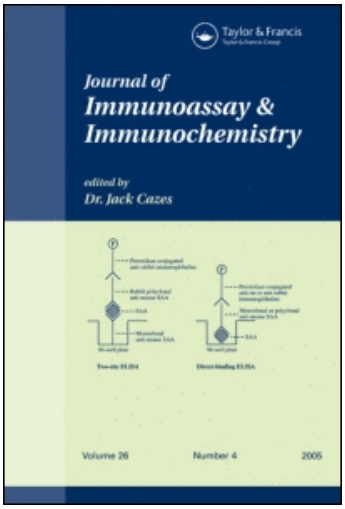


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# How an Improved Immunoassay Sensitivity Can Be Achieved by Gamma Irradiation: Modification, Application and Characterization of Polystyrene Surface for Anti HIV-1 ELISA

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**Abstract:** Polystyrene microtitre plates modified by $^{60}$Co $\gamma$-ray irradiation were used in an indirect enzyme-linked immunosorbent assay (ELISA) for detection of anti-human immunodeficiency virus type 1 (anti HIV-1). The plates with 9 kGy (optimum dose) irradiation showed 2-5-fold higher detection sensitivity in serodiagnosis tests compared to untreated ones, and a 3-fold lower enzyme concentration than the control used was still detectable. Adsorption/desorption experiment results, atomic force microscopy (AFM) images, and X-ray photoelectron spectroscopy (XPS) analysis provide the reason for this improvement. The oxidized surface formed during irradiation presented much more binding affinity for coating antigens and could adsorb a larger amount (1.5-3-fold) of protein uniformly.

**Keywords:** ELISA, Gamma irradiation, HIV-1, Polystyrene surface, Protein adsorption

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## INTRODUCTION

Polystyrene (PS) microtitre plates are usually used as solid substrate for protein adsorption in the enzyme-linked immunosorbent assay (ELISA), which is widely employed in biological science and medical laboratory diagnositics as an established technique.[1,2] Since this assay is based on immunological reaction on solid phase, the adsorption of bioactive compound to the plate is of great importance.[3] However, low molecular weight antigens/antibodis can be hardly immobilized on PS surface by random adsorption due to the fact that small molecules have few binding sites.

The weak interaction between PS surface and small protein limits the sensitivity of ELISA assay.[4–6] The immobilization of this type of targets on PS surface commonly involves covalent coupling procedures, which are time-consuming and may change the epitopes as a result of unsuitable presentation and orientation of protein.[7–9] To maintain their conformation and biological activity, different methods, such as biotin-streptavidin bridge[10–12] and affinity ligand[13–15] have been used for oriented immobilization. In this study, we pretreated commercially available PS microtitre plates with certain doses of $^{60}$Co gamma irradiation and the modified plates were applied for adsorption of the low molecular weight HIV-1 recombinant antigen (MW: 32 kDa). We evaluated the effect of irradiation on the performance of PS plates by detecting anti HIV-1 in a typical indirect ELISA system. Moreover, adsorption/desorption experiment, atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS) were adopted for further research of protein adsorption on PS surface.



## EXPERIMENTAL

### Irradiation Treatment

Under the air flow rate of 3 $m^3$/min, PS 96-well microtitre plates (Nunc, Denmark) were irradiated with a $^{60}$Co source (Academy of Fujian Agricultural Science, China) at a dose rate of 1 kGy/h to levels of 0, 3, 6, 9, 12, and 15 kGy, respectively.

