Streptavidin conjugated ZnO nanoparticles for early detection of HIV infection

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Received: 23 March 2016, Revised: 19 August 2016 and Accepted: 13 October 2016

DOI: 10.5185/amlett.2017.6579
www.vbripress.com/aml

Abstract

Streptavidin labelled fluorescent ZnO nanoparticles have been surface engineered to develop a fluorescent ZnO nanoparticle linked immunoassay (FZLIA) for the sensitive detection of HIV infection. ZnO nanoparticles were synthesized by a single step chemical precipitation method. Cysteine was used to graft carboxyl groups on to the surface of nanoparticles in a single step. Cysteine capped ZnO nanoparticles exhibited fluorescence at 546 nm when excited with 358 nm and FESEM confirmed the particle size to be 50-70 nm. FTIR and TGA confirmed the functionalisation of carboxyl groups by cysteine. The amount of cysteine grafted on the ZnO nanoparticles calculated as 68.1% from TGA analysis indicated the presence of large amount of carboxyl groups. ZnO nanoparticles were conjugated to streptavidin and the same were deployed as fluorescent probes in the development of the FZLIA platform for the early and accurate detection of HIV infection. The linear dose dependent detection range was from 25 pg/mL to 1000 pg/mL. HIV positive and HIV negative plasma samples were tested using FZLIA for the presence of HIV-1 p24 antigen. This immunoassay exhibited no false positive and false negative results with the clinical samples tested. This highly sensitive HIV-1 p24 antigen assay may be useful to improve blood safety by reducing the antibody negative window period in blood donors in resource limited settings where nucleic acid testing is not practical or feasible. This technology can be transferred to a lab-on-chip platform for use in resource limited settings and can also be easily adopted for the detection of other antigens.

Keywords: ZnO nanoparticles, fluorescence, immunoassay, HIV-1, p24 antigen, streptavidin.

Introduction

Infectious diseases are the major causes of death in children and one of the leading causes of deaths in adults [**1**]. Although a wide range of diseases such as malaria, tuberculosis, respiratory infections prevail, a major disease which has alarmed the world is the acquired immune deficiency syndrome (AIDS). AIDS is a set of symptoms caused by immunodeficiency virus (HIV) which kills or impairs cells of the immune system and progressively destroys the body's ability to fight infections and certain cancers. HIV has infected 36.9 million people globally and does not have complete cute at present [**2**]. With no cure at present, prudence can save thousands of people who have yet to be exposed to the virus. The antiretrovirals (ARVs) and their use in combination, “cocktails,” have dramatically reduced mortality and morbidity and improved the lives of sufferers. However, 95% of people with HIV/AIDS live in developing countries, where access to these medicines remains unacceptably limited and the early diagnosis is unimaginable [**3**]. Early recognition and diagnosis of acute HIV-1 infection can provide important benefits at the individual level. The potential for early initiation of treatment may allow for preserved immune-system, control of the virus, and at the public health level because the risk of transmission may be reduced [**4**] Accurate and early detection of recent HIV infection is critical for therapy and preventing new infections. However, diagnostic tools for an early and accurate detection have not yet been developed and remain an unmet public health need.

Thus, the situation highlights the need for efficient, ultrasensitive and inexpensive diagnostic kits easily deployable in any part of the world which would prevent its further transmission to healthy, at-risk individuals and also for the timely intervention of treatment. p24 is HIV viral caspid protein which is detectable in blood earlier than HIV antibodies during infection. Many HIV antigen assays measure p24 because of its abundance in the early stages of infection which is due to the initial burst of virus replication and associated with high levels of viremia

during which the individual is highly infectious [**5**]. However, when antibodies to HIV become detectable, however, p24 antigen is often no longer demonstrable, most likely due to antigen-antibody complexing in the blood [**6**]. When detected, p24 antigen is highly specific for infection. Testing for p24 can be of high value in detecting early HIV infection, screening blood, diagnosing infection in the newborn and monitoring antiviral therapy [**7**]. p24 antigen is found in serum in either free form or bound by anti-p24 antibody. Free p24 can be measured with enzyme immunoassays whereas detection of bound p24 requires pretreatment with an acid to dissociate the complex. Procedures to dissociate antigen-antibody complexes have improved the sensitivity of the p24 test [**8**, **9**]. Antibody remains detectable throughout infection, whereas p24 antigen characteristically appears early and late during infection.

Immunoassays are widely used high-throughput diagnostic tools across the globe for screening various diseases. Among them ELISA is the most commonly used technique for the detection of various biomolecules and pathogens. This technique has been widely applied for HIV detection due to its simplicity. Even though these conventional ELISA detection methods are available with 99% sensitivity they mainly rely upon the HIV antibodies detection [**10**]. These antibodies based tests are not of much use for the early diagnosis of the individual since it takes up to 3 months for the antibodies to become detectable in the plasma [**11**]. But measuring the antigen i.e. p24 antigen can directly tackle the problem for early diagnosis. Also current ELISA technique can detect only up to 15pg/mL while TSA-mediated signal amplification-boosted ELISA can detect up to 5 pg/mL [**12**]. Their sensitivity is always limited by affinity, nonspecific binding and activity of the label. For fluorescence based immunoassays the sensitivity is also limited by background to signal ratio due to their high fluorescence yield. Sandwich-type immuno-sensors and immunoassay methods were developed for detection of antigen with more than one epitope due to the use of matched antibodies. High-affinity antibodies and appropriate labels were usually employed for the amplification of detectable signal [**13**].

