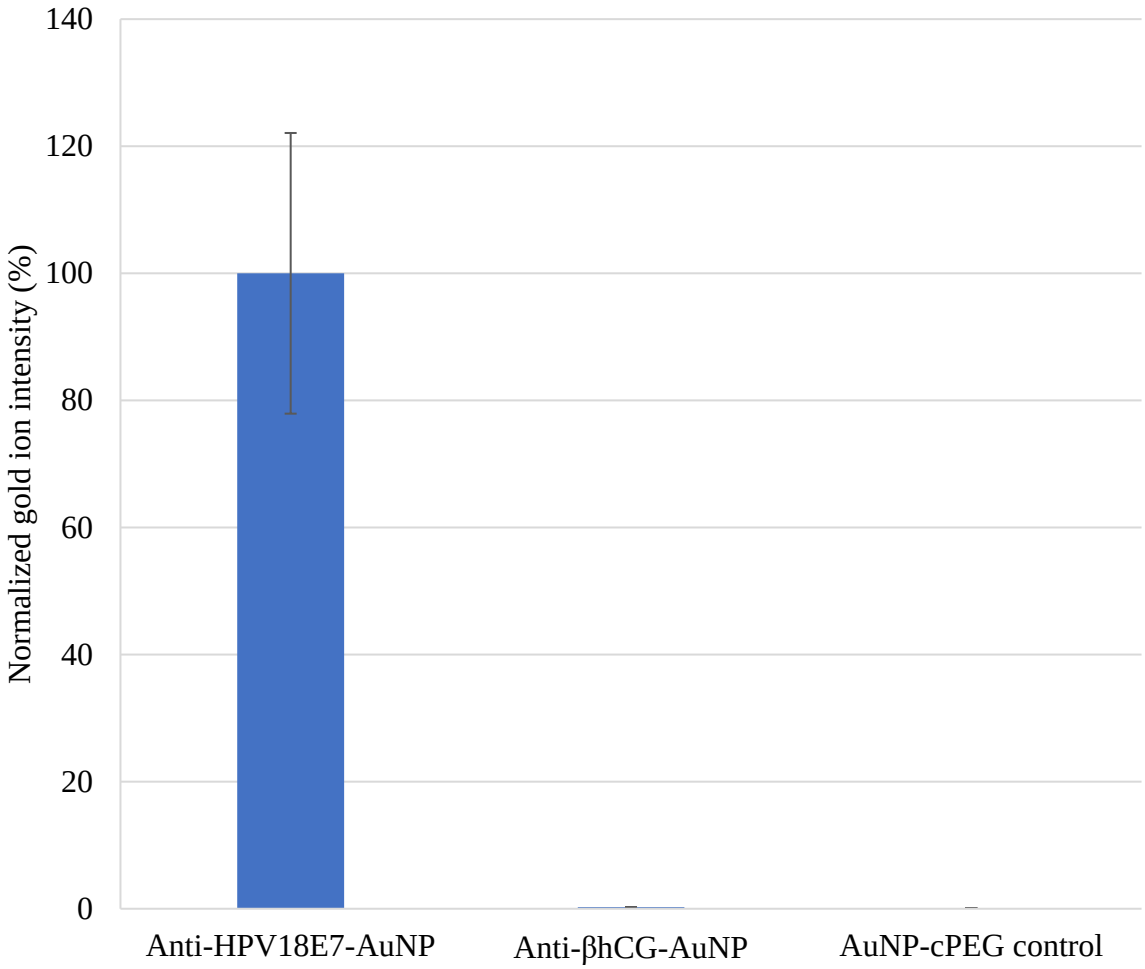

Fig. S4 Graph depicting the relative total gold ion intensity generated from anti-HPV18E7-AuNPs, anti-βhCG-AuNPs and AuNP-cPEG controls in the detection of 4.5 fmol HPV18E7 recombinant protein. Conjugates were prepared at a ratio of 100:1 cPEG per Ab-PEG, and at the PEG loading amount of 34μg per 100μL.

