DESIGN AND DEVELOPMENT OF A FIELD APPLICABLE GOLD NANOSENSOR FOR THE DETECTION OF LUTEINIZING HORMONE

Ajit Zambre[1], Nripen Chanda[1], Sudhirdas Prayaga[2], Rosana Almudhafar[3], Zahra Afrasiabi[3*] Anandhi Upendran[4,5*], and Raghuraman Kannan[1*]

Departments of [1]Radiology and [5]Physics, University of Missouri, Columbia, MO 65212, USA; [2]Antibody Research Corporation, St Charles, MO 63304, USA; [3]Department of Life and Physical Sciences, Lincoln University, Jefferson City, MO 65101, USA; [4]Nanoparticle BioChem. Inc., Columbia, MO 65211, USA

*Raghuraman Kannan, Tel: +001-573-882-5676, Fax: 001-573-884-5679, email: Kannanr@health.missouri.edu; Anandhi Upendran, Tel: +001 573-884-0162, Fax: +001 573-884-4904, e-mail:upendrana@missouri.edu; Zahra Afrasiabi, Tel: +001 573-681-5131, Fax: +001 573-681-5944, e-mail: afrasiabiz@lincolnu.edu.

Experimental Section

Materials: The components used in the synthesis of AuNPs were procured from standard vendors: $NaAuCl_4$ was purchased from Alfa-Aesar, nitrocellulose membrane and all buffer reagents from Sigma-Aldrich. PEG-750 was procured from Rapp Polymer, Germany. Sheep Luteinizing Hormone (LH) and rabbit polyclonal *anti*-Luteinizing Hormone (*anti*-LH) was purchased from Harbor-UCLA Research and Education Institute. All other chemicals were used as received and all aqueous solutions were prepared in doubly distilled water.

Instrumentation: Transmission electron microscope (TEM) images were obtained on a JEOL 1400 transmission electron microscope (TEM), JEOL, LTD., Tokyo, Japan. TEM samples were prepared by placing 5 µL of gold nanoparticle solution on the 300 mesh carbon coated copper grid and the solution allowed to sit five minutes. Excess solution was removed carefully and the grid was allowed to dry an additional five minutes. The average size and size distribution of AuNPs synthesized were determined by the processing of the TEM image using image processing software such as Adobe Photoshop (with Fovea plug-ins). The core size of the nanoparticles was also measured on a DC 24000, CPS Instruments Inc., USA. The hydrodynamic diameter and zeta potential were obtained using a Zetasizer Nano S90 (Malvern Instruments Ltd. USA). The absorption measurements were done using a Varian Cary50 UV-vis spectrophotometer with 1 mL of gold nanoparticle solution in disposable cuvettes with 10 mm path length. The HPLC was performed on a Schimadzu reverse-phase HPLC/ 4.6x250mm, Phenomenex C18-5µ 300Å column using two gradient solvent systems (A and B), where A is 0.1%TFA in 100% acetonitrile and B is 0.1%TFA in 100% water. Mass spectrum was recorded by electrospray ionization-mass spectrometry (ES-MS). X-ray photoelectron spectroscopy was performed using a Kratos Axis HSi XPS instrument. The gold content was determined by neutron activation analysis (NAA).

Identification and synthesis of Lutenizing Hormone Peptide (LHP): The sequence of LHP was identified based on the bioinformatics analysis. Bioinformatics analysis of ovine LH protein was performed to determine the following criteria: (a) homology to rabbit LH and other mammalian LH, (b)

hydropathy, and (c) immunogenicity. BLAST and ClustalW analysis showed that the C-terminal 12 amino acid peptide of ovine LH has minimum homology to LH of rabbit (8 out of 12 amino acids are identical), suggesting better immunogenicity of this peptide in rabbit. Detailed *in vivo* immunogenicity analysis of the peptide in rabbit model supported our prediction. LHP was synthesized by standard F-moc chemistry. The purity of the LHP was determined using HPLC (**Figure S2, SI**) and ES-MS (**Figure S3, SI**). LHP was conjugated to Keyhole Limpet Hemocyanin (KLH) and used for immunization of rabbits to raise polyclonal antibodies to LHP.