The recent advances in nanotechnology and nanomaterials have been integrated into analytical chemistry for the design of large numbers of fluorescent chemical and biological probes. Fluorescent nanomaterials extended their applicability to numerous fields including environmental and life sciences due to their unique optical properties such as narrow emission bands and other advantages over traditional organic fluorophores [**14**]. Fluorescent nanoparticles are gradually replacing traditional organic fluorophores to function as simple fluorescent reporters in many areas such as immunoassays [**15**], microarrays, fluorescent imaging applications [**16**], sensing [**17**] and other sensor platforms. When combined with techniques such as microfluidics and fluorescence spectroscopy, the detection limit can be further enhanced to very low level and possibly to single molecule level. Semiconducting fluorescent nanoparticles with their unique physical and chemical properties are ideal for bio-diagnostic applications. Nanoparticles, in particular semiconducting nanoparticles with their high intensity optical emission, the cost effective, fast and reliable synthesis and simple functionalisation procedures are key features for their application in the field of biosensors and bio-imaging and drug delivery applications [**18**, **19**]. They pave way for improvements in sensitivity, selectivity and multiplexing capacity over conventional fluorophores.

ZnO nanoparticles are highly attractive for biomedical applications because of their low cytotoxicity, inexpensive nature and good stability against air and sunlight. Because of their large band gap and large excitation binding energy, ZnO nanoparticles can also be used for new applications in bio-imaging after careful surface modifications [**20**]. However, synthesis of water soluble fluorescent ZnO nanoparticles is still a challenge for researchers. It is mainly because in ZnO nanoparticles the visible fluorescence is caused by transitions involving trapped levels which are the result of surface oxygen vacancies. These defects can be destroyed by water molecules surrounding the ZnO nanoparticles. Apart from the defects, even the surface morphology and size of ZnO nanoparticles influence the luminescent property of ZnO nanoparticles [**21**]. Passivation with ligands or proper surface modifications are necessary to reduce the surface trap densities which may cause enhancement in the quantum yield and also increase in photo stability. The high surface to volume ratio of ZnO nanoparticles results in more surface defects which play an important role in its luminescence for various applications [**22**].

Present work is aimed at developing a rapid nanoparticle based sandwich immunoassay assay for accurate detection of HIV infection at an early stage. In an attempt to exploit the potential applications of the highly fluorescent nanoparticles, fluorescent ZnO nanoparticles have been surface engineered with streptavidin to develop a fluorescent ZnO nanoparticle linked immunoassay. Fluorescent ZnO nanoparticles were synthesised by chemical precipitation method and the carboxyl groups were grafted with cysteine on ZnO surface in a single step. EDC/NHS method was employed for the bio-conjugation of streptavidin with carboxylated fluorescent ZnO nanoparticles. These surfaces engineered streptavidin labelled fluorescent ZnO nanoparticles were deployed for the early and accurate detection of HIV-1 p24 antigen by fluorescence immunoassay. FZLIA exhibited a linear detection range between 25 to 1000 pg/mL in a linear dose dependent manner. The sensitivity of the FZLIA developed was enhanced over conventional ELISA. The specificity of the immunoassay was 100% when tested with plasma samples that were negative for HIV-1 p24 antigen and those infected with other viruses such as HBV and HCV. The study results presented in this report may be useful for people in resource limited settings for the early diagnosis of HIV where nucleic acid testing is not feasible. FZLIA transferred to lab on chip platform can pave the way for new methodologies for the simultaneous detection of multiple antigens.

Experimental

Materials

Lithium hydroxide ($LiOH.H_2O$, 99%), zinc acetate (99% pure), L-cysteine (97% pure) were purchased from Sigma-Aldrich, USA. Absolute Ethanol (99.9%) was supplied by S D FINE Chemicals, India. MicroFluor black color plates (cat. No. 7605), Caesin Block buffer (1% (w/v)) and wash buffer (0.1 % tween-20 in PBS, PBST) were supplied by Thermo Scientific, USA. Phosphate buffer saline (PBS; 100 mM, pH=7.2), Carbonate – bicarbonate buffer (500 mM, pH 9.5) were prepared in the lab by following established procedures. Double distilled water was used for all the experiments. HIV-1 p24 antigen (Virogen, USA), C65690M anti-p24 antibody from mouse (Prospec, USA) and biotinylated HIV-1 detector antibody (Perkin – Elmer, USA) were purchased. Streptavidin was supplied by Scripps labs, USA. All the chemicals were used as received without any further purification.

