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Immunosensor for HIV-1 Diagnostics Based on Immobilization of the Antigenic Peptide p24-3 Into Liposomes

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In this work we developed an immunosensor for HIV-1 diagnostics that exploits the biorecognition between the antibody anti-p24 and the antigenic peptide p24-3 (AMATLRAEQASQEVKNWMTETL-LVQNA) derived from the HIV-1 p24 protein. p24-3 was encapsulated in phospholipid liposomes and immobilized in layer-by-layer (LbL) films produced with polyethyleneimine (PEI). The incorporation of p24-3 into liposomes was investigated using circular dichroism (CD) spectroscopy, from which an increase in the alpha helix conformation could be noted. The maximum fluorescence emission for p24-3 occurred at 340 nm in solution, compatible with the tryptophan residue being exposed to the solvent, and at 335 and 322 nm when in liposomes and PEI/p24-3-liposome LbL films, respectively. This blue shift is consistent with the tryptophan being in a partially buried environment. With the preserved structure in the LbL films, p24-3 could recognize the anti-p24 antibody in impedance spectroscopy measurements. Therefore, LbL films containing p24-3 may be suitable for detecting HIV-1 in a low-cost, easy-to-use immunosensing assay.

Keywords: HIV Immunosensor, Layer by Layer Films, Antigenic Peptide, Liposome.

1. INTRODUCTION

The development of rapid, easy-to-use methods for HIV diagnostics has been a goal for considerable time.1–8 HIV detection is mostly performed in specialized laboratories, with methods such as enzyme-linked immunosorbent assay (ELISA), Western blot and immunofluorescence assay (IFA).9 Reports have been made of rapid tests, including agglutination assays, membrane immunocentrization devices, solid phase tests (ImmunoDot) and immunochromatography.9 However, the latter methods are based on detecting antibodies against HIV proteins or components of the virus which are easily susceptible to structural changes and may thus lose their function.10 One alternative route to design immunosensors involves antigenic peptides, which are advantageous for their low cost and stability.5–8,11 The success of this route requires specific arrangements for the immunogen. For example, when the epitope is localized in β-turns or loop regions of a protein, a cyclic peptide could better mimic the native secondary structure than a linear peptide, and be more efficiently recognized by specific antibodies.12 Alternatively, the epitope region could be synthesized using an optimal size peptide, with amino acids that induce such structure.12 Another strategy for increasing antigen–antibody binding consists in incorporating the peptide into phospholipid layers or liposomes,5,11 for these matrices are known to help preserve the native structure of proteins and peptides.13

The use of antigenic peptides in immunosensors may be exploited in conjunction with platforms using nanostructured films and electrochemistry or impedance spectroscopy as the principle of detection.5,14 Recent examples

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of immunosensing and biosensors made with ultrathin films are given in Refs. [15–19]. In this paper, we produced an immunosensor for HIV-1 diagnostics that exploits the biorecognition between the antibody anti-p24 and the antigenic peptide p24-3 (AMATLRAEQASQEVKNWMTETLLVQNA) that corresponds to the residues 171–194 from the HIV-1 p24 protein.\textsuperscript{20} The antigenic peptide p24-3 was encapsulated in phospholipid liposomes and then used to build layer-by-layer (LbL) films with polyethyleneimine (PEI). The interactions between p24-3 and liposomes were investigated using circular dichroism (CD) and fluorescence spectroscopy. The ability of the films to interact with the anti-p24 antibody was investigated with electrochemistry, electrical, contact angle and atomic force microscopy measurements. These approaches not only provide a better understanding of the antigenic structure of peptides, but may also contribute to developing methodologies such as artificial vaccines or diagnostic reagents.