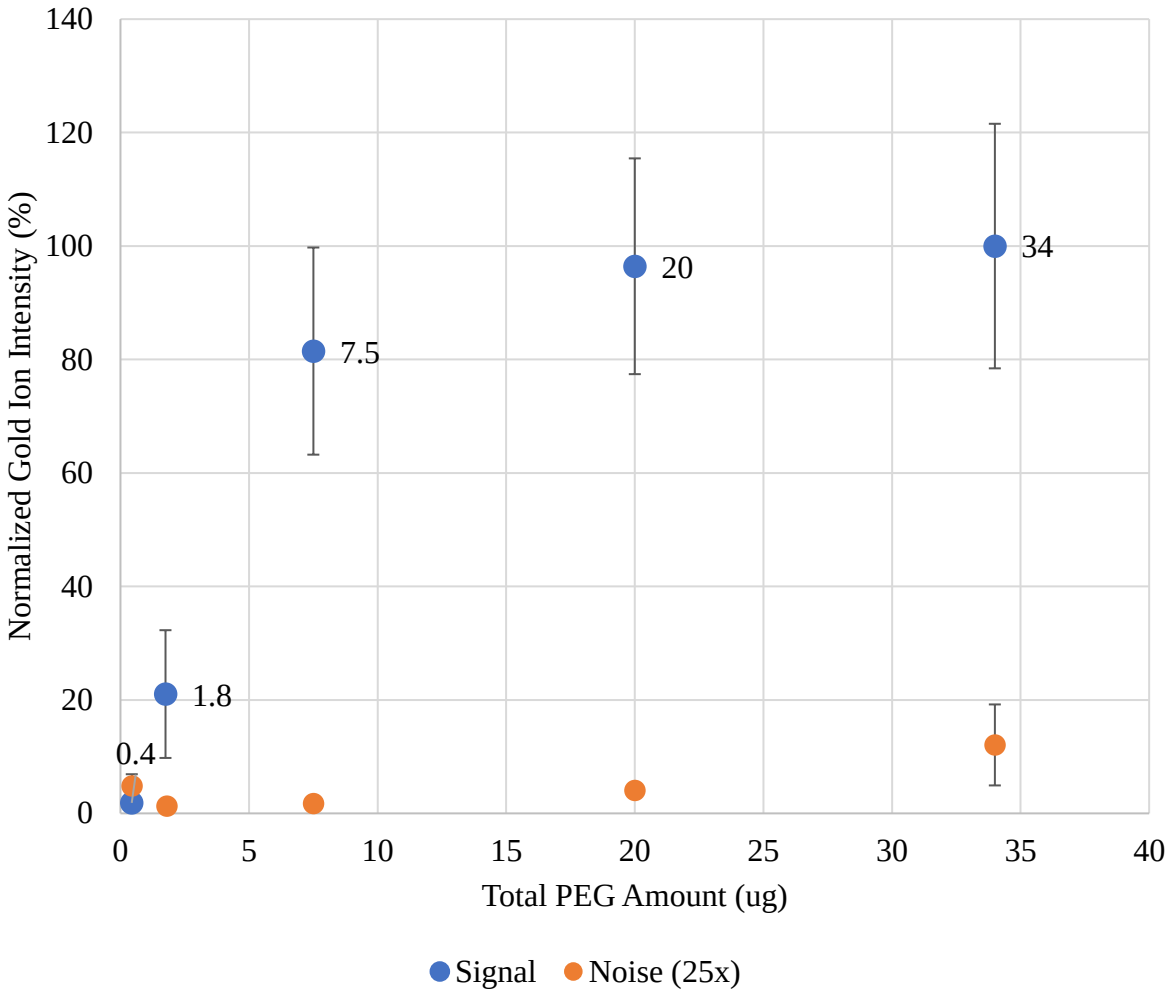

Fig. S5 Supplementary data for Fig. 3(b), showing the effect of varying PEG loading amount on the measured Signal (blue) and Noise (orange) at 4.5 fmol of recombinant HPV18E7 for 20nm AuNP mass tags at a fixed ratio of 202:1 (cPEG per Ab-PEG). The relative total gold ion intensity across for the measured signal (N=4) and noise (N=4) across duplicated independent assays are depicted. The intensity of the noise has been multiplied by 25 times for better visual clarity.

