Bioconjugation of AuNPs with HPV 16/18 E6 antibody through physical adsorption technique

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Abstract

Gold nanoparticle (AuNP) bioconjugates are increasingly being utilised in biomedicine due to their low toxicity on biological tissues and unique electronic and chemical properties. They have been utilised in several biological applications namely manufacture of nanomaterials, biosensing, electron microscopy and drug delivery systems. Particularly, immuno-assays often employ gold nanoparticles (AuNPs) to enhance detection of a biological component. This paper presents a study on the bioconjugation of AuNPs with an antibody against an oncoprotein that is overexpressed in cervical carcinoma progression through physical adsorption. The study successfully developed a HPV 16 E6/18 E6-HRP (CP15)-AuNPs bioconjugate. The study also demonstrated that the antibody pI, gold colloidal solution pH and amount of antibody determine the generation of stable Antibody–AuNPs bioconjugates. These results further demonstrate that physical adsorption can be used to produce functional Antibody–AuNPs that could be employed in diagnostic immunoassays.

Introduction

HPV 16/18 E6 oncoprotein has been evaluated as a useful biomarker with prognostic abilities as it can detect pre-cancer and cancerous states of cervical cancer progression (1). This is endorsed by results obtained from a collaborative pilot clinical study conducted by Arbor Vita Corporation, PATH, and the Chinese Academy of Sciences that showed a correlation of E6 oncoprotein positivity with both severity of cervical histopathology and risk for progression into invasive carcinoma using the AVantage HPV 16/18/45/31/33/52/58 E6 lateral flow visual rapid test (2)

Arbor Vita corporation in another study has developed an E6 rapid immunoassay that detects protein from human cells using a PDZ domain protein that binds all high-risk HPV-E6 proteins and monoclonal antibodies against E6 subtypes. It's able to detect E6 protein in about 60000 cervical cancer cells. From a conference proceeding in 2011, the Arbor Vita Corporation's OncoE6 (HPV 16/18) test showed 99.0% specificity and 69.6% sensitivity for HSIL (CIN3+) precancerous lesions (3). In China, a HPV16/18/45 E6 test produced sensitivity and specificity of CIN2+ as 42.8% and 94.3% respectively and for CIN3+ as 54.2% and 93.8% respectively (4,5).

A positive E6 assay, indicates high correlation to the cervical cancerous phenotype, not the potential for cervical cancer, thus high specificity in triaging patients during screening (6–8). E6 levels of expression associate directly with severity of CIN lesions and risk of progression to invasive carcinoma (9).

Although, the current interventions utilizing the E6 oncoprotein from high risk HPV strains have high specificity, the sensitivity is relatively low. Arbor Vita Corporation's OncoE6 test showed 69.6% sensitivity for HSIL (CIN3+) precancerous lesions (3). HPV16/18/45 E6 test produced sensitivity and specificity of CIN2+ as 42.8% and 94.3% respectively and for CIN3+ as 54.2% and

93.8% respectively (4,5). More advanced methods of detecting E6 oncoprotein are therefore required to produce a sensitivity between 76.3 %-97.2% which is indicative of diagnostic test quality (10)

Nanobiotechnology is being used in cancer diagnostics to enhance detection (11). It uses nanosized tools and systems in the study of biological phenomena for example in prevention (nanovaccines), diagnosis (in vivo and in vitro diagnostics) and in treatment (drug delivery) (12). The types of nanomaterials include metal based (gold (Au) and silver (Ag) nanoparticles), oxides (super paramagnetic iron oxide (SPIO) nanoparticles, silver (Ag) oxide nanoparticles), liposomes, dendrimers, quantum dots, nanosphere, carbon nanotube, nanofibers, nanolayers like graphene sheets, biopolymers among others (13).

Early cervical cancer diagnostics has been improved by application of nanotechnologies (16). For example; the ultra-bright fluorescent mesoporous silica nanoparticle to detect folate (Fa) receptors on cervical cells surface. The brighter the fluorescence, the more advanced the cervical carcinoma (17). Secondly, monodispersed inorganic silica nanoparticles in organic dyes detect HR-HPV DNA using DNA microarrays (18). Thirdly, in detection of proteins for example the piezoelectric (contains quartz crystals) immuno-sensor that detects protein $p16^{INK4a}$ for early cervical cancer detection (19).