### ELISA Protocol

After irradiation, the plates were incubated overnight at 4°C with 100 μL/well of HIV-1 recombinant antigen (gp120′-gp41′, kindly provided by Dr. Dayong Jia) diluted in 10 mM phosphate buffered saline (PBS),

pH 7.2 (this procedure usually known as coating). Next, the plates were washed with PBS containing 0.05% Tween-20 (v/v), and blocked with blocking solution (PBS containing 2.5 mg/mL BSA) at 37°C for 2 h. 100 μL of diluted serum sample (positive samples containing anti HIV-1 antibodies, obtained from National Center for Clinical Laboratory, China) was added to each well of the plates. After 0.5 h incubation at 37°C, the plates were washed and added with horseradish peroxidase-conjugated goat anti-human IgG (HRP-IgG, Sigma, USA) at an appropriate concentration, followed by another 0.5 h incubation. The wells were washed again to remove any unbound conjugate and developed with the substrate tetramethylbenzidine (Sigma, USA) for 15 min. The colorimetric reaction was terminated by addition of 50 μL of 2 M $H_2SO_4$ and optical density (OD) was measured at 450 nm by using a model 550 microplate reader (Bio-Rad, USA). All tests were repeated four times and the arithmetic mean of absorbance values ($OD_{450\,nm}$) was calculated.



### Desorption Experiment

Each coated well was added with 100 μL of 100 mM $KNO_3$ solution (pH 7), incubated at 37°C for 2 h with vigorous shaking. After the solution was collected, the amount of desorbed HIV-1 antigens was determined from the protein concentration in the solution. Desorption experiment was repeated with 99 mM $KNO_3$-1 mM $HNO_3$ (pH 3) and 90 mM $KNO_3$-10 mM KOH (pH 12). Protein concentration was determined using micro BCA protein assay kit (Pierce, USA).

### AFM and XPS

Well bottom of coated plates was applied for AFM and XPS measurement before blocking procedure. AFM was performed with a Nanoscope IIIa multimode scanning probe microscope (Digital Instruments, USA) in the contact mode. XPS was carried out on a SSX-100 spectrometer (Surface Science Instruments, USA) with a monochromatised $AlK_\alpha$ X-ray source (1486.6 eV) run at 150 W.

## RESULTS AND DISCUSSION

### Immunoassay Characteristics

The effects of various doses of irradiation were assessed on ELISA sensitivity. Figure 1 reveals that the absorbance value was gradually

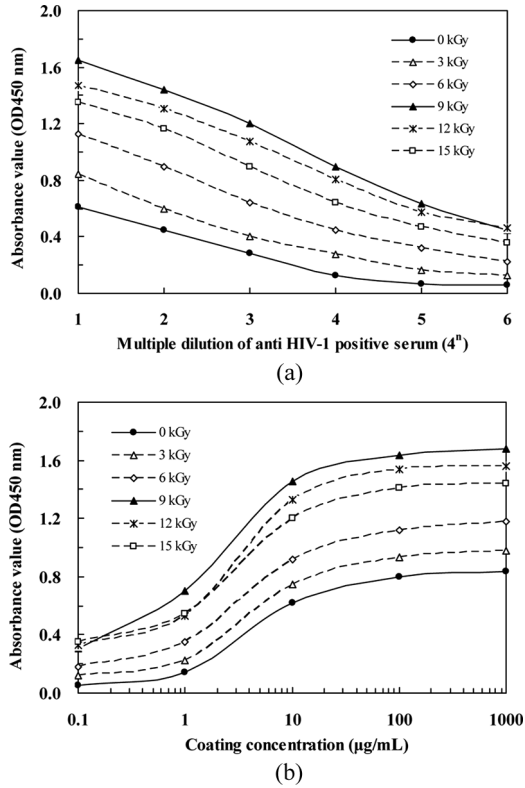


**Figure 1.** Immunoassay characteristics of HIV-1 recombinant antigen adsorption on PS plates pretreated with various doses of irradiation. (a) The concentrations of coating antigen were all 40 μg/mL, while the concentrations of anti HIV-1 were varied in series multiple dilutions. (b) The same anti HIV-1 (1:10 dilution of a positive serum sample) was used in tests, while the concentrations of coating antigen were varied in the range of 0.1–1000 μg/mL. 1:2400 diluted HRP-IgG was used in (a) and (b).