Synthesis, purification and characterization of Luteinizing Hormone Peptide (LHP): A 12 amino acid peptide sequence **("CDHPPLPDILFL")** from the native sheep luteinizing hormone was synthesized using standard Fmoc chemistry. HPLC results show a purity >98% for LHP. Fmoc-IIe-Wang resin was used as the solid support for the synthesis. Fmoc protected amino acids were activated using one equivalent of 0.45M HBTU/HOBt solutions and two equivalents of DIEA. The aminoacids were Fmoc deprotected using piperidine, and coupled using NMM.HBTU. Following the coupling of all the amino acids in the appropriate sequence, cleavage of the peptide from the resin was performed using TFA. This cleavage step also removes the amino acid side chain protecting groups. The peptide was dissolved in DMSO and the mass spectrum was recorded to check the final sequence (Waters ZQ 2000). The peptide was purified on a reverse phase HPLC/C18 column. The material was eluted using 0.1% TFA in 100% acetonitrile and 0.1%TFA in 100% water, mixed using a linear gradient of 45-70% with 10ml/min flow rate. A column size of 4.5x250mm (Kromosil, C18-5) was used. The purity was determined using HPLC and ES-Mass spectroscopy. ES-MS, MW (measured) 1380.69; 1379.1(calc'd.) HPLC profile showed a single peak at 220nm with a retention time of 6.5min.

Raising antibody against LHP in rabbits: The polyclonal antibody of the lutenizing hormone peptide (anti-LHP) was raised in rabbit, purified from the serum and obtained from Antibody Research Corporation.

Quantification of LHP by HPLC Analysis: The amount of LHP bound to AuNPs was quantified by HPLC analysis. A standard calibration curve using pure LHP (1.5, 2.0, 2.5, 3.0, 3.5 4.0 mg/ml) was constructed based on HPLC retention time measurements. AuNP-LHP was synthesized by addition of LHP to pegylated AuNPs. After the reaction was complete, AuNP-LHP was isolated by centrifuging the reaction mixture three times and the supernatants were collected. HPLC was recorded for the supernatant solutions and compared with standard calibration curves to quantitatively determine the amount of peptide bound to AuNPs.

XPS Analysis: We have established the structural details of AuNP- LHP through detailed XPS analysis. Using XPS measurements, we have determined relative elemental composition of C, N, O and S in AuNP-LHP. AuNP-LHP was dried onto the silicon wafer pieces and samples were measured at a 90^{o} take-off- angle (TOA) yielding a sampling depth of ~10nm. The analysis area was ~500μm diameter. Analyses were performed with a monochromatic Al k* X-ray source powered at 15kV and 15mA. Charge neutralization of the sample surface was achieved with the use of a low-energy electron flood gun. Energy scales of the spectra are references to the C 1S C-C/C-H signal at 285.0eV. The quantification method assumes that the sampling volume is homogeneous. High-energy solution XPS analyses of the C1s, S 2p and Au 4f regions were also performed on the sample. The 4f region of the spectrum shows two different signals for gold with a spin orbit coupling of 4.4 eV and 4.3 eV respectively, corresponding to core gold atom Au (0) and surface gold with partial Au (I) character (**Figure S5A**). The sulfur atom of the cysteine residue of the peptide covalently binds to AuNPs resulting in partial Au (I) character on the surface of the nanoparticle. The signal corresponding to the binding energies of ~85 eV and 88 eV for Au peaks in 4f region are similar to peptide conjugated AuNPs reported in literature, suggesting that Au is bound to S of cysteine amino acid residue of LHP [1,2]. The XPS hi-res spectrum of C1s region shows various O-C=O, C-O, C-N, C-C, and C-H regions as expected from conjugated LHP and the experimental atomic ratios are listed in **Table S1**. Analysis of the XPS hi-res spectra for the sulfur 2p region suggests that there are two different S states. The sulfur 2p region, exhibits two distinct

sulfur peaks at 164.1eV and 165.3eV corresponding to Au-S (sulfur coordinated to AuNPs) and S-C (sulfur present in the methyl cysteine amino acid, S-CH_3) respectively, in the LHP (**Figure S5B**). Each class of sulfur peak was further split into doublets, 2p3/2 and 2p1/2 with coupling of 1.2 eV, which is in agreement with literature.[1,3] These data confirm that, LHP is conjugated to AuNPs covalently through the thiol linkage. The S-Au and S-C At% ratios are 40:60 respectively. Based on the ratios, we propose that of the total 40% of S-Au, 30% is accounted for LHP conjugated to the surface of AuNPs through Au-S bond and 10% is accounted for the PEG present as a protective layer. Of the total 60 At% of C-S bonds, 30% can be attributed to S-C present in LHP through Au-S-cysteine bond directly conjugated to AuNPs and the remaining 30% can be accounted for a subsequent LHP layer bound to inner LHP present on gold surface. These results suggest the presence of two layers of LHP on AuNPs. This postulation is well supported by literature evidence in which the existence of a two-layer boundary in cysteine conjugated AuNPs has been well documented.[4,5]