This study developed a bioconjugate of gold nanoparticles with HPV 16 E6/18 E6-HRP (C1P5) antibody that can be utilised to sensitively detect E6 oncoprotein an early biomarker in cervical cancer screening.

Materials and methods

Bioconjugation preparation

The pH of 500 µl mouse IgG_1 HPV 16 E6/18 E6-HRP (CP15) monoclonal antibodies (Santa Cruz Biotechnologies, USA Cat# sc-460) was determined using a pH Meter. The pH of 1000 µl 20nm colloidal spherical monodispersed citrate stabilized gold nanoparticles (Sigma Aldrich) was adjusted to a pH unit of 0.5 higher than the iso-electric point (IEP) of the antibody molecule to be adsorbed using 20 mM borate buffer pH 8.7. Using Jenway UV- VIS Spectrophotometer Model 6800, the lambda maximum of the colloidal gold was measured before bioconjugation.

Bioconjugation optimization

A bioconjugation optimization protocol was carried out to determine the amount of antibody needed for maximum bioconjugation. This was accomplished by diluting 200 µg/ml HPV 16 E6/18 E6-HRP (CP15) antibodies in 1X PBS buffer pH 7.4 (Beijing Solarbio Science & Technology Co. Ltd) a range of 0mg/ml to 0.012mg/ml Table 1. To each dilution 100 µl of the gold solution was added. This mixture was incubated for 2 hours to allow for passive adsorption (20). The absorption spectra of each mixture was then determined to confirm the concentration at which bioconjugation took place using Jenway Model 6800 UV- VIS Spectrophotometer (21).

Table 1. Showing dilutions of HPV 16 E6/18 E6-HRP (CP15) Monoclonal antibodies with 1X PBS buffer

Concentration of antibody (µg/ml)	Volume of antibody Stock solution (µl)	Volume of PBS Buffer (µl)	Final volume (µl)
0	0	1000	1000
0.3	1.5	998.5	1000
0.6	3	997	1000
0.9	4.5	995.5	1000
1.2	6	994	1000
2.0	10	990	1000
4.0	20	980	1000
6.0	30	970	1000
8.0	40	960	1000
10.0	50	950	1000
12.0	60	940	1000

The dilutions of HPV 16 E6/18 E6-HRP (CP15) Monoclonal antibodies was determined using: $C_1V_1=C_2V_2$

C1- Concentration of Stock/initial Solution

V1- Volume of stock/initial solution

C2- Concentration of final Solution

V2- Volume of final solution

Bioconjugation confirmation using Spectrophotometer

The cuvette was then filled with the sample, surfaces rinsed and placed in the spectrophotometer. The absorbance spectrum was obtained for all dilutions through a scan within the standard range of wavelengths 400-900nm. From the collected absorbance spectrum, the absorbance maximum (λ_{max}) was determined.

Verification of Bioconjugation

Bioconjugation procedure was repeated at 4.0 µg/ml antibody concentration to verify a shift in lambda max at this concentration of antibody. Bioconjugation was confirmed by determining the absorption spectrum using spectrophotometry, to determine the lambda maximum shift.

Preparation of HPV 16 E6/18 E6-HRP (CP15)-AuNPS conjugate for downstream processes

About 100 µl of AuNPs was added into an Eppendorf tube, the pH was adjusted to pH 7 using 1X PBS at pH 7.4. 200 µg/ml of antibody was diluted to 4µg/ml by adding 20 µl of stock to 980 µl 1X PBS buffer to make 4 µg/ml concentration of the antibody. 900 µl of the antibody concentration obtained above, was added to the 100 µl AuNPs to make a final solution of 1000 µl. This was incubated for 2 hours at 4°C to allow passive adsorption. The solution was centrifuged at 2500rpm for 1 minute to remove unbound antibody and supernatant discarded. The pelleted HPV 16 E6/18 E6-HRP (CP15) -AuNPS conjugate is resuspended in 1X PBS buffer pH 7.4. The centrifugation was repeated and the pelleted conjugate stored undiluted at 4°C. It can be stored for up to 12-18months.