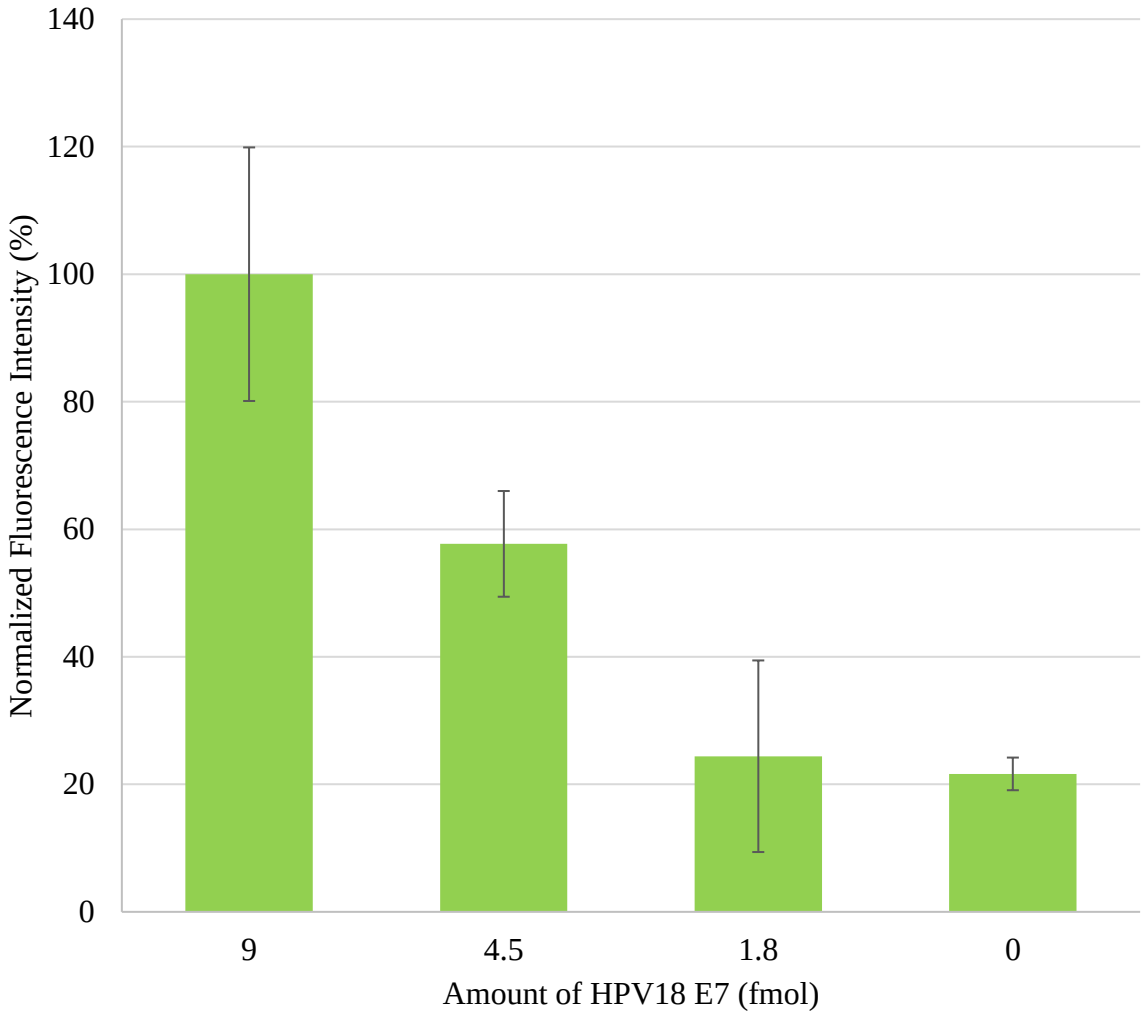

Fig. S6 Graph showing the relative fluorescence intensities for various amounts of recombinant HPV18E7 measured via immuno-capture assay with fluorescein-conjugated anti-HPV18E7 (N=3).

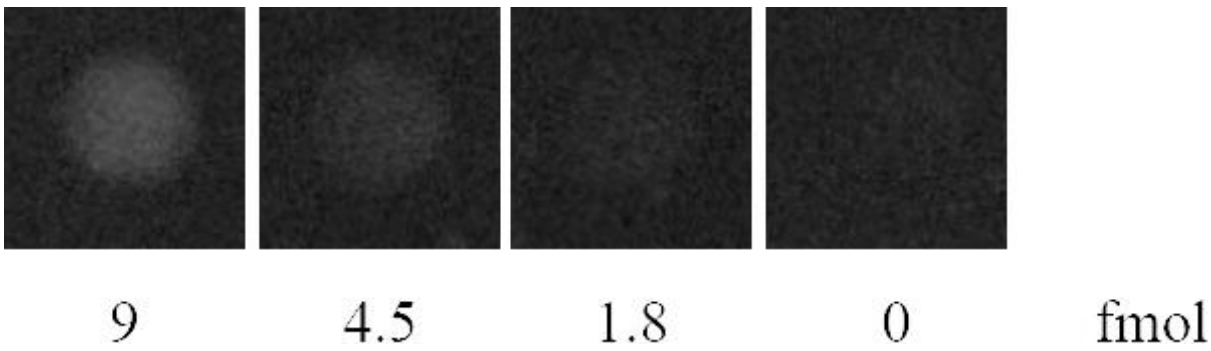

Fig. S7 The corresponding fluorescence images of sample spots at 9, 4.5, 1.8, and 0 fmol of recombinant HPV18E7 shown in Figure S6. The contrast and brightness for this set of images have been digitally adjusted for better visual clarity.

