*2013 International Nuclear Atlantic Conference - INAC 2013*
*Recife, PE, Brazil, November 24-29, 2013*
*ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN*
*ISBN:* **978-85-99141-05-2**

# SURFACE GRAFTED POLYMER BRUSHES: POTENTIAL APPLICATIONS IN DENGUE BIOSENSORS

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## ABSTRACT

A polymer brush membrane-based ultrasensitive biosensor for dengue diagnosis was constructed using poly(hydroxyethyl methacrylate) (PHEMA) brushes immobilized onto low density polyethylene (LDPE) films. LDPE surface films were initially modified by $Ar^+$ ion irradiation to activate the polymer surface. Subsequently, graft polymerization of 2-hydroxyethyl methacrylate onto the activated LDPE surface was carried out under aqueous conditions to create patterned polymer brushes of PHEMA. The grafted PHEMA brushes were characterized by Fourier transform-infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM) and contact angle analysis. The SEM observations showed that selective surface activation with $Ar^+$ implantation and graft polymerization on the selectively activated surface had occurred. The PHEMA brushes were electrically characterized in the presence of concentrations of human immunoglobulin (IgG). The proposed amperometric biosensor was successfully used for determination of IgG in physiologic samples with excellent responses.

## 1. INTRODUCTION

Dengue (DEN) is an acute febrile infectious disease widespread in the world and is classified as neglected diseases [1]. Actually there are more than 50 million annual infections that occur in 100 countries where dengue is endemic and more than 2.5 billion people living in risk areas can contract the virus of the disease [2]. In according to WHO a rapidly spreading and a 30-fold increase in global incidence of DEN over the past 50 years have been observed [2].

The diagnosis of acute infection with DEN can be made by isolating the virus or by detecting viral genome or antigen. Serologically, a primary infection with dengue virus results in detectable levels of IgM and IgG antibodies by the third afebrile day after infection. The IgM antibodies persist for 1-2 months after infection. IgG antibodies are detected approximately 14 days after onset of primary infection. Secondary infections with DEN virus are characterized by a rapid increase in IgG antibody levels [3-5]. Nowadays, the detection of immunoglobulin’s IgM and IgG antibodies through enzyme linked immunosorbent assays (ELISA) in human serum or plasma is the main technique for the laboratory diagnosis of DEN [6-8]. Thus, the use of a reliable and sensitive rapid serological test that can simultaneously detect the presence of IgG and IgM antibodies is of great clinical utility.

Recently, polymer brushes were employed as a large surface area matrix for immobilization of a variety of biomolecules including proteins and enzymes [9-10], DNA fragments [11] and antibodies [12]. In the present work, a simply modified biosensor for the electrochemical detection of humman IgG based on anti-IgG-modified poly(hydroxyethyl methacrylate) (PHEMA) brushes grafted onto low density polyethylene (LDPE) films was reported.

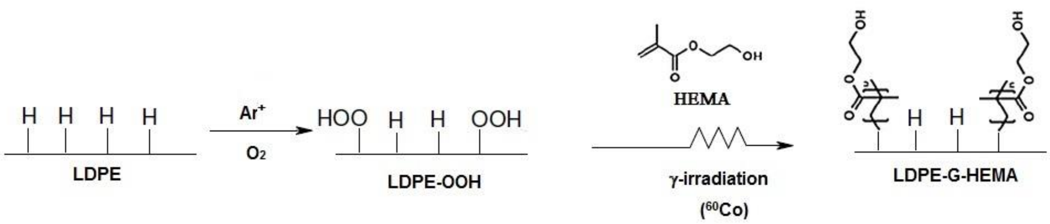
The new random copolymer, denoted as LDPE-G-HEMA, was synthesized by radical polymerization induced by gamma rays from $^{60}$Co. The successful immobilization of anti-IgG biomolecules through the polymer adlayer and the performance of the biosensor for IgG detection was demonstrated. The electrochemical monitoring of biorecognition of IgG provides a simple and cost-effective method for DEN detection.

## 2. MATERIALS AND METHODS

Low density polyethylene (LDPE, 3M) of 40 μm thickness films were placed between two polished 4'' silicon wafers and heated for 15 min at 100 ºC in a hot press under a pressure of 200 N/cm$^2$ in order to obtain flat surfaces with roughness below 20 nm. 2- Hydroxyethyl methacrylate (HEMA) was purchased from Sigma-Aldrich Co and was used without further purification.