Clinical samples

Sri Sathya Sai Institute of Higher Medical Sciences, Prasanthigram, India has provided all the clinical samples (HIV positive, HIV negative, HBV and HCV) required for the study after due approval from the Institutional Ethics Committee. All the samples were tested for HIV by both third generation Microlisa-HIV Microwell ELISA kit (J.Mitra &Co Pvt ltd, India) and fourth generation Microlisa HIV Ag^+ Ab Elisa kit (J.Mitra & Co Pvt Ltd, India).

Characterization

Fourier transform infrared (FTIR) spectroscopy

Functionalisation of cysteine to ZnO nanoparticles was confirmed by Fourier Transform Infrared (FTIR) spectroscopy and measurements were carried out by SHIMADZU IR Affinity-1 FTIR spectrophotometer. All the spectra were collected in transmission mode in the range of 400-4000 cm^{-1} with an accumulation of 32 scans and 2.0 cm resolution.

UV-vis absorption spectroscopy

Absorbance spectrum was obtained by using Shimadzu UV-2450 absorbance spectrophotometer. Samples were dispersed in alcohol and diluted to obtain the spectrum with absorbance below 0.35.

Photoluminescence spectroscopy

Photoluminescence excitation and emission spectra were recorded on Perkin Elmer LS 45 fluorescence spectrophotometer equipped with Xe lamp. Spectramax M5 spectrophotometer, plate reader was used to measure the fluorescence in the end point format for the detection of antigen. All the measurements were done in endpoint mode.

Field emission scanning electron microscopy

The morphology and size of the ZnO nanoparticles were investigated by Field Emission Scanning Electron Microscope (FESEM), using Zeiss Gemini Ultra 55 FESEM, operated at 5 kV. The samples were gold sputtered prior to microscopy.

Thermogravimetric analysis

Thermogravimetric analysis (TGA) of cysteine and cysteine functionalized ZnO nanoparticles was carried out in air with a heating rate of 10 K min^{-1} till 800 ^oC using Mettler Toledo, TGA/ DSC 1. The molar quantity of cysteine molecules grafted on to ZnO was calculated from weight loss percentage by the following equation.

$$\text{Molar quantity of cysteine} = \frac{\text{weight loss percentage of cysteine}}{\text{Molecular wt.of cysteine X 100}} \quad (1)$$

Therefore, the grafted cysteine ratio, which is the mol % of cysteine molecules immobilized on the surface of ZnO, can be expressed by the following equation

$$\text{Graft ratio \%} = \frac{\text{Molar quantity of cysteine X Weight of cysteine capped ZnO}}{1.23\ X\ 10^{-4}\ moles}\ X\ 100 \quad (2)$$

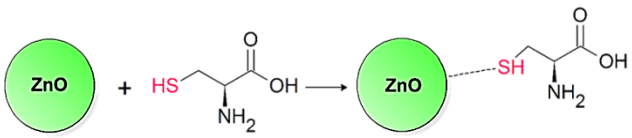
where $1.23x10^{-4}$ moles is the initial moles of added cysteine

Synthesis of ZnO nanoparticles

ZnO nanoparticles were synthesised by precipitation method in a single step [**23**]. Firstly, 220 mg of zinc acetate was dissolved in 100 mL of absolute ethanol with vigrous stirring at room temperature to obtain clear solution. 120 mg of Lithium hydroxide as precipitating agent was dissolved in 100 mL of absolute ethanol.This lithium hydroxide solution was added drop by drop to zinc acetate solution with vigrous stirring and the pH of the solution was maintained at pH 8. After 2 hours of the reaction the solution became turbid indicating the formation of ZnO nanoparticles. Finally, ZnO nanoparticles were washed four times with ethanol to remove unreacted compounds and finally dried under vaccum at 60 ^oC.

Functionalisation of ZnO nanoparticles with cysteine

To graft carboxyl groups on to the surface, 30 mg of ZnO nanoparticles were mixed with 15 mg of cysteine in 30mL of water. The reaction was continued for 2 hours with vigorous stirring. The mixture was washed 3 times with double distilled water and dried under vaccum at 60^oC. The cysteine functionalised ZnO particles obtained were successfully employed for the conjugation of streptavidin. The functionalisation is depicted in **scheme 1.**



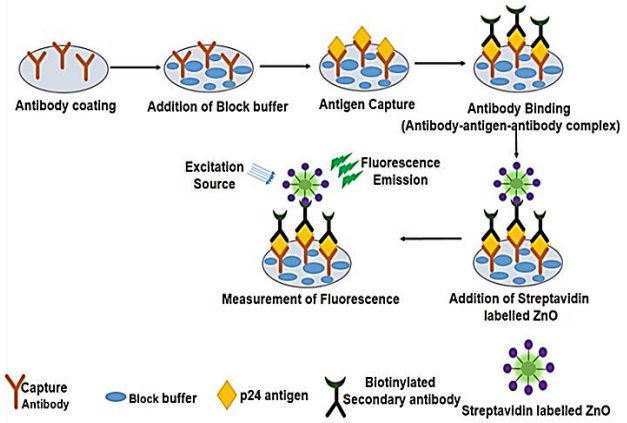
Scheme 1. Functionalisation of cysteine molecules to ZnO NPs.