increased with the increase of irradiation dose, and was up to maximum at 9 kGy, further irradiation would reduce the value to some extent. Compared to untreated ones, the 9 kGy irradiated plates showed 2–5-fold higher detection sensitivity at serial dilutions (4–$4^6$-fold) of anti HIV-1 (Figure 1a) or over a wide range (0.1–1000 μg/mL) of coating concentrations (Figure 1b). Table 1 shows the Signal-to-Noise Ratio (SNR, the ratio of the difference between the mean absorbance value of positive and negative samples to that of negative ones) of ELISA using 9 kGy irradiated plates was as high as 25.9, and a 3-fold lower HRP-IgG concentration than the control used was still detectable. In contrast,

**Table 1.** Sensitivity/Specificity comparison of ELISA detection using untreated and 9 kGy irradiated plates

| Plates & HRP-IgG conc. | SNR | Mean absorbance value (n = 12)/CV (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Anti HIV-1 (+) sample | | | Anti HIV-1 (–) sample | | |
| Untreated-1:2400[a] | 7.7 | 0.162/6.9 | 0.353/9.8 | 0.612/9.5 | 0.003/7.3 | 0.047/8.5 | 0.079/8.6 |
| Irradiated-1:2400[b] | 25.9 | 0.741/3.6 | 1.276/4.1 | 1.702/3.2 | 0.005/3.6 | 0.050/2.1 | 0.083/2.7 |
| Untreated-1:800[c] | 6.5 | 0.662/9.6 | 1.280/8.8 | 1.643/9.4 | 0.082/9.6 | 0.156/7.3 | 0.242/6.7 |

3 known anti HIV-l positive serum samples were previously confirmed by Western blot analysis and 3 negative ones were collected from healthy donors who had no HIV serological markers. The plates were coated with 40 μg/mL HIV-1 recombinant antigen. Absorbance values of negative/positive samples suggest that test (a) and (b) were kept at the same specificity level, while test (b) and (c) were kept at the same sensitivity level. Higher sensitivity could be achieved by increasing the concentration of HRP-IgG, but the detection specificity lossed simultaneously. The Signal-to-Noise Ratio (SNR) is defined as the ratio of the difference between the mean absorbance value of positive and negative samples to that of negative ones.

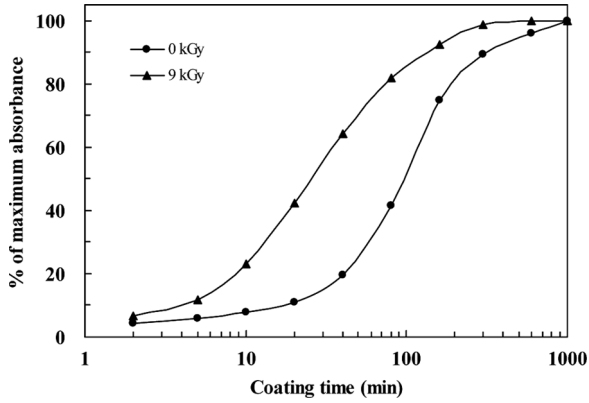


**Figure 2.** Influence of coating time on ELISA detection using untreated and 9 kGy irradiated plates. The plates were coated with 40 μg/mL HIV-1 recombinant antigen for time of 2–1000 min. The same anti HIV-1 (1:10 dilution of a positive serum sample) and 1:2400 diluted HRP-IgG were used in tests.



the SNR of assay using untreated plates was rather poor, only 7.7 and 6.5 when using 1:2400 and 1:800 diluted HRP-IgG respectively. Figure 2 shows that protein adsorption on 9 kGy irradiated plates went more quickly and could reach the equilibrium earlier than that on untreated ones.