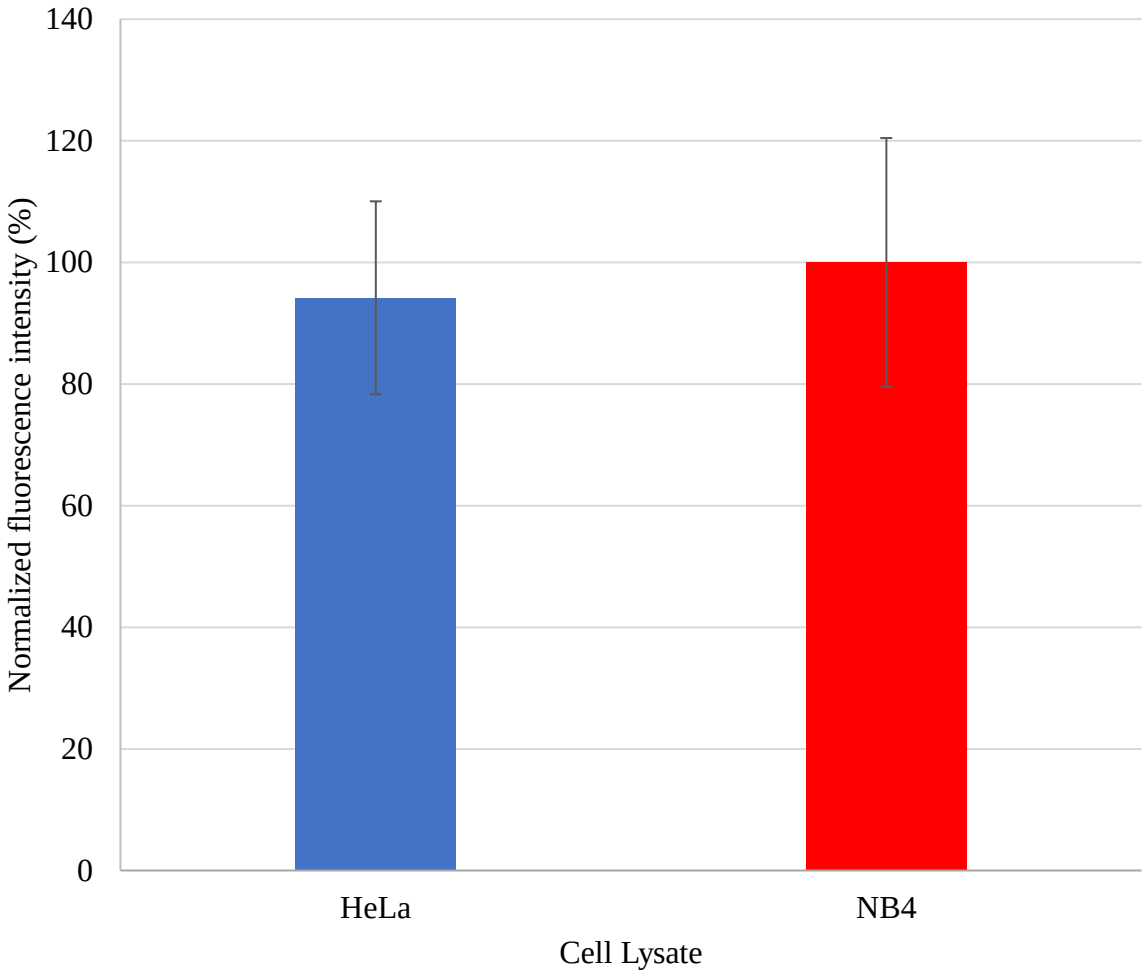

Fig. S8 Graph showing the relative intensities of fluorescence signal using fluorescein-conjugated antibodies for the detection of HPV18E7 in HeLa cell lysate and NB4 cell lysate control at 12,500 cells/well via immuno-capture assay. 8 replicate measurements were performed for each cell lysate. As determined in Fig. 6 of the manuscript, the total amount of HPV18E7 proteins was only 127 attomoles in 12,500 HeLa cells, which is far below the detection limit of fluorescence detection. Thus, no significant signal differences were observed between the HeLa cell lysate and NB4 cell lysate.