Conjugation of streptavidin to carboxyl functionalised ZnO nanoparticles

The carboxyl groups present on the ZnO nanoparticles were covalently conjugated with primary amines of streptavidin using EDC/sulfo –NHS chemistry [**24**]. In the initial step, 20 mg of ZnO nanoparticles dispersed in 10 mM PBS were washed in a NanoSep centrifugal ultrafiltration device (MWCO 300 kDa, Pall Life Sciences, Ann Arbor, MI, USA). After washing, the carboxyl groups present on the ZnO nanoparticles were activated with 10 mM of EDC and 20 mM sulpho-NHS in PBS buffer for 30 min. After washing these activated particles with glycine buffer, 50 µL of 1 mg/mL streptavidin in the carbonate buffer (pH=9.0) solution was added. The mixture was incubated for 24 hours at room temperature followed by washing 5 times with glycine buffer. The resultant streptavidin coated ZnO nanoparticles were diluted to 0.1 mg/mL in PBS and stored at 4 ºC for immunoassay experiments.

Fluorescent ZnO linked immunosorbent assay (FZLIA) for p24 antigen detection

We adopted the sandwich immunoassay format shown in **Scheme 2** to capture p24 antigen.



Scheme 2. Depictions of the fluorescent ZnO nanoparticle linked immunosorbent assay (FZLIA).

Antibody coating and blocking

Capture antibody (Ab_1) was diluted to 2 µg/mL with carbonate-bicarbonate buffer (100 mM, pH 9.6). 55 µL of this diluted capture antibody was coated on to microplates and incubated for 24 hours at 4 ºC followed by washing 5 times with PBST after which 300 µL/well casein blocking buffer was added to each well and discarded. The excessive binding sites were blocked by caesin block buffer (250 µL/well) at 37 ºC for 30 minutes to avoid the nonspecific adsorption of antigen.

Antigen capture

Hundred microliters (100 µL) of various concentration of p24 antigen diluted with caesin block buffer/diluted plasma was added to each well and incubated at 37 ºC with shaking for 1 hour. All the wells were washed 3 times with PBST.

Antibody binding

1 µg/mL of detector antibody conjugated to biotin (Ab_2, 100 µL/well) was added and incubated with shaking for 1 hour at 37 ºC. The plates were drained and washed with PBST for 3 times. To this sandwich immunocomplex, 100 µL of Streptavidin conjugated ZnO nanoparticles (0.1 mg/mL) were added to each well and incubated with shaking for 30 minutes at 37 ºC followed by washing 5 times with PBST and tapped dry to reduce background noise.

Signal collection

The fluorescent signals from sandwich immunocomplexes were read in end point assay format (with exitation at 358 nm and emission at 546 nm) on a plate reader. All the experiments were performed in triplicates. The fluorescence intensity values were plotted against the concentration of p24 antigen.

We also applied this immunoassy to assess the recoveries of p24 antigen from clinical samples spiked with different concentrations of p24 antigen standard.

Results and discussion

Characterization of functionalized ZnO nanoparticles

In this study we synthesied green fluorescent ZnO particles and functionalised with cysteine for carboxyl groups. The functionalisation with cysteine offered both the aqueous solubility and the surface passivation required for the immunoassay application. FESEM analysis of cysteine functionalised ZnO nanoparticles confirmed the size of these nanoparticles to be between 50-70 nm **(Fig. 1)**. These nanoparticles were found to be spherical in shape. The carboxyl, thiol and amino functional groups present on ZnO nanoparticles could influence short range particle interactions.

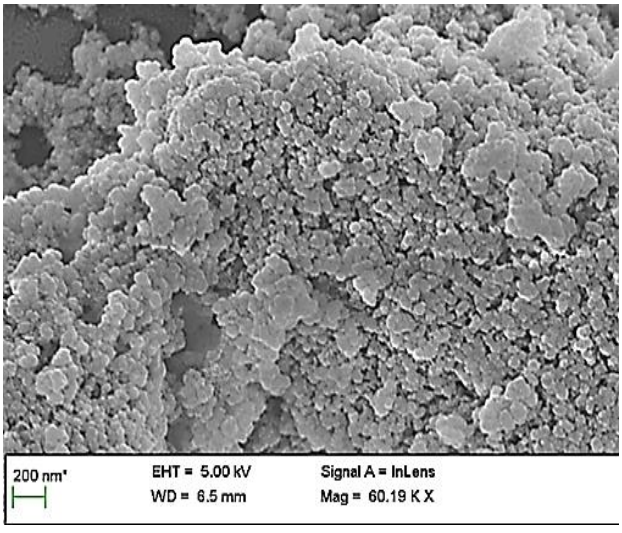


Fig. 1. FESEM image of cysteine functionalised ZnO nanoparticles.

The induced agglomeration seen in the image might be due to the intermolecular interaction caused mainly by hydrogen bonding among cysteine molecules.