Results

The pH of 500µl HPV 16 E6/18 E6-HRP (CP15) antibody was obtained as pH 7.05. The pH of 1000 µl of colloidal gold solution was adjusted to pH 7.83 a pH unit of about 0.5 higher than the iso-electric point (IEP) of the antibody molecule using 20 mM borate buffer pH 8.7. The maximum absorption for the 20 nm, 0D 1 gold nanoparticles with a concentration of 6.54 x 10^{11} particles/mL, a peak SPR wavelength of 518-524 nm and extinction coefficient, ε of 9.21 x 10^{8} (M^{-1} cm^{-1} before conjugation was at 523nm as shown on Fig 1.

Fig 1. Absorption Spectra of Gold Nanoparticles before conjugation using Jenway Model 6800 UV- VIS Spectrophotometer.

After two hours incubation, the Lambda max of 0mg/ml to 0.012mg/ml range of antibody solutions were obtained as outlined in Table 2.

Table 2. Showing Absorption Spectra of AuNPs after 2hour incubation of different concentrations of HPV 16 E6/18 E6-HRP mAbs with 100µl of AuNPs using Jenway Model 6800 UV-VIS Spectrophotometer.

HPV 16 E6/18 E6-HRP antibody concentration (µg/ml)	Absorption Maximum after 2 hours incubation
0	523.0
0.3	519.5
0.6	520.0
0.9	520.5
1.2	521.0
2.0	522.0
4.0	523.5
6.0	522.0
8.0	522.0
10.0	521.0
12.0	521.5

From these absorption spectra, a red shift of lambda maximum by 0.5nm was observed at antibody concentration 4.0µg/ml from the initial 523.0 nm. This is further illustrated in Fig 2.

Fig 2. Absorption Spectra of Gold Nanoparticles after conjugation with 4.0µg/ml concentration of antibody using Jenway Model 6800 UV- VIS Spectrophotometer

Verification of Bioconjugation

A lambda maximum of 527nm was obtained after verification and further optimization of bioconjugation protocol at 4.0µg/ml concentration of HPV 16 E6/18 E6 (C1P5)–HRP antibody. The spectra obtained is as illustrated in Fig 3.

Fig 3. Absorption Spectra of Gold Nanoparticles after conjugation with 4.0 µg/ml antibody concentration using Jenway Model 6800 UV- VIS Spectrophotometer

Discussion

Bioconjugation occurred upon successful binding of the monoclonal antibodies to the AuNPs surface, causing the localized surface plasmon resonance (LSPR) spectrum to red-shift by a few nanometers but within 400nm-900nm (22). The red shift indicated in this study was 0.5nm. After further verification and optimization, the red shift recorded was 4nm. A red shift indicates an increase in wavelength. This is attributed to the fact that the antibody replaces the negative charge citrate capping on the surface of citrate stabilized colloidal gold nanoparticles. This involved both electrostatic and hydrophobic interactions involved in passive adsorption as in Fig 4 (20).

Fig 4. Showing bioconjugate: HPV 16 E6/18 E6-HRP (CP15)-AuNPs

It comprises 20nm citrate stabilized monodispersed spherical AuNPs passively adsorbed to horseradish peroxidase (HRP) enzyme labelled HPV 16/18 E6 (C1P5) mouse monoclonal antibodies through (A) electrostatic and (B) hydrophobic interactions

To ensure optimal bioconjugation, there were critical steps involved. Firstly, there was adjustment of the pH of colloidal gold solution to pH 7.83, a pH unit of about 0.5 higher than the iso-electric point (IEP) of the antibody molecule to be adsorbed whose isoelectric point is about pH 7.00 (23). This is critical because hydrophobic interactions are maximal at IEP of antibody hence maximal bioconjugation was achieved (24,25).