Stability Studies: *In vitro* stability of AuNP-LHP conjugate was monitored using UV-vis spectral measurements. Typically, 1 mL of gold nanoconjuate solution was added to glass vials containing 0.5 ml of 3 % NaCl, 0.5 % cysteine, 0.2 M histidine, 0.5 % HSA and BSA solutions, respectively, and incubated for 30 min. The stability and the identity of the nanoparticles were measured by recording UV-vis absorbance at 0.5 hours as well as after 24 hours. The *in vitro* stability with various phosphate buffers (pH 7 and 9) were also observed following the similar procedure. *In vitro* stability of AuNP-LHP in ELISA medium was also determined. AuNP-LHP at different concentrations (1.0, 10.0, 25.0, 50.0 µg/ml) was incubated with ELISA medium (1x PBS containing 0.05% Tween20) and the stability was monitored by UV-visible plasmon resonance at 0.5 hours and 24 hours.

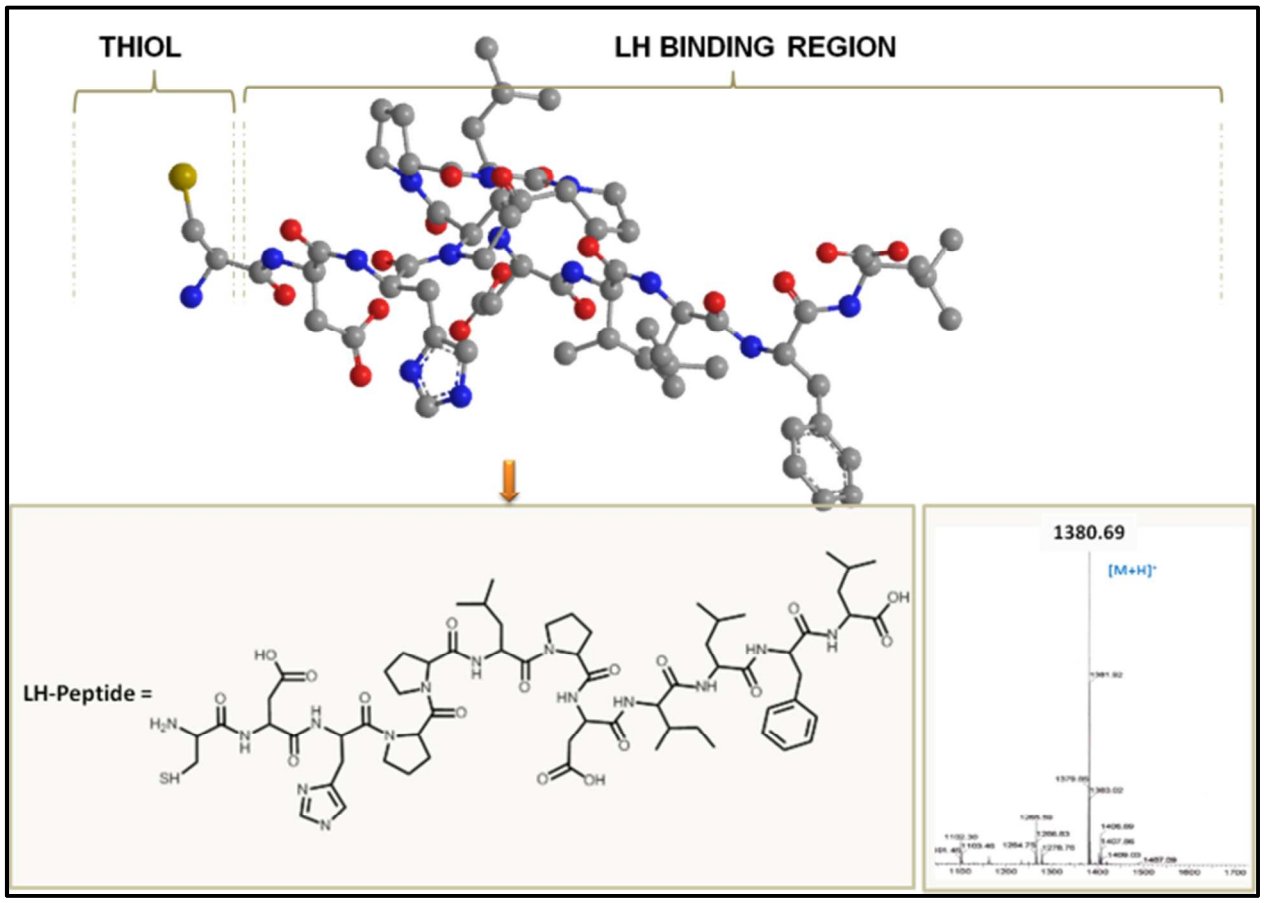

Figure S1. Amino acid sequence of the peptide (LHP) of the native sheep hormone LH as identified by bioinformatics analysis.