Fig. 2 shows the UV-visible absorbance spectra of the cysteine-capped ZnO nanoparticles The absorption peak with maxima at 358 nm could be due to the surface plasmon absorption of ZnO nanoparticles. In metal oxide nanoparticles the surface plasmon absorption is caused when the incident electromagnetic radiation is absorbed for the collective oscilltion of free conduction band electrons [**25**]. The sharp peak indicates the uniform size of the ZnO nanoparticles. ZnO nanoparticles dispersed in alcohol exhibited green fluorescence which could be observed under UV lamp (365 nm).

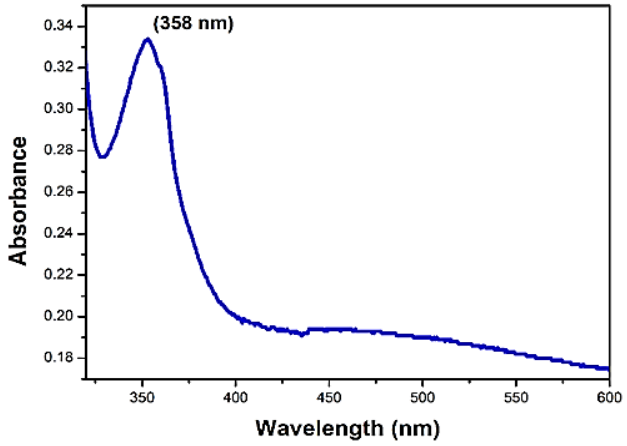


Fig. 2. UV-visible absorbance spectrum of the cysteine-capped ZnO nanoparticles.

The Photoluminescence spectrum of the cysteine capped ZnO nanoparticles dispersed in ethanol exhibited broad emission at 546 nm when excited with 358 nm as shown in the **Fig. 3**. The observed green luminiscence originates from oxygen defect sites present on the ZnO nanoparticles [**26**].

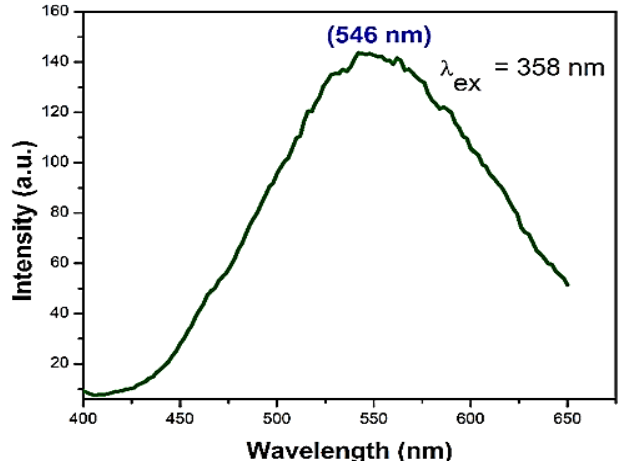


Fig.3. Emission spectra of ZnO nanoparticles.

The regulation of pH to basic medium has strong oxidizing capabilities which influenced the formation of more oxygen vacancies during the reaction. The cysteine functionalisation caused increase in particle size resulting in the red shift of PL slightly. Their fluorescence remained unchanged for more than 3 months exhibiting their good colloidal stability which is highly needed for the immunoassay applications.

The prescence of functional groups arising from cysteine were confirmed by FTIR spectroscopy **(Fig. 4)**. The presence of bands at 1407 and 1602 cm^{-1} correspond to the symmetric and asymmetric stretchings of carbonyl groups of cysteine. The broad band at 3150 cm^{-1} could be attributed to the N-H stretching vibration and the bands at 2870 and 2930 cm^{-1} can be assigned to the C−H stretching mode. The peaks at about 3350 and 1102 cm^{-1} can be ascribed to the characteristic absorption bands of the −OH stretching vibration mode. The spectra showed a standard sharp peak at 450 cm^{-1} which is attributed to the stretching vibration of Zn-O bonds. The confirmation of carboxyl groups on the surface of ZnO nanoparticles makes the conjugation of streptavidin realised for adoption in the present FZLIA.

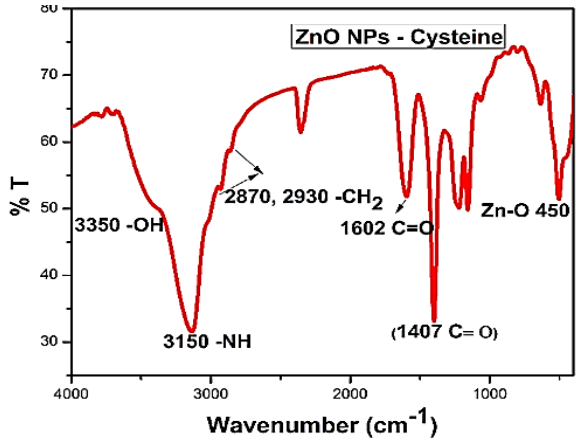


Fig. 4. FTIR spectra of cysteine capped ZnO nanoparticles.