Secondly, the concentration of antibody needed was optimized through titrations of the antibody as too little antibody adsorbed to the gold surface, will cause aggregation upon addition of electrolytes present in standard buffers. Bioconjugation occurred at 4.0µg/ml HPV 16 E6/18 E6-HRP (CP15) antibody concentration**.** The bioconjugate formed was prepared and pelleted conjugate stored undiluted at 4ºC for use in E6 oncoprotein biomarker detection in cervical cancer screening. It can be stored for 12-18months.

The absorption spectrum obtained after verification was fairly smooth (Figure 3) due to possible antibody contamination with small contaminants such as sodium azide. Dialysing the antibody with a buffer solution is highly recommended in subsequent analysis. It could also arise from contamination of gold colloidal solution during pipetting hence aliquoting the gold solution during use is to be maintained. Despite the challenges, passive adsorption of gold nanoparticles to antibody was achieved, and testing the antibody sensitivity on E6 protein will further verify the findings.

Early cervical cancer diagnostics have been improved by application of nanotechnologies (16). Particularly, immuno-assays often employ gold nanoparticles (AuNPs) to enhance detection of a biological component. Gold nanoparticle (AuNP) bioconjugates are increasingly being utilised in biomedicine due to their low toxicity on biological tissues and unique electronic and chemical properties such as unique surface characteristics, optical properties and consistency (26). The Bioconjugate herein, uses an antibody conjugated to HRP thus conjugation to AuNPs will further enhance visual enzyme linked immunosorbent assay (ELISA) based colorimetric detection of the E6 oncoprotein analyte (27).

Conclusion

It was possible to conjugate mAbs against HPV 16/18 E6 oncoprotein to 20nm citrate stabilized gold nanoparticles through passive adsorption. The bioconjugate formed was HPV 16 E6/18 E6-HRP (CP15)-AuNPs. Currently, there is no report on the development a gold nanoparticle bioconjugate with mAbs against the E6 protein of HPV 16/18 to enhance the signal of the immunoassay involving the E6 protein and the antibody thereof. This HPV 16 E6/18 E6-HRP (CP15)-AuNPs conjugate can be utilized in immunoassays for further verification of the bioconjugate's bio-functionality.