## Adsorption/Desorption Behavior

In order to identify whether this improved ELISA sensitivity was primarily owing to the increased adsorption ability of modified surface or owing to the better stereo presentation of coated antigens, untreated and 9 kGy irradiated plates were coated with antigens and then reacted with HRP conjugated rabbit anti HIV-1 (polyclonal antibody, produced in rabbits by injection of HIV-1 recombinant antigen) in a direct ELISA system (named adsorption experiment here). If the improved ELISA sensitivity was primarily owing to the increased adsorption ability of modified surface rather than the better stereo presentation of coated antigens, the absorbance value of direct ELISA using irradiated plate should be increased because polyclonal antibodies could recognize all coated antigens regardless of their stereo presentation. It was found that, at all coating concentrations tested (8, 40 and 200 μg/mL) in adsorption experiment, the absorbance value of direct ELISA using 9 kGy irradiated plate was about 2–4-fold greater than that using untreated one. The conclusion drew from this experiment was in accordance with XPS results of the following.

The stability of the coating was evaluated by desorption experiment. When the coating concentration was 40 μg/mL, HIV-1 antigens adsorbed on 9 kGy irradiated surface were not removed with 100 mM $KNO_3$ at pH 7 or with 90 mM $KNO_3$-10 mM KOH at pH 12 while on untreated one were removed 11% and 29% under the same conditions. About 6%/48% of antigens coated on irradiated/untreated plate were removed from PS surface at pH 3. Similar results could be obtained at other coating concentrations. Furthermore, it was found that antigens could be adsorbed on modified surface with scarcely suffering from interference by coexisting surfactants and salts during coating process. No significant loss of immune reactivity was observed even after the addition of 5% Tween-20 (v/v) and 0.5 M NaCl. These findings suggest that, compared with untreated plate, antigens were preferentially adsorbed on irradiated one and not readily desorbed in a wide range of pH.



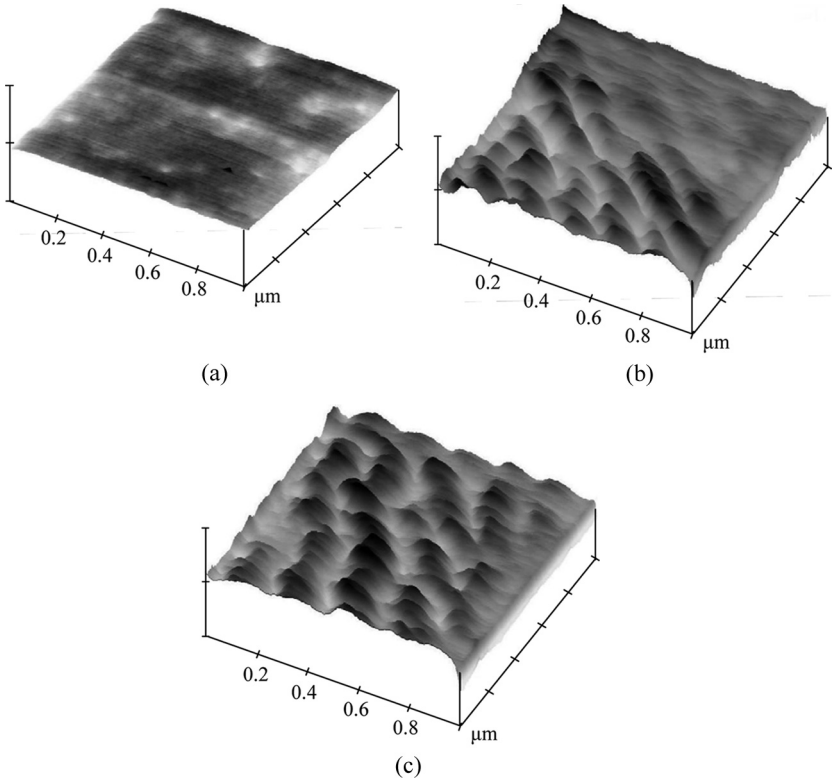


**Figure 3.** Plate surfaces imaged by AFM. (a) Background of PS surface before coating. (b) and (c) Untreated and 9 kGy irradiated plates coated with 40 μg/mL of HIV-1 recombinant antigen, respectively. Each image represents 1 μm × 1 μm of the surface.