Thermal gravimetric analysis (TGA) was performed to assess the extent of surface modification. As shown in **Fig. 5**, the weight loss for bare ZnO nanoparticles sample is only 4.2 % and 41 % for the cysteine capped ZnO nanoparticles.

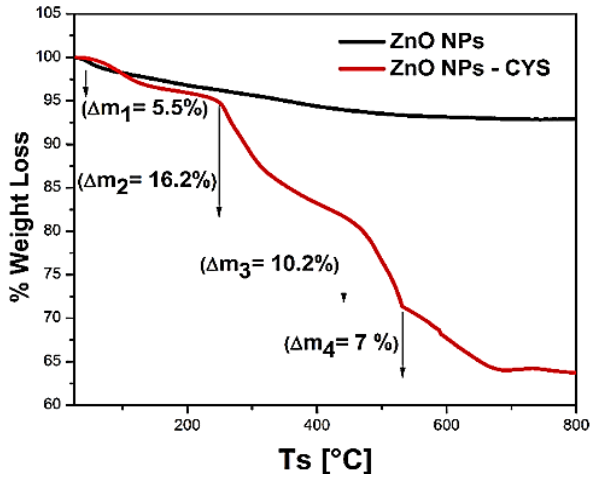


Fig. 5. TGA analysis of ZnO and Cysteine capped ZnO nanoparticles.

The weight loss of bare ZnO nanoparticles is because of the removal of adsorbed water onto the sample. Cysteine capped ZnO nanoparticles exhibited four step weight loss which is similar to earlier reports [**27**]. The first (until 120 ^{o}C) and second (120-250 ^{o}C) weight losses 5.5 and 16.2 % were caused by the removal of physically adsorbed water and organic components in the sample and this constitutes 60 % of the total removed amount. The third weight loss, 10.2 % (up to 460 ^{o}C) was due to the removal of physisorbed and chemisorbed molecules of cysteine. Final weight loss, 7 % was due to the complete decomposition of organic moieties which ended at 700 ^{o}C. From the equation (1) we calculated the amount of cysteine grafted on the ZnO nanoparticles to be 0.274 mmol/g from the weight loss attributed to removal and decomposition of cysteine. From equation (2) the grafting ratio was calculated as 68.1 % confirms the immobilization of cysteine onto ZnO nanoparticles. This high grafting ratio could contribute to the efficient conjugation of carboxyl groups of cysteine and primary amine groups of streptavidin.

FZLIA for sensitivity assessment

The sensitivity of FZLIA was evaluated by measuring HIV-1 p24 antigen standards. In this study, we adopted sandwich immunoassay format to form an antibody-antigen-antibody sandwich complex in which detector antibody was biotinylated. Streptavidin conjugated fluorescent ZnO nanoparticles were used to detect p24 antigen. As shown in **Fig. 6** a standard curve for the purified HIV-1 p24 antigen was established to find the lower detection limit for the FZLIA.

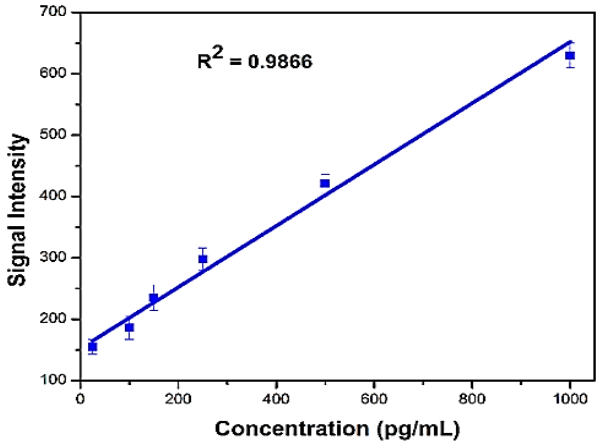


Fig. 6. Calibration curve of FZLIA for the detection of purified p24 antigen.

The FZLIA method allowed for p24 antigen determination in a large linear working range of 25 pg/mL to 1000 pg/mL, which allows the detection of HIV both in the earlier and later stages of disease development. It is evident from the graph with increase in the concentration of p24, the signal intensity also increased linearly. It is generally believed that for ZnO nanoparticles luminescence efficiency strongly depends on the nature of the surface and since nanoparticles have large surface-to-volume ratios cause surface defects which greatly effect small particles' emission peak [**28**]. From the linear fit of the data a good correlation between the concentrations of p24 and the signal intensity by p24 FZLIA assay was calculated to be R^2= 0.9866, suggesting that it is a linear dose dependent system. The present FZLIA can effectively detect the presence of relatively high levels of p24 in blood during the early stages of HIV infection. This range of detection can help in both in diagnosis and monitoring of the disease progression. Thus, FZLIA turned out to be more sensitive and convenient method which can also be extended as a platform for the efficient detection of other pathogens.

Effect of BSA as an interfering protein on FZLIA

In order to study the effect of any interference for other proteins, a control experiment was performed with bovine serum albumin as the interfering protein. Five concentrations of BSA were taken in the range of 0.5 μg/mL–10 μg/mL, and its effect on the fluorescence intensity was noted at the p24 antigen concentration of 1 ng/mL. With the above parameters, FZLIA was performed on the samples in triplicate and the effect of the interfering protein was studied.