2. EXPERIMENTAL DETAILS

2.1. Materials and Solutions

The antigenic peptide p24-3 was purchased from Bio-Synthesis Inc. The sequence NH2-TLRAEQASQEVKNWMTETLLVQNA-\textsubscript{OH} was purified and isolated by HPLC with purity > 90%, being confirmed with mass-spectral analysis according to procedures described by Bio-Synthesis Inc. The anti-p24 (antibody specific to HIV-1 protein p24), the polyelectrolyte poly(ethylene imine) (PEI) and the phospholipid dipalmitoyl phosphatidyl glycerol (DPPG) were purchased from Santa Cruz Biotechnology Inc., Sigma-Aldrich and Avanti Polar Lipids, respectively. p24-3, PEI and liposome solutions were prepared with ultrapure water from a Milli-Q system at concentrations of 0.1 mmol·L\textsuperscript{−1} and 1 mmol·L\textsuperscript{−1}, respectively. The antibody was diluted in phosphate buffered saline (PBS) solution at concentrations ranging from 0.01 to 1 μg·mL\textsuperscript{−1}, which were then used in electrochemical and electrical measurements.

2.2. Incorporation and Immobilization of p24-3

Scheme 1 shows the procedures for preparing liposomes and immobilizing them in LbL films, consisting in the following steps: (a) incorporation of p24-3 into DPPG liposomes was performed using the method described by Petri et al.\textsuperscript{2} (b) The DPPG liposomes containing p24-3 were immobilized in LbL films alternated with PEI layers onto a solid substrate. The latter was initially immersed in PEI solution for 3 min, while adsorption of liposomes was performed during 10 min, which was determined as optimized time from an adsorption kinetics study.\textsuperscript{3} After each deposition step, the films were rinsed with Milli-Q water.

The incorporation and secondary structure of p24-3 in the DPPG liposomes were confirmed with fluorescence spectroscopy and circular dichroism (CD) measurements. The multilayer growth of PEI/p24-3-containing liposomes was monitored with UV-vis and fluorescence spectroscopies. The CD spectra were collected with a quartz cell of 1 mm light path, in a Jasco spectropolarimeter, model 815, with bandwidth of 1 nm, response time of 0.5 s and scanning speed 100 nm/min. CD spectra were obtained by averaging eight scans. Fluorescence measurements of p24-3 free in solution and incorporated into DPPG liposomes were performed with a Shimadzu spectrometer model RF-5301PC, with excitation at 280 nm.

2.3. Atomic Force Microscopy and Contact Angle of the LbL Films

The morphology of LbL films made with PEI/p24-3-containing liposomes bilayers in the absence and presence of anti-p24 was studied with a NanoSurf Instruments atomic force microscope EasyScan II in the tapping mode (512 × 512 pixels) under ambient conditions. This mode was selected instead of the contact mode because it is less damaging to the surfaces under investigation,\textsuperscript{21,22} since the transferred energy is less and the tip does not exert a significant lateral force on the surface. A sample area of 5 μm\textsuperscript{2} was scanned. Film roughness was determined using the NANOSURF Instruments software. The contact angle was measured on films using the sessile drop technique, with water, ethylene glycol and diiodomethane as solvents. Several sessile solvent drops (2 μL) were dispensed on the same sample with a precision microsyringe. Digital pictures of the drops were taken from above, using KSV Cam 200. The contact angle values were used to calculate the surface free energy with the Owens–Wendt method.\textsuperscript{23}

2.4. Electrochemical and Electrical Measurements

Cyclic voltammetry measurements were performed with a μ Stat 400 from DropSens using screen-printed carbon electrodes. The electrochemical cell consisted of a working electrode (4 mm diameter), a carbon counter electrode and a silver reference electrode. The working electrode was modified through deposition of LbL films of either PEI/p24-3-containing liposomes or PEI/liposomes.
The experiments were conducted in a PBS solution (at pH 7.4) at room temperature (22 °C) with a scan speed of 0.05 V/s and potential range from −0.6 to 0.6 V. Cyclic voltammograms were obtained in the absence and presence of the anti-p24 antibodies. The anti-p24 solution in PBS was placed on top of the LbL film for 10 min and washed with PBS prior to the measurements.