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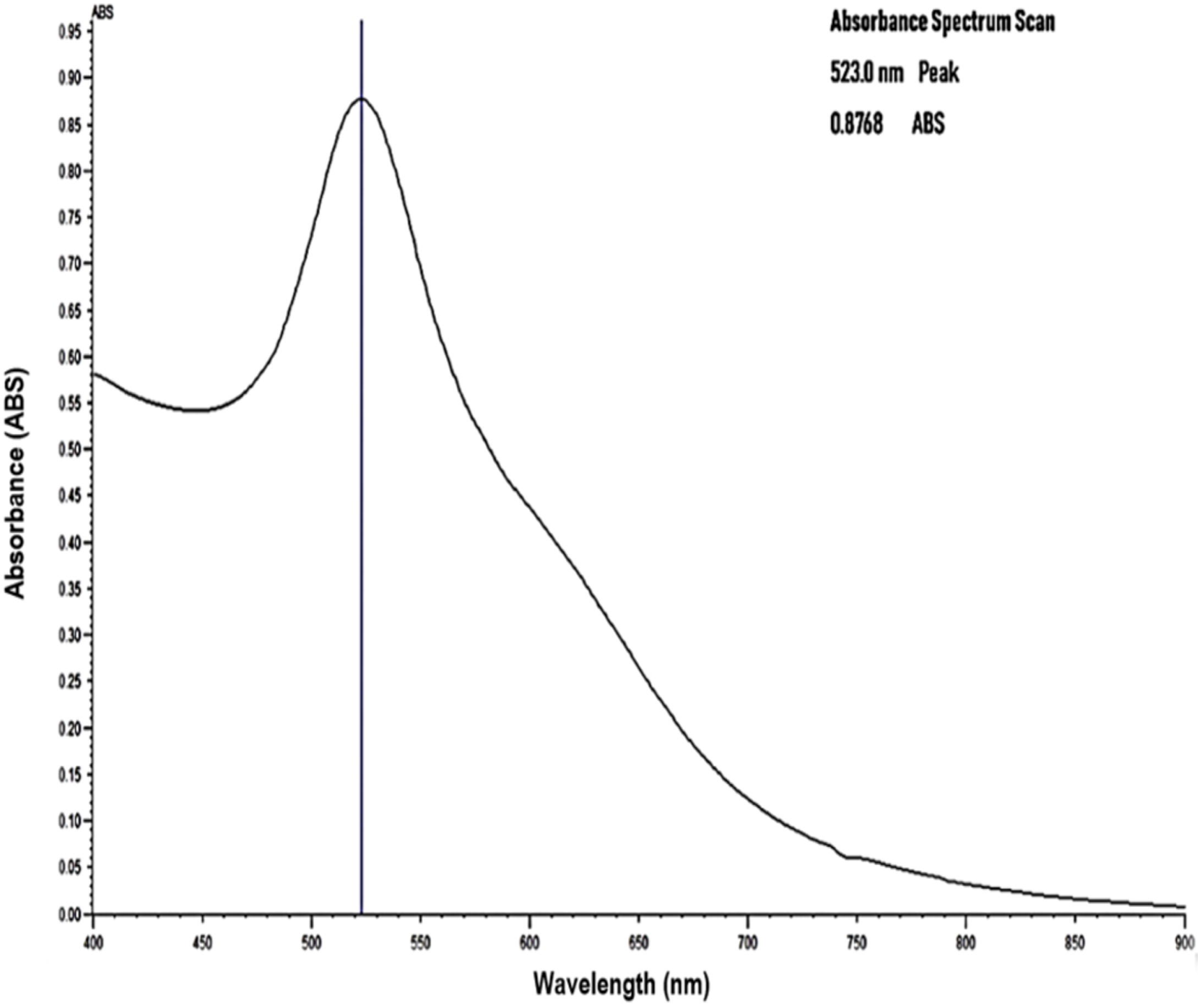
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