All the concentrations of BSA, as an interfering protein has hardly any effect on their fluorescence intensities for fixed concentration of p24. From **Fig. 7** it is evident that protein such as BSA had negligible effect on the fluorescence measurements of FZLIA which confirms the stability of the assay.

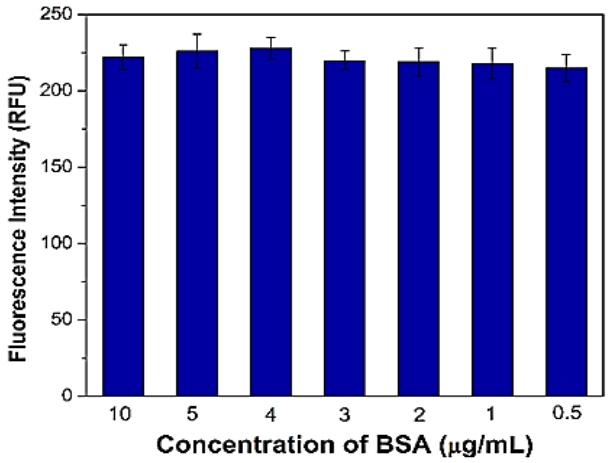


Fig. 7. Effect of the presence of BSA as an interfering protein on FZLIA.

Following the FZLIA for the detection of p24 antigen, we evaluated the feasibility of FZLIA for qualitative detection of HIV in plasma samples. Always a specific dilution is necessary to avoid the matrix effects of plasma samples for the analysis.

In this study, we diluted the HCV negative plasma sample to study the effects of varying concentration on the fluorescence intensities of FZLIA. The results of the FZLIA performed on the p24 concentration of 50 pg/mL are depicted in the **Fig. 8**. It is very evident that the

dilution of plasma sample to 100 times have significant effect on the fluorescence intensities of p24 detection. This would also reduce the requirement of the sample for the analysis by FZLIA.

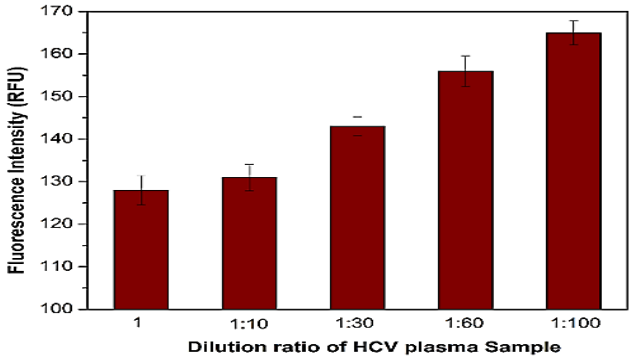


Fig. 8. Effect of varying HCV sample dilution on the fluorescence intensities of FZLIA for p24 detction.

Evaluation of FZLIA in human plasma samples for specificity

In order to evaluate the accuracy of our method, FZLIA was applied to access the recoveries of p24 antigen from HIV-1 p24 negative plasma samples spiked with known concentrations of HIV-1 p24 antigen. The plasma was diluted to obtain 50, 100, 200, 400 and 500 pg/mL concentration of p24 antigen in the sample. The signal response was directly correlated with the concentration from the standard curve **(Fig. 6)** of the purified p24 antigen. The recoveries ranged from 86.6 to 99.3%. **(Table 1)**.

Table 1. FZLIA with plasma samples from HIV negative plasma samples spiked with various concentrations of purified HIV-1 p24 antigen.

Sample	Spiked concentration (pg/mL)	Measured concentration (pg/mL)	Recovery (%)
1	50	48.2	97.6
2	100	97.5	99.3
3	200	184.87	92.4
4	400	398.79	98.7
5	500	443.3	86.6

The recoveries are in good agreement with the actual amounts spiked. The results suggested that the established FZLIA method is reliable and is suitable for the analysis of p24 antigen in plasma samples. Any small variation in the recoveries could be due to the slight increase in the background noise from other plasma proteins.

We evaluated the specificity of the FZLIA using plasma samples from HIV positive and negative individuals. All 12 plasma samples tested from confirmed HIV infected patients showed no false negative results as shown in the **Fig. 9**.

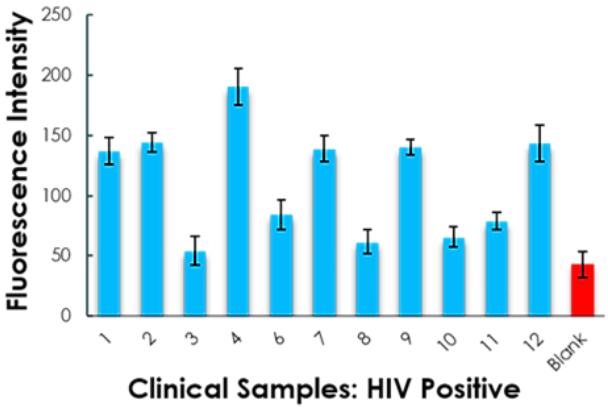


Fig. 9. FZLIA applied to 12 HIV positive samples and compared with the results from blank and a HIV negative sample. The *red bar* denotes the blank.