## Surface Characterization

To investigate the improved performance of the irradiated plates, surface characterization was examined by AFM and XPS. The surface morphologies of coated plates as determined by means of AFM are shown in Figure 3. HIV-1 antigens were present as aggregates that were approximately 0.1 μm wide and 0.05 μm high with average spacing of 0.15 μm. Compared to modified surface, the distribution of antigens on untreated one was uneven and completely absent in some regions. It appears to be the reason for the difference of coefficient variation (CV, see Table 1) and ELISA sensitivity between untreated and 9 kGy irradiated plates.

Before protein adsorption, only carbon and oxygen were detected in XPS analysis of PS surface. Untreated plates contained 1.7% oxygen (might have come from additives and sterilization treatments), and the content went up to 13.1% after irradiated with optimum dose of 9 kGy, indicating the development of oxygenic groups which might include hydroxyl, carbonyl, carboxyl group, etc.[16,17] So, the surface oxidation during irradiation with a corresponding increase in hydrophilicity was probably the main cause of improved performance of modified plates. After coating of HIV-1 antigens, the amount of protein immobilized on PS surface could be deduced from the amount of nitrogen detected by XPS (the average nitrogen content of proteins being about 16%). Figure 4 shows that, at all coating concentrations tested (1.6–1000 μg/mL), the amount of protein adsorbed on 9 kGy irradiated plates was approximately 1.5–3-fold greater than that on untreated ones. This



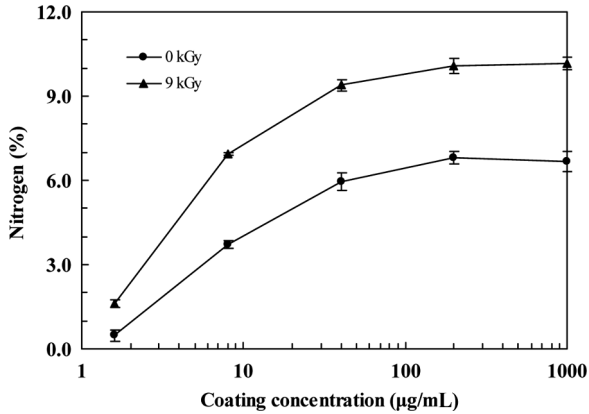


**Figure 4.** XPS analysis of the relative amounts of protein adsorbed on untreated and 9 kGy irradiated plates. The concentrations of coating antigen were varied in the range of 1.6–1000 μg/mL. Mean % nitrogen ± SD was given (n = 4).

was in accordance with ELISA results mentioned above, and demonstrated that PS plates pretreated with appropriate dose of $^{60}Co$ irradiation indeed exhibited much more binding affinity for coating proteins.

## CONCLUSION

Low molecular weight HIV-1 recombinant antigen could be directly adsorbed on PS surface pretreated with 9 kGy (optimum dose) $^{60}Co$ $\gamma$-ray irradiation, and the resulting coating showed high levels of sensitivity, specificity and homogeneity in ELISA detection. The results of adsorption/desorption experiments, AFM and XPS analysis suggest that the hydrophilic surface formed during irradiation presented high binding affinity for coating antigens and could adsorb protein more quickly and more firmly. The assay used in this study is well suited for ELISA and might be applied to other detection techniques using PS as support matrix.



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## REFERENCES

1. Butler, J.E. Enzyme-linked immunosorbent assay. J. Immunoassay Immunochem. **2000**, *21*, 165–209.
2. Lequin, R.M. Enzyme immunoassay (EIA)/Enzyme-linked immunosorbent assay (ELISA). Clin. Chem. **2005**, *51*, 2415–2418.
3. Butler, J.E.; Ni, L.; Brown, W.R.; Joshi, K.S.; Chang, J.; Rosenberg, B.; Voss, E.W. The immunochemistry of sandwich ELISAs-VI. Greater than 90% of monoclonal and 75% of polyclonal anti-fluorescyl capture antibodies (CAbs) are denatured by passive adsorption. Mol. Immunol. **1993**, *30*, 1165–1175.
4. Suzuki, C.; Ueda, H.; Mahoney, W.; Nagamune, T. Open sandwich enzyme-linked immunosorbent assay for the quantitation of small haptens. Anal. Biochem. **2000**, *286*, 238–246.
5. Zhao, J.; Li, G.; Yi, G.X.; Wang, B.M.; Deng, A.X.; Nan, T.G.; Li, Z.H.; Li, Q.X. Comparison between conventional indirect competitive enzyme-linked immunosorbent assay (icELISA) and simplified icELISA for small molecules. Anal. Chim. Acta. **2006**, *571*, 75–89.