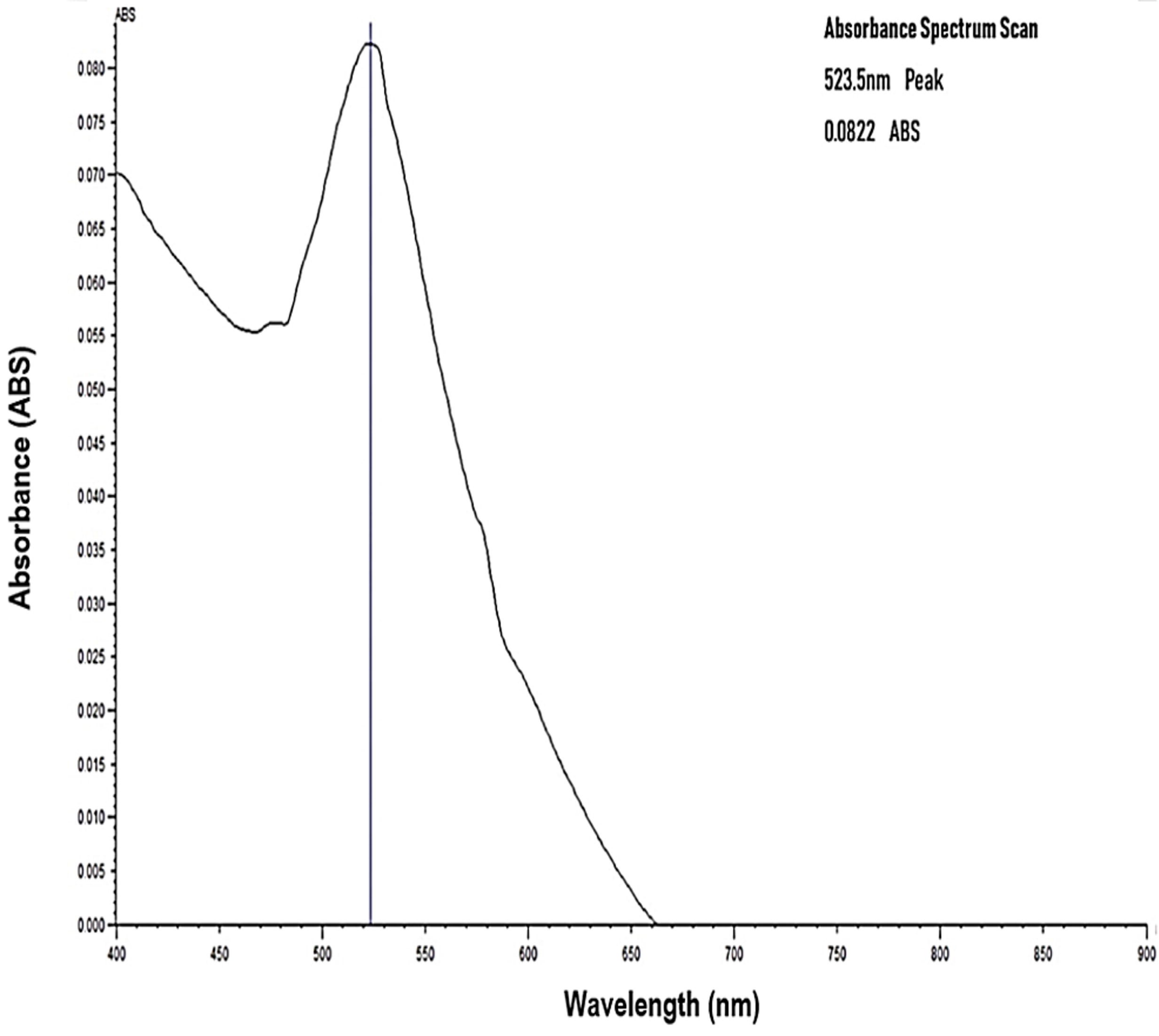
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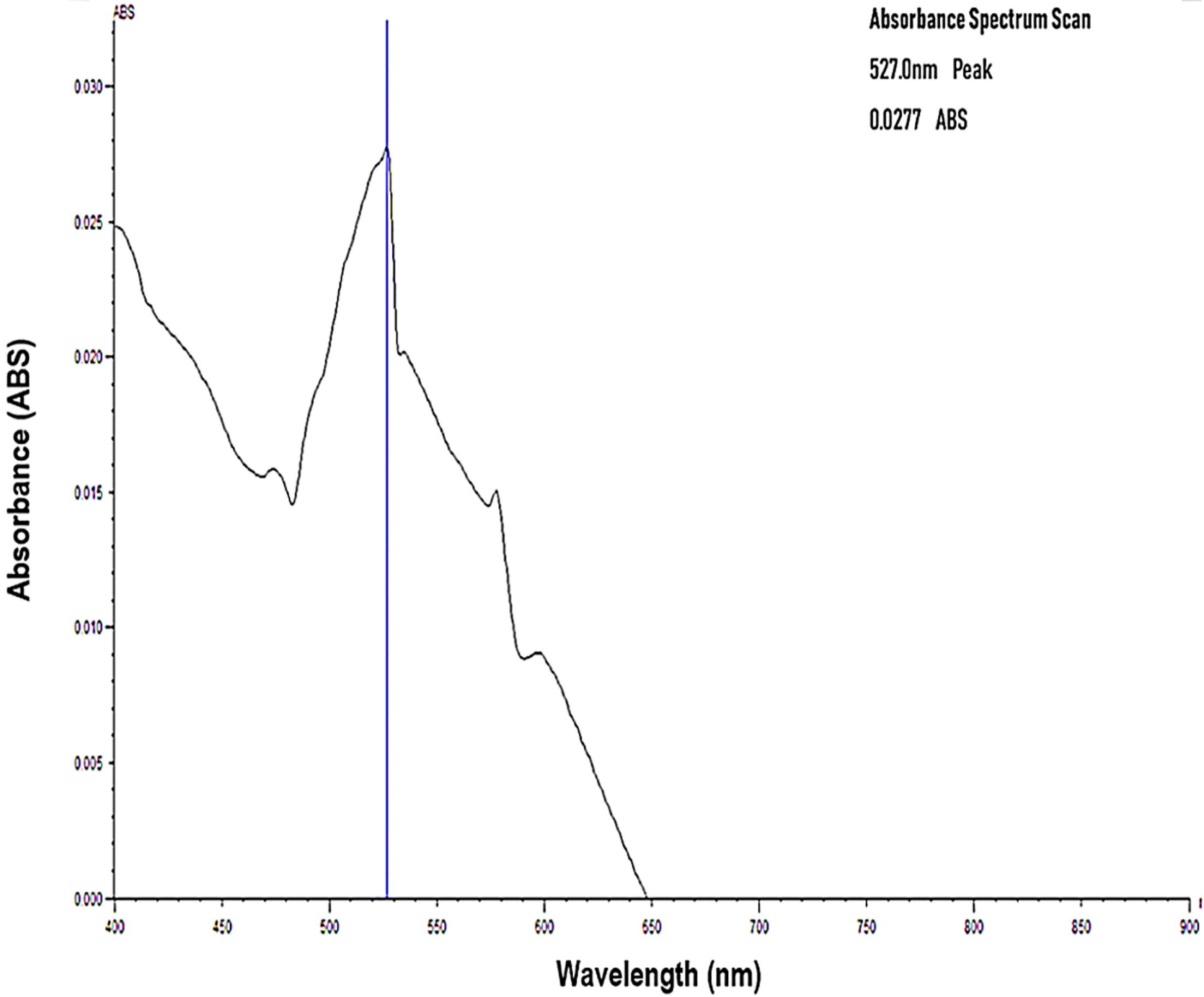