Inset showing mass spectrum of LHP.

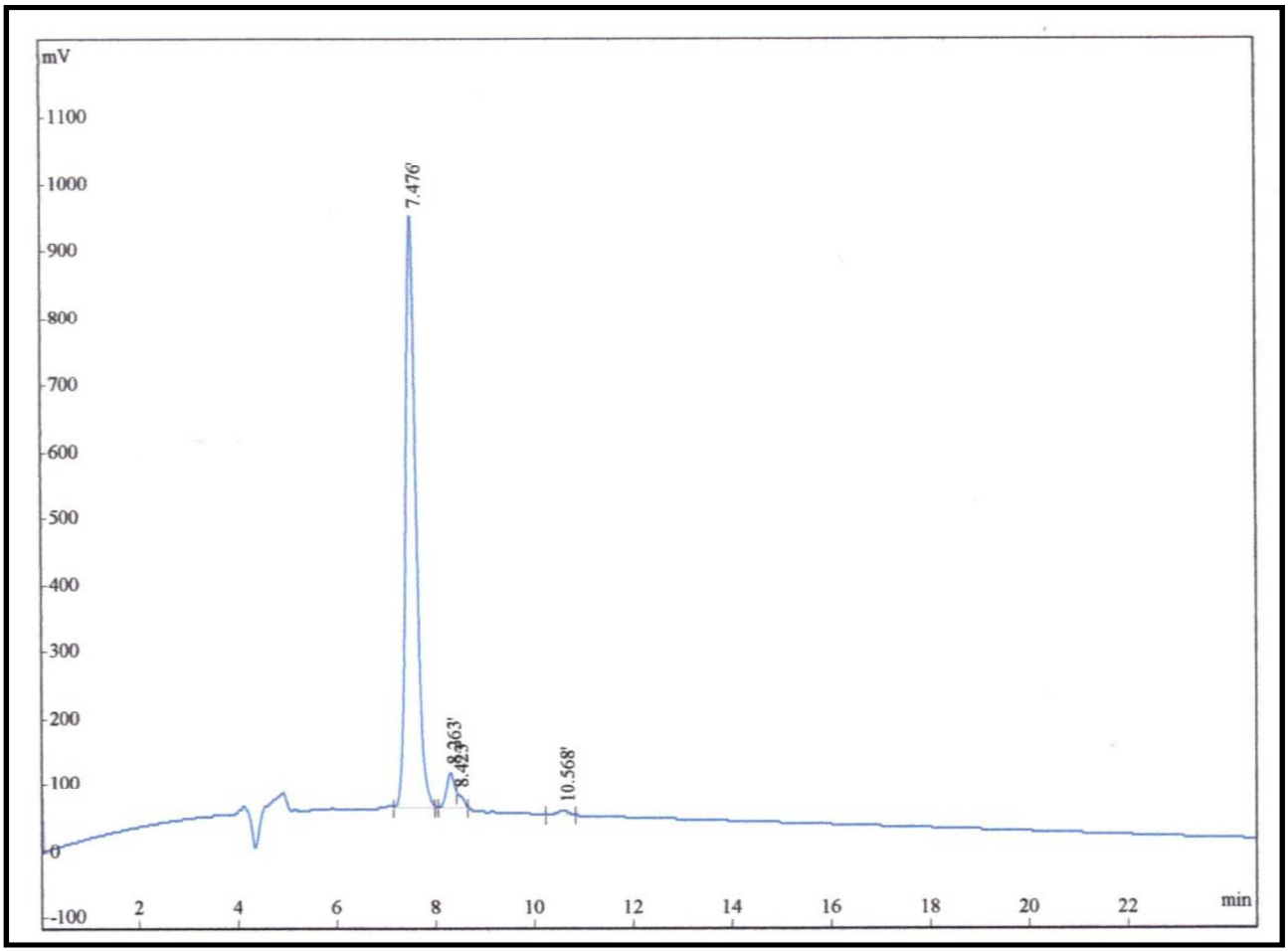

Figure S2: HPLC profile of Luteinizing hormone peptide (LHP) using solvent gradient A:0.1% TFA in Acetonitrile, B: 0.1% TFA in water with flow rate 1.0ml/min (Column: 4.6*250mm, Kromasil C18-5; Detector wavelength: 220nm, Volume of Injection 10µl)