The mean ratio of samples over the cut-off ratio of the negative control for these samples was 250.8 ± 197.54. We also tested 15 plasma samples from confirmed HIV negative individuals. The signal to cut off ratio was considerably less than 57.21 (36.60 ± 3.62) and none of them were falsely positive **(Fig. 10)**. The specificity of the assay was further evaluated by 4 HBV positive/HIV negative and 4 HCV positive/HIV negative plasma samples for the cross reactivity in the detection of HIV-1 p24. The mean ratios of samples over the cut-off value of the negative control for samples from the patients HBV positive/ HIV negative, HCV positive/HIV negative infected plasma samples were 48.96 ± 32.48, 49.47 ± 38.25 respectively. The absence of false negative results in this study clearly indicates that there is no cross reactivity of HIV-1 p24 with HBV, HCV viruses. Based on the above findings FZLIA had a specificity of 100 % (15/15). No cross-reactivity was observed with HBV positive (4/4) and HCV positive (4/4) samples. Thus, based on these results this platform can be reliably used for the detection and also quantification of p24 antigen in

Table 2. Comparison of FZLIA with other immunoassays.

S.no.	Target analyte	Concentration detected	Method	References
1.	Human α-fetoprotein	10 ng/mL	CLISA	29
2.	HIV-1 p24	5 pg/mL	μENIA	30
3.	Carcinoma embryonic antigen	1 pg/mL	ELISA	13
4.	Dopamine	0.05 μM	Fluorescent ZnO NPs	31
5.	Carbohydrate Antigen 19-9 (CA 19-9)	0.1 μM	ZnO quantum dot labelled immunosensor	32
6.	Anthrax Toxin	0.01 ng/mL	ENIA	33
7.	HIV-1 p24	250 pg/mL	CDPIA	34
8.	HIV-1 p24	25 pg/mL	FZLIA	Present work

clinical samples. The comparison of FZLIA for HIV detection were compared with other antigens by other detection methods **(Table 2)**.

This signal based amplification assay could achieve a sensitivity level close to PCR techniques which require sophisticated instruments, costly reagents and are very prone to contamination. FZLIA has worked out to be a very sensitive immunoassay because of its stable signal and high signal to noise ratio.

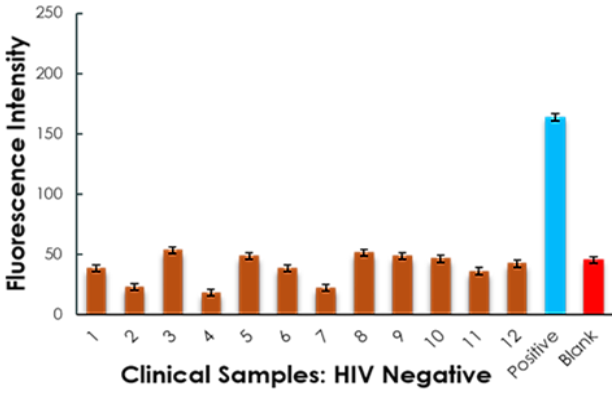


Fig. 10. FZLIA of 12 HIV negative samples which are compared with the result from a blank and a HIV positive sample. The brown, red and blue bars denote the negative, blank and the lowest measured positive samples, respectively.

The short incubation time and flexible platform could facilitate use in a POC setting. FZLIA does not involve enzymatic reactions for detection, specific instrumentation for conducting reactions and special storage conditions for reagents. Due to the high specificity of streptavidin conjugated ZnO nanoparticles, FZLIA could be developed into a universal labelling technology by replacing capture and detection antibodies for detection of various antigens. There is no need for specific training of technicians to handle FZLIA as it is similar to traditional ELISA which is widely practiced across various laboratories and clinics. Upon further optimization and simplification, FZLIA could be developed into a rapid and ultrasensitive testing platform for clinical diagnosis and laboratory research in resource limited settings.

Conclusion

Our work has shown that the use of fluorescent ZnO nanoparticles in infectious disease assay development can result in highly enhanced assay sensitivity. Although there are similar assays, this assay is capable of detecting HIV p24 antigen in the pg/mL range which will be useful in identifying early HIV infection cases. The findings described in this paper are important for those developing highly sensitive assays for HIV incidence estimation and detection of acute infection cases as part of the strategy towards an AIDS-free generation. This highly sensitive p24 assay can also help improve blood safety by reducing the antibody negative window period in blood donors in resource limited settings where nucleic acid testing is not practical or feasible.

Acknowledgements

Authors are grateful to Sri Sathya Sai Baba for his constant inspiration. We also thank the UGC, Govt. of India for BSR fellowship to L.A. Avinash Chunduri. Author Aditya Kurdekar acknowledges DST, Govt. of India for the DST-Inspire Fellowship.

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