For the electrical impedance measurements, 1 and 5-bilayer PEI/liposomes and PEI/p24-3-containing liposomes LbL films were built onto 50 pairs of gold interdigitated electrodes with 10 µm width, 10 µm apart from each other. The measurements were carried out with a Solartron 1260A impedance/gain phase analyzer in the frequency range from 102 to 105 Hz. The experiments were placed on top of the LbL film for 10 min and washed with PBS prior to the measurements.

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### 2.5. Data Analysis

In order to distinguish between samples with distinct concentrations of anti-p24, the real component of the impedance was plotted using a multidimensional projection technique, referred to as IDMAP,25 with procedures established by Paulovich et al.26–29 In this method, an initial mapping of the samples is performed, which is then improved by using the Force Scheme strategy.30 With the latter, the placement is improved by mimicking a mass-spring system with attraction and repulsion forces, in a similar fashion to well-known graph-layout approaches.31 Mathematically, this strategy consists in computing for each projected sample \( y_i \in Y \) a vector \( \vec{v}_{ij} = (y_i - y_j) \), \( \forall y_i \neq y_j \). Then, \( y_i \) is moved on the direction of \( \vec{v}_{ij} \) by an amount given by

\[
S_{IDMAP} = \frac{\delta(x_i, x_j) - \delta_{\min}}{\delta_{\max} - \delta_{\min}} - d(y_i, y_j) \quad (1)
\]

where \( \delta_{\min} \) and \( \delta_{\max} \) are the minimum and maximum distances between the samples. The difference \( |\delta(x_i, x_j) - d(f(x_i, x_j))| \), \( \forall x_i, x_j \in X \) is reduced after successive applications of this procedure. In other words, the placement of the points is such as to preserve the distances in the original space. In the present problem, \( \delta(x_i, x_j) \) is the Euclidean distance between the electrical impedances of samples \( x_i \) and \( x_j \) for \( m \) different frequencies. Each dataset is then an \( m \)-dimensional vector embedded into an \( m \)-dimensional space. Also used was a standardization procedure25 to avoid possible bias from impedance values measured on different scales (depending on the frequency). Standardization was performed as follows. For \( n \) sample measurements, where \( \bar{x}_j = (1/n) \sum_{i=1}^{n} x_{ij} \) is the average computed for the \( j \)-th measurement and \( \sigma_j = \sqrt{\left(1/n\right) \sum_{i=1}^{n} (x_{ij} - \bar{x}_j)^2} \) is the corresponding standard deviation, we used \( x_{ij}' = (x_{ij} - \bar{x}_j) / \sigma_j \) for \( 1 \leq i \leq n \) and \( 1 \leq j \leq m \). Such procedures in multidimensional projections are aimed at preserving the similarity of the samples in the original data space when they are projected into the 2-dimensional plot.

### 3. RESULTS AND DISCUSSION

#### 3.1. Incorporation of p24-3 Into DPPG Liposomes

Figure 1(A) shows the CD spectrum for p24-3 free in an aqueous solution, featuring a minimum at 217 nm characteristic of a β-sheet conformation. In contrast, the spectrum for p24-3 incorporated into DPPG liposomes displays two minima at 204 and 225 nm, indicating an α-helix conformation for p24-3, which was expected from X-ray diffraction for the residues 171–194 from the native p24 protein.33 Thus, DPPG liposomes induced p24-3 to adopt a structure closer to the native one. This structuring has been reported by several authors, where the bioactive conformation of a peptide is generally induced

![Figure 1](image-url)

**Figure 1.** CD (A) and fluorescence (B) spectra of p24-3 free in aqueous solution (solid curve) and incorporated in DPPG liposomes (dashed curve).
Figure 2. Increase in the absorption at 280 nm for an LbL film of PEI/p24-3-containing liposomes as a function of the number bilayers. Inset: Fluorescence spectrum of the film with excitation at 280 nm.

Figure 3. AFM images and height profiles for 1 layer of PEI (A) and 1-bilayer LbL film of PEI/p24-3-containing liposomes in the absence (B) and presence of anti-p24 antibody (C).