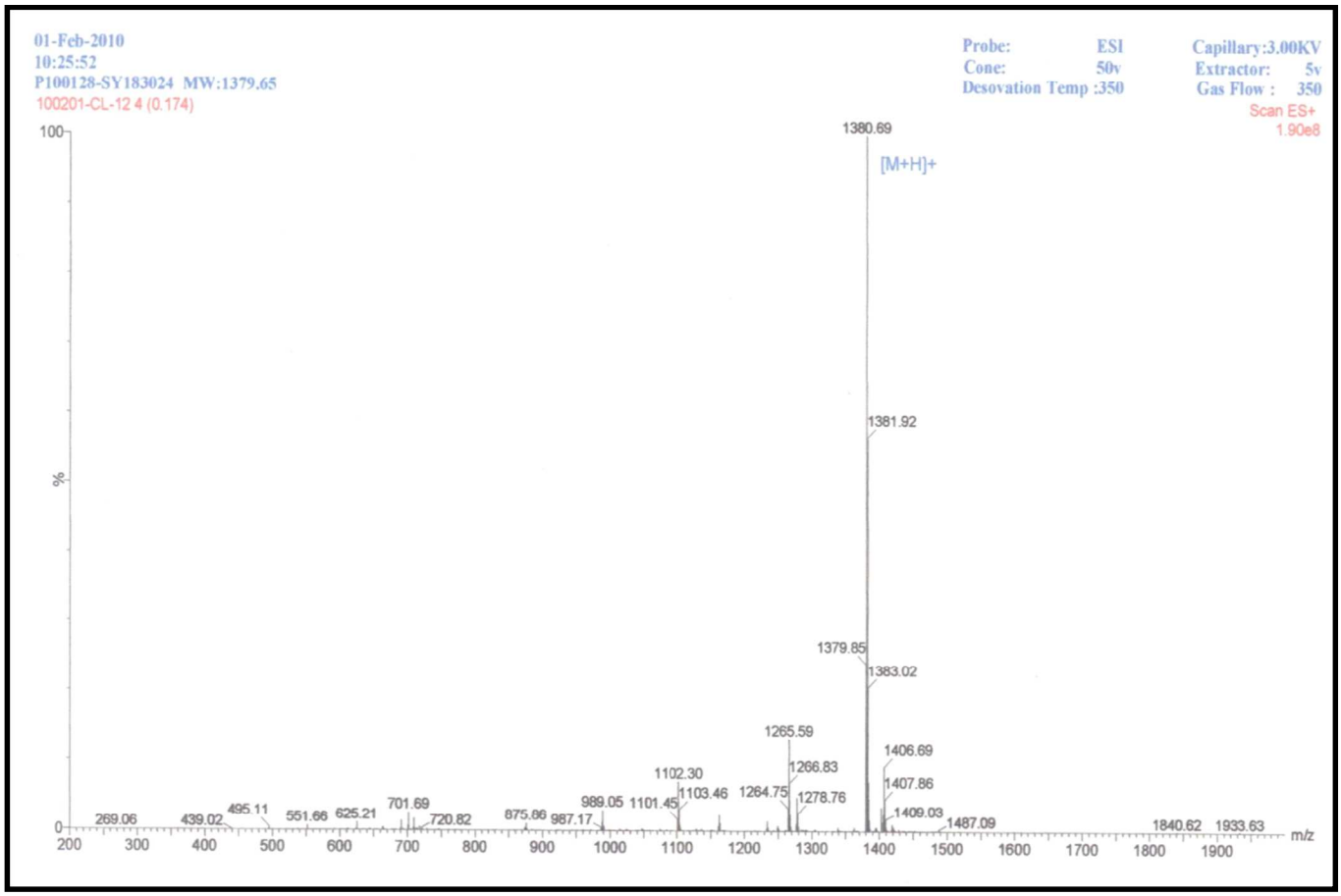

Figure S3: ES-MS of LHP Molecular Weight: 1379.65

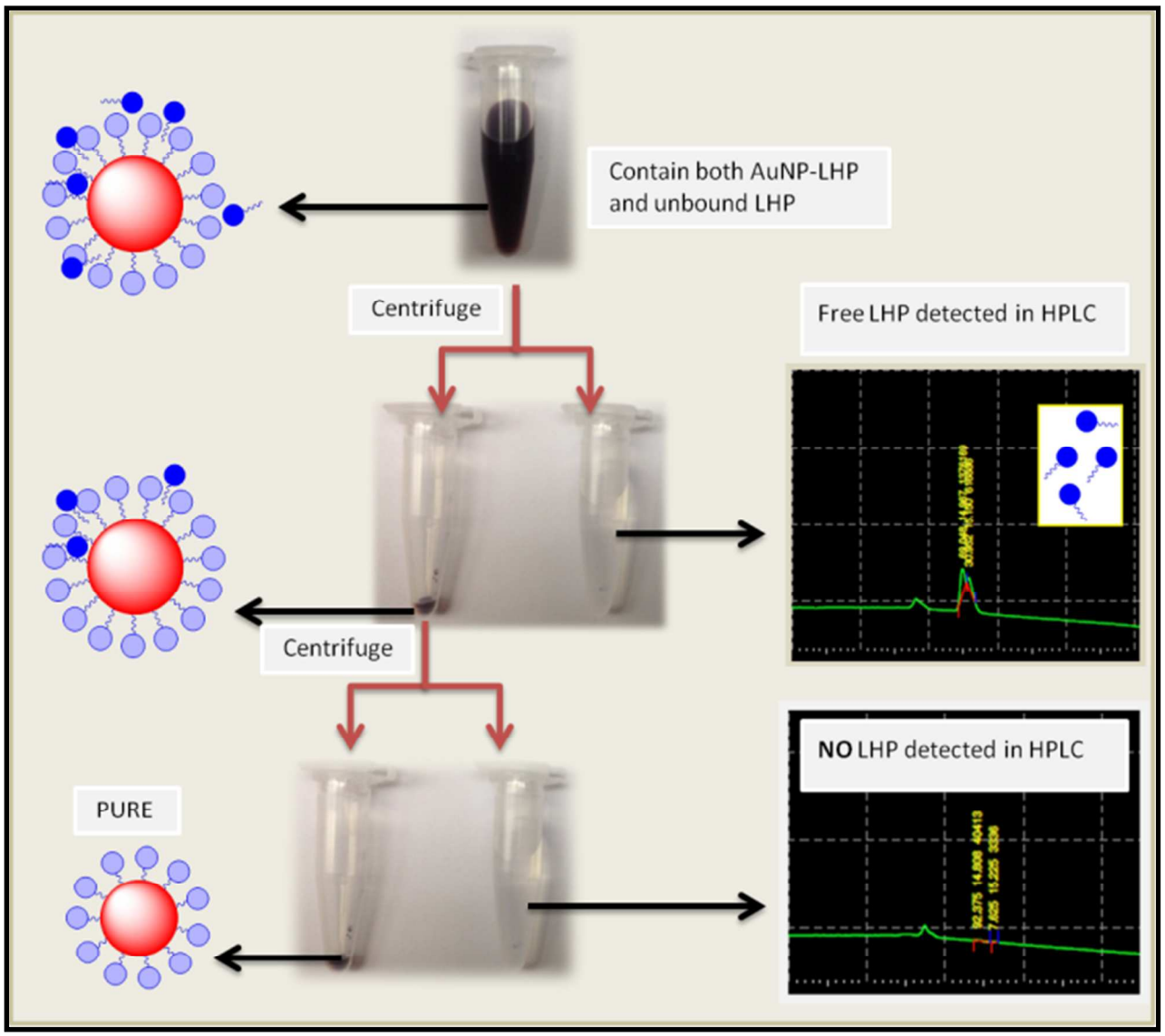

Figure S4. Purification of AuNP-LHP by centrifugation process and quantification of LHP conjugated to AuNPs by reverse phase HPLC analysis.