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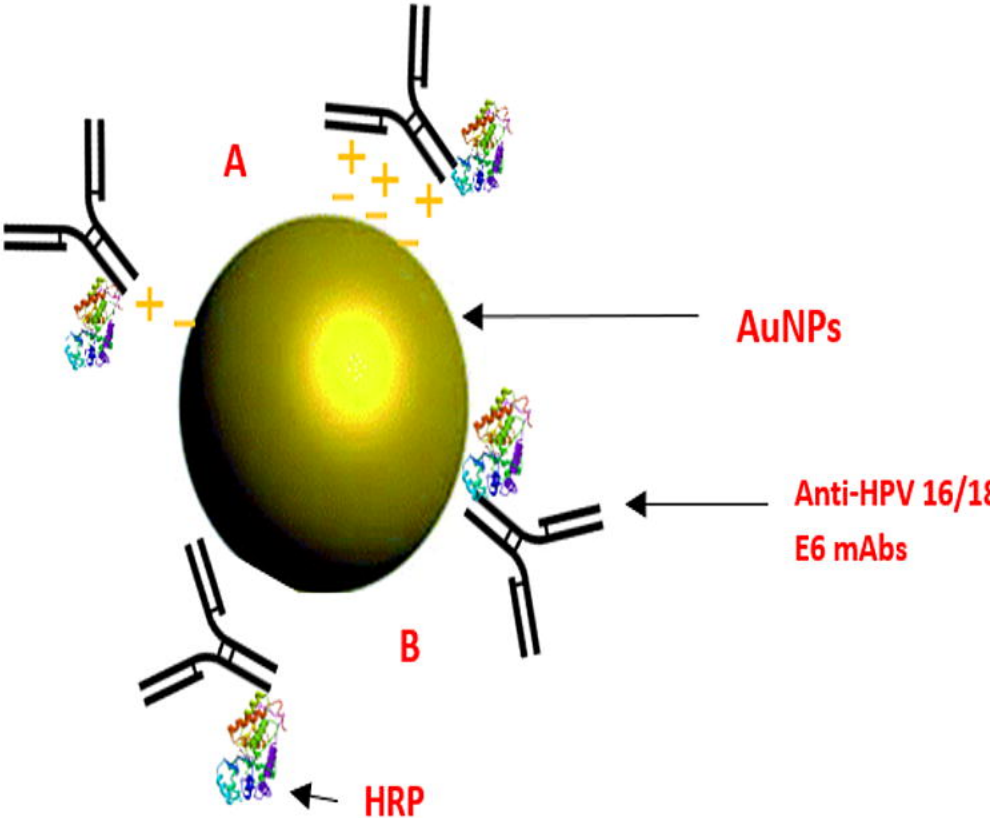
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