3.2. Layer-by-Layer Films Containing the Antigenic Peptide p24-3

Attempts to produce LbL films with solutions of p24-3 failed, probably because the peptide molecules were not protected, similarly to what was observed in an earlier work for the peptide p17-1. Therefore, all the LbL films reported here were obtained with p24-3 encapsulated into DPPG liposomes. Figure 2 displays the absorption by conformational changes through its interaction with biomembranes.

The fluorescence spectra in Figure 1B corroborate the CD results, with a blue shift of 5 nm being observed in the emission maximum, from 340 nm for p24-3 free in solution to 335 nm for p24-3 in liposomes. The blue shift is explained by the high sensitivity of tryptophan (W) to solvent polarity, i.e., emission of W may be blue shifted when buried within a native structure.
maximum at 280 nm as a function of number of bilayers of PEI/p24-3-containing liposomes, indicating that film growth appears exponential for the first few bilayers, as in many LbL films made with biomolecules. The spectrum in the inset features a maximum at 322 nm, which can be assigned to p24-3 buried in a hydrophobic environment, probably with its structure preserved. The shoulder at 360 nm can be due to aggregates formed upon the film adsorption.

Figure 3 shows AFM images and height profiles (cross-section of the images) for 1 layer of PEI and 1-bilayer PEI/p24-3-containing liposomes films, in the absence and presence of anti-p24 antibody. The images were taken in a scan window of 5 μm × 5 μm. A clear globular structure is identified in the analysis of the height profiles. The immobilization of p24-3 on the PEI layer can also be confirmed by the increase in film roughness (RMS), which increased from 1.99 nm for the PEI layer to 7.83 nm after adsorption of p24-3. The interaction with the anti-p24 antibody caused appreciable increase in size of the globules and film roughness, as shown in Table I.

From the height profiles one may infer the film growth direction. Figure 4 shows that the mean diameter of the globular structures increased linearly with the height for the LbL film in the absence of anti-p24, while an exponential dependence was observed for the film interacting with anti-p24, where larger globular features were observed. There is a maximum size for the globules at ca. 600 nm, which may be associated with saturation of the binding sites for the antigen–antibody interaction.

Table I shows roughness, contact angle and surface energy values for PEI/p24-3-containing liposomes films in the presence and absence of anti-p24, with the contact angle measurements corroborating the AFM results in that interaction with anti-p24 altered the surface properties. The surface of p24-3-containing liposome films is hydrophobic, so that a drop of polar solvent provides a surface tension which prevents its diffusion in the pores. After adsorption of antibodies, the film with antigen p24-3 became more hydrophilic, with the contact angle for water decreasing from 70.90° to 57.79°, i.e., the film had its hydrophilicity or wettability increased. The surface free energy in Table I was calculated using the method of Owens and Wendt from the contact angle values obtained for water, ethylene glycol and diiodomethane. The results indicate an increase in surface energy owing to the antigen–antibody interaction. The surface free energy is important for biosensors, because it provides information on the possible adhesion of proteins for cell growth, ensuring compatibility between the template and the coating materials.

3.3. Immunosensors Made with LbL Films

Cyclic voltammograms for 1-bilayer PEI/DPPG liposome and PEI/p24-3 in DPPG liposome LbL films were obtained in a PBS buffer as electrolyte solution in the presence and absence of anti-p24 (1 μg mL⁻¹). For the 1-bilayer...
Figure 6. Capacitance and loss versus frequency curves for a 5-bilayer LbL film of PEI/p24-3 in DPPG liposome, in the absence and presence of different concentrations of anti-p24.

Figure 7. Capacitance and loss versus frequency curves for a 5-bilayer PEI/p24-3 into DPPG liposome-containing LbL film, in the presence (dashed curve) and in the absence (solid curve) of anti-HCV.