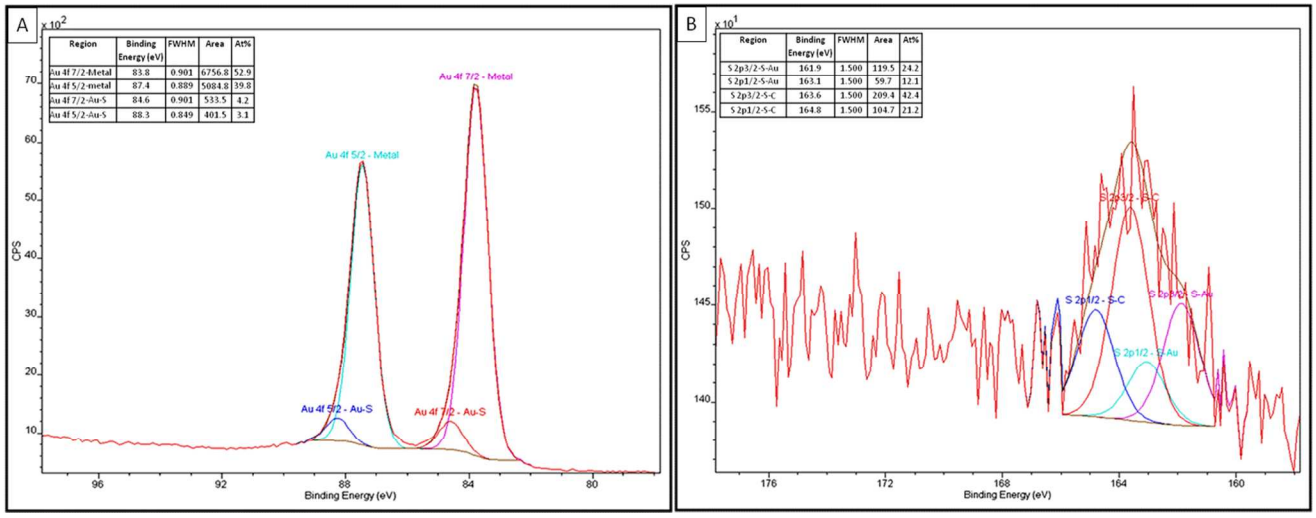

Figure S5. XPS hi-resolution spectrum of AuNP-LHP: (A) Au 4f region; (B) sulfur S 2p region.

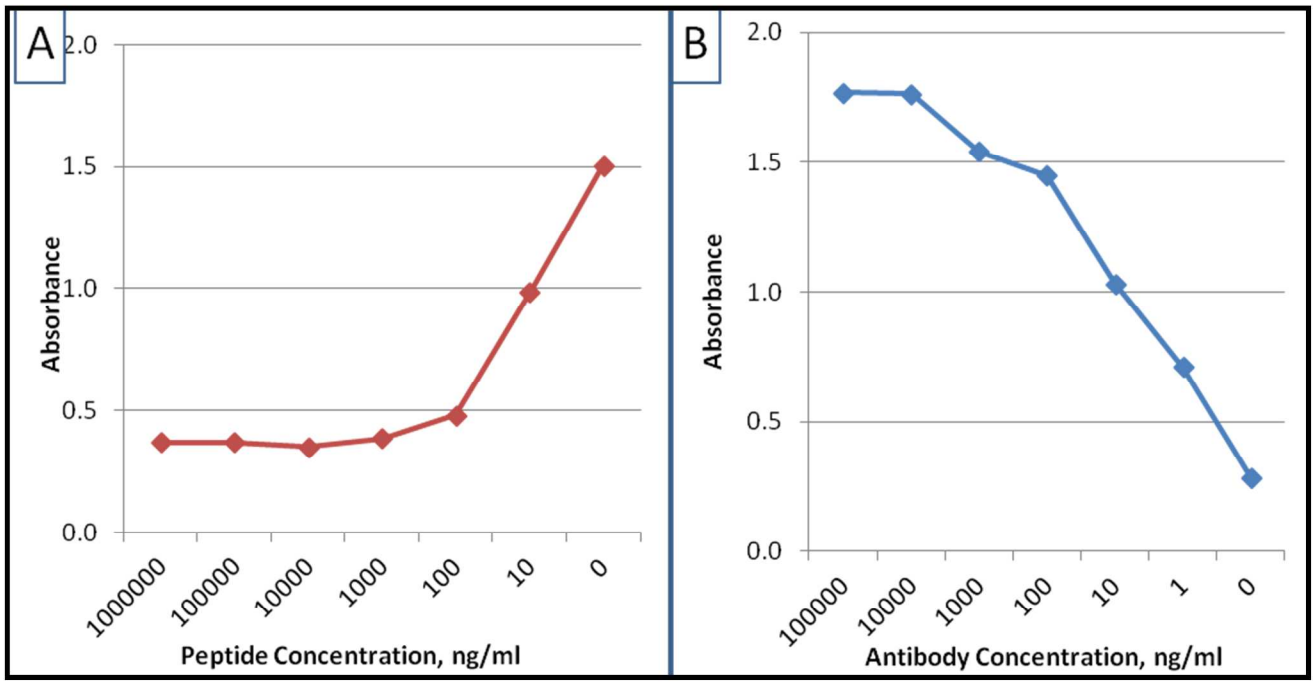

Figure S6. A Competitive ELISA binding studies for LHP vs anti-LHP in the presence of AuNP-LHP; B. Binding of LHP vs anti-LHP (serves as a control). Results shown are true representative of at least 3 independent experiments. Data points are given as mean absorbance of 3 independent experiments.

Table S1. Relative elemental compositions and probable peak assignments for carbon species (C 1S region) of AuNP-LHP as determined by XPS [Atom %]

Construct	C	N	O	F	Na	Si*	S	Au	C-C, C-H	C-O, C-N	O-C=O
AuNP-LHP	63	6.2	21	0.5	1.0	2.3	0.9	4.5	44	45	11

*Silicon detected due to incomplete coverage of the silicon wafer with the sample.