6. Winger, L.A.; Dessi, J.L.; Self, C.H. Enhanced specificity for small molecules in a convenient format which removes a limitation of competitive immunoassay. J. Immunol. Meth. **1996**, *199*, 185–191.
7. Bora, U.; Chugh, L.; Nahar, P. Covalent immobilization of proteins onto photoactivated polystyrene microtiter plates for enzyme-linked immunosorbent assay procedures. J. Immunol. Meth. **2002**, *268*, 171–177.
8. Frederix, F.; Bonroy, K.; Reekmans, G.; Laureyn, W.; Campitelli, A.; Abramov, M.A.; Dehaen, W.; Maes, G. Reduced nonspecific adsorption on covalently immobilized protein surfaces using poly(ethylene oxide) containing blocking agents. J. Biochem. Bioph. Meth. **2004**, *58*, 67–74.
9. Holthues, H.; Pfeifer-Fukumura, U.; Sound, I.; Baumann, W. Evaluation of the concept of heterology in a monoclonal antibody-based ELISA utilizing direct hapten linkage to polystyrene microtiter plates. J. Immunol. Meth. **2005**, *304*, 68–77.
10. Cho, I.H.; Paek, E.H.; Lee, H.; Kang, J.Y.; Kim, T.S.; Paek, S.H. Site-directed biotinylation of antibodies for controlled immobilization on solid surfaces. Anal. Biochem. **2007**, *365*, 14–23.
11. Vynios, D.H.; Papadas, Th.A.; Faraos, A.; Mastronikolis, N.S.; Goumas, P.; Tsiganos, C.P. A solid phase assay for the determination of heparan sulfate and its application to normal and cancerous human cartilage samples. J. Immunoassay Immunochem. **2001**, *22*, 337–351.
12. Nara, S.; Tripathi, V.; Chaube, S.K.; Rangari, K.; Singh, H.; Kariya, K.P.; Shrivastav, T.G. A novel enzyme-linked immunosorbent assay for cortisol using a long-chain biotinylated cortisol-3-CMO derivative. J. Immunoassay Immunochem. **2008**, *29*, 390–405.
13. Kumada, Y.; Tokunaga, Y.; Imanaka, H.; Imamura, K.; Sakiyama, T.; Katoh, S.; Nakanishi, K. Screening and characterization of affinity peptide tags specific to polystyrene supports for the oriented immobilization of proteins. Biotechnol. Prog. **2006**, *22*, 401–405.
14. Wang, J.; Bhattacharyya, D.; Bachas, L.G. Improving the activity of immobilized subtilisin by site-directed attachment through a genetically engineered affinity tag. Fresenius' J. Anal. Chem. **2001**, *369*, 280–285.
15. Feng, B.; Dai, Y.; Wang, L.; Tao, N.; Huang, S.; Zeng, H. A novel affinity ligand for polystyrene surface from a phage display random library and its application in anti-HIV-1 ELISA system. Biologicals **2009**, *37*, 48–54.
16. Onyiriuka, E.C.; Hersh, L.S.; Hertl, W. Surface Modification of polystyrene by gamma-radiation. Appl. Spectrosc. **1990**, *44*, 808–811.
17. Onyiriuka, E.C. The effects of high-energy radiation on the surface chemistry of polystyrene: a mechanistic study. J. Appl. Sci. **1993**, *47*, 2187–2194.

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