Figure 8. Data for the real component of the impedance projected with the IDMAP technique for the various samples (as specified in the inset). Note that the samples containing anti-p24 antibodies are distinguished according to their concentration, with a trend toward the right for decreasing concentrations. The exception is the sample with the highest concentration. The data for the non-specific antibody, namely anti-HCV, are close to the PBS buffer for that series of experiments. No labels are assigned to the axes in the plots because in this type of multidimensional projection the distance between data points represents dissimilarity among the samples.

Electrical measurements with the LbL films deposited onto interdigitated gold electrodes were performed in the presence and absence of anti-p24 and anti-HCV (hepatitis C virus) antibodies. For the 1 and 5-bilayer LbL films containing PEI and DPPG liposomes (with no antigens) there was no significant change in the capacitance and loss curves when anti-p24 was added to the buffer (result not shown). In contrast, noticeable differences appeared in Figure 6 for a 5-bilayer LbL film made with PEI/p24-3 in DPPG liposomes, where the curves for the samples containing different concentrations of anti-p24 differ from that of the buffer.

In order to check the selectivity of the biosensor, we immersed the electrode containing a 5-bilayer LbL film of PEI/p24-3 in DPPG liposomes in a liquid sample with anti-HCV antibody (1 µg · mL⁻¹). No significant change in the capacitance and loss curves was observed, as can be seen in Figure 7, thus demonstrating the specificity of the immunosensor. Furthermore, for LbL films containing PEI/DPPG liposomes, but with no antigenic peptide film containing p24-3, the anti-p24 antibody affected the voltammogram with the appearance of an oxidation peak at ca. 0.1 mV, as seen in Figure 5. This electrochemical response occurs through biorecognition toward the anti-p24 antibody, where the latter probably adsorbed on the p24-3 immobilized on the electrode, thus generating electroactive species. For the 5-bilayer PEI/p24-3 LbL films no significant change was observed in the voltammogram with anti-p24-3, probably because charge transfer was blocked due to the film thickness. For the bare electrode, the voltammogram did not show the oxidation peak with anti-p24 eliminating the hypothesis of recognition sites between carbon electrode and antibody (results not shown).
p24-3, no oxidation peak or change in capacitance and loss curves were observed as the electrodes interacted with anti-p24, for any number of bilayers (results not shown). This indicates lack of molecular recognition in the absence of p24-3.

In subsidiary experiments, we also observed that if the LbL film contained only one layer the differences would not be significant, probably because the coverage of the electrodes was not sufficient to reach high sensitivity, in contrast to the electrochemical sensor where one bilayer sufficed for detection of anti-p24.

We should mention that with both the electrochemical and the impedance spectroscopy methods, detection of the anti-p24 antibody was straightforward. In order to make the study more quantitative we employed the multidimensional projection technique IDMAP25 to treat the real component of the impedance for all samples. The results are depicted in Figure 8, which shows a clear separation from the samples containing anti-p24 antibodies from those of the buffer and from a “negative” antibody from non-specific anti-HCV. Also important is to note the trend toward the right in the plot for decreasing concentrations of anti-p24, with the exception of the highest concentration (1 μg mL⁻¹). For such high concentrations, the impedance signal was probably affected by saturation in the specific molecular recognition interactions. Using multidimensional projection as in Figure 8 is advantageous because all the data for the various samples can be analyzed in a single plot.

4. CONCLUSIONS

We demonstrated that the structure of the p24-3 peptide can be preserved when immobilized in liposomes, with which layer-by-layer (LbL) films could be produced. The biorecognition interaction between p24-3 and the anti-p24 antibody was found to cause important changes in the film morphology, with increases in the size of the globules and in film roughness. Such changes were reflected in an increase in surface energy for the films containing p24-3. This antigen–antibody interaction was exploited in sensing anti-p24 with both cyclic voltammetry and impedance spectroscopy measurements. Therefore, the results presented here amount to a proof-of-principle demonstration that LbL films made with peptides incorporated into liposomes can be used in routine assays for HIV detection.

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References and Notes


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