Table S2: Direct ELISA binding assay plate map for LHP against anti-LHP and anti-LH (sheep); LH (sheep) against anti-LHP and anti-LH; AuNP-LHP against anti-LHP and anti-LH (sheep). Antibody was added in a serial fold dilution from 10 to 10,000 folds.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B				LHP 400ng Vs anti-LHP 1:10	LHP 400ng Vs anti-LH 1:10	LH 400ng Vs anti-LHP 1:10	LH 400ng Vs anti-LH 1:10	AuNP-LHP 400ng Vs anti-LHP 1:10	AuNP-LHP 400ng Vs anti-LH 1:10			
C				LHP 400ng Vs anti-LHP 1:100	LHP 400ng Vs anti-LH 1:100	LH 400ng Vs anti-LHP 1:100	LH 400ng Vs anti-LH 1:100	AuNP-LHP 400ng Vs anti-LHP 1:100	AuNP-LHP 400ng Vs an-ti-LH 1:100			
D				LHP 400ng Vs anti-LHP 1:1000	LHP 400ng Vs anti-LH 1:1000	LH 400ng Vs anti-LHP 1:1000	LH 400ng Vs anti-LH 1:1000	AuNP-LHP 400ng Vs anti-LHP 1:1000	AuNP-LHP 400ng Vs anti-LH 1:1000			
E				LHP 400ng Vs anti-LHP 1:10000	LHP 400ng Vs an-ti-LH 1:10000	LH 400ng Vs anti-LHP 1:10000	LH 400ng Vs anti-LH 1:10000	AuNP-LHP 400ng Vs anti-LHP 1:10000	AuNP-LHP 400ng Vs an-ti-LH 1:10000			
F				LHP 400ng H2O	LHP 400ng H2O	LH 400ng H2O	LH 400ng H2O	AuNP-LHP 400ng H2O	AuNP-LHP 400ng H2O			

Table S3: Plate map of Competitive ELISA binding assays.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Coat LHP; Sample Rb-Anti-LHP 1mg/mL	Coat LHP; Sample Rb-Anti-LHP 1ug/mL + AuNP-LHP 1mg/mL										
B	Coat LHP; Sample Rb-Anti-LHP 100ug/mL	Coat LHP; Sample Rb-Anti-LHP 1ug/mL + AuNP-LHP 100ug/mL										
C	Coat LHP; Sample Rb-Anti-LHP 10ug/mL	Coat LHP; Sample Rb-Anti-LHP 1ug/mL + AuNP-LHP 10ug/mL										
D	Coat LHP; Sample Rb-Anti-LHP 1ug/mL	Coat LHP; Sample Rb-Anti-LHP 1ug/mL + AuNP-LHP 1ug/mL										
E	Coat LHP; Sample Rb-Anti-LHP 100ng/mL	Coat LHP; Sample Rb-Anti-LHP 1ug/mL + AuNP-LHP 100ng/mL										
F	Coat LHP; Sample Rb-Anti-LHP 10ng/mL	Coat LHP; Sample Rb-Anti-LHP 1ug/mL + AuNP-LHP 10ng/mL										
G	Coat LHP; Sample Rb-Anti-LHP 1ng/mL	Coat LHP; Sample Rb-Anti-LHP 1ug/mL + AuNP-LHP 1ng/mL										
H	Blank	Blank										

References:

(1) Chanda, N.; Kattumuri, V.; Shukla, R.; Zambre, A.; Katti, K.; Upendran, A.; Kulkarni, R. R.; Kan, P.; Fent, G. M.; Casteel, S. W.; Smith, C. J.; Boote, E.; Robertson, J. D.; Cutler, C.; Lever, J. R.; Katti, K. V.; Kannan, R. *Proc Natl Acad Sci U S A* **2010**, *107*, 8760.

(2) *Handbook of X-ray Photoelectron Spectroscopy*; Moulder, J. F.; Stickle, W. F.; Sobol, P. E.; Bomben, K. D.; Chastin., J.; King, J., R. C. , Eds.; Physical Electronics, Inc. Eden Prairie, MN, 1995.

(3) Patra, C. R.; Bhattacharya, R.; Wang, E.; Katarya, A.; Lau, J. S.; Dutta, S.; Muders, M.; Wang, S.; Buhrow, S. A.; Safgren, S. L.; Yaszemski, M. J.; Reid, J. M.; Ames, M. M.; Mukherjee, P.; Mukhopadhyay, D. *Cancer Res* **2008**, *68*, 1970.

(4) Abraham, A., Mihaliuk, E., Kumar, B., Legleiter, J., Gullion, T. *J. Phys. Chem., C* **2010**, *114*, 18109.

(5) Mocanua, A., Cernica, l., Tomoaiac,G., Bobosa, L. D., Horovitz, O., Tomoaia-Cotisel, M. *Colloids and surfaces. A: Physicochem. Eng. Aspects* **2009**, *338*, 93.