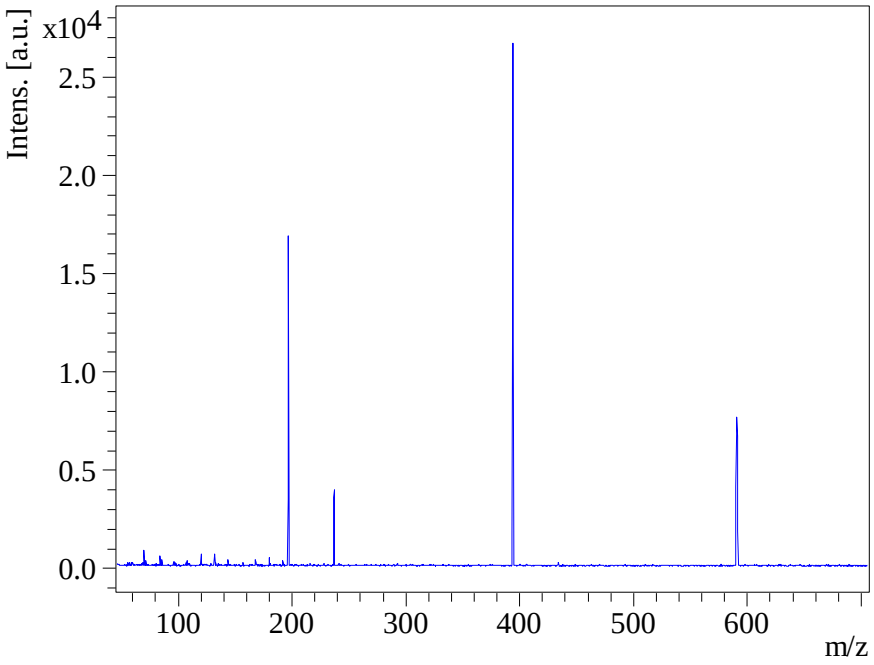

Fig. S9 Mass spectrum of gold cluster ions generated in immuno-capture LI-MS assay for the detection of HPV18E7 protein in HeLa cell lysate (12,500 cells per well).

VIII. References

1. Haiss, W.; Thanh, N. T.; Aveyard, J.; Fernig, D. G. Determination of size and concentration of gold nanoparticles from UV− Vis spectra. *Anal. Chem*. **2007**, 79, 4215-4221.

2. Day, E.S., Bickford, L.R., Slater, J.H., Riggall, N.S., Drezek, R.A., West, J.L. Antibody-conjugated gold-gold sulfide nanoparticles as multifunctional agents for imaging and therapy of breast cancer. *Int. J. Nanomedicine*. **2010**, 5, 445–454.

3. Billingsley, M.M., Riley, R.S., Day, E.S. Antibody-nanoparticle conjugates to enhance the sensitivity of ELISA-based detection methods. *PLoS One*. **2017**, 12(5), e0177592.

4. Hanson, C., Israelsen, N.D., Sieverts, M., Vargis, E. Fabricating a UV-Vis and Raman Spectroscopy Immunoassay Platform. *J. Vis. Exp*. **2016**, 117, 54795.

5. Xie H, Diagaradjane P, Deorukhkar AA, et al. Integrin αvβ3-targeted gold nanoshells augment tumor vasculature-specific imaging and therapy. *Int. J. Nanomedicine*. **2011**, 6, 259–269.

6. Yasuhiro, N., Kazuma, I., Yosuke, S., Atsushi, S. The pH-Dependent Formation of PEGylated Bovine Lactoferrin by Branched Polyethylene Glycol (PEG)-*N*-Hydroxysuccinimide (NHS) Active Esters. *Biol. Pharm. Bull*, **2009**, 32(3), 523-526.

7. Chattopadhyay, N., Cai, Z., Pignol, J.-P., Keller, B., Lechtman, E., Bendayan, R., Reilly, R. M. Design and characterization of HER-2-targeted gold nanoparticles for enhanced X-radiation treatment of locally advanced breast cancer. *Mol. Pharm.*, **2010**, 7(6), 2194-2206.

8. Cheng, Y.-H.; Cheung, Y.-F.; Tam, T. S.-C.; Lok, C.-N.; Sun, H.; Ng, K.-M. Plasmonic metal nanoparticles as efficient mass tags for ion signal amplification and ultrasensitive detection of protein markers. *Anal. Chim. Acta* **2019**, 1055, 1-6.