LDPE films were cut into 3x1.5 cm$^2$, washed ultrasonically in ethanol and dried in an oven (-600 mmHg) at room temperature (25 ºC) for 12 h before use. After drying, the LDPE films were irradiated through the mask of micron filter stainless steel wire mesh (500 µm) at 25 ºC with 150 keV $Ar^+$ ions in a plasma chamber operating at approximately $10^{-5}$-$10^{-6}$ Torr and ion density of about 1.0 µA/cm$^2$ and ion fluence ranging from $10^{14}$ to $10^{15}$ ions/cm$^2$. The objective of $Ar^+$ irradiation was to deposit selectively a self-assembled monolayer of hydroperoxide initiators on LDPE polymeric surface. Then, the hydroperoxide species was used for the preparation of patterned PHEMA lines onto LDPE films.

For the preparation of patterned PHEMA brushes, LDPE films were weighed and then immersed in an solution of 40% HEMA (v/v) in an ethanol:water solution (1:1). The direct γ-irradiation ($^{60}$Co) grafting was carried out at dose 40 kGy (dose rate 6 kGy/h) under nitrogen atmosphere. The grafted LDPE-G-HEMA films were removed and then soaked overnight in ethanol: water solution (1:1) to extract the residual monomers and the homopolymer in the membranes. Afterwards the LDPE-G-HEMA films were then washed thoroughly with ethanol: water solution (1:1) in an ultrasound bath and drying in oven at room temperature (25 ºC) until a constant weight. The overall grafting yield ($G_t$) was gravimetrically calculated. The key idea of grafting PHEMA brushes onto LDPE is shown in Scheme 1.




**Scheme 1- Illustration of the reaction path for the preparation of patterned PHEMA brushes onto LDPE films.**

The chemical state environment of the ion implanted and grafted LDPE polymer surface was investigated by using X-ray photoelectron spectroscopy (XPS, ESCA 650B) employing Mg-Kα radiation. The applied power was 14.5 keV and 20 mA.

The surface morphologies of the grafted PHEMA brushes were observed by using scanning electron microscopy (SEM, Shimadzu model SS550).

The chemical microstructure of grafted LDPE sample films were verified by Fourier transform infrared (FT-IR) spectroscopy in the attenuated total reflectance mode (ATR). A Perkin Elmer model Spectrum One spectrophotometer was used to examine the functional group of PHEMA brushes at grafted LDPE surface and 32 scans were cumulated at a resolution of 4 $cm^{-1}$.

The wettability of grafted LDPE film surface was characterized by assessing water contact angle using an optical contact angle goniometer. Drops of purified water (5μL) were dropped onto the grafted LDPE surface, and the direct microscopic measurement of the contact angles was done with a goniometer. The contact angle of was averaged from tests at five different points.

The covalent immobilization of the anti-rabbit IgG with horseradish peroxidase (anti-IgG/HRP) onto PHEMA brushes was performed using carbodimide/succinimide chemistry (EDC-NHS) [13]. It is well known that EDC catalyzes the formation of amide bonds between carboxylic acids and amines by activating the carboxylic groups. The EDC reaction proceeds at room temperature and physiological pH. The covalent binding of anti-IgG occurs via lysine residues under formation of amide groups with the activated carboxylic terminal groups of the polyHEMA brushes.

The activated carboxylic groups of PHEMA brushes reacted with terminal amino groups of the antibody giving rise to stable peptide bonds. The LDPE-G-PHEMA films were incubated in a solution containing a 4:1 molar ratio of 1-ethyl- 3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Sigma-Aldrich) and N-hydroxysuccinimide-ester (NHS-ester, Sigma-Aldrich). Afterwards the activated carboxylic groups were left to react with anti-IgG/HRP (Sigma-Aldrich, 100 μg $mL^{-1}$ solution prepared in 0.1 M phosphate buffer pH 7.2 (PBS) at 4ºC overnight. The concentration was chosen considering the affinity of anti-IgG/HRP to IgG [14].

The biosensor response of the prepared biosensor to antibody was evaluated through the exposure of the PHEMA brushes containing anti-IgG/HRP to IgG in PBS solutions at concentrations ranging of 2-32 mg/mL. These concentrations cover the whole range of the normal level of IgG in human blood. IgG levels above 16 mg/mL characterize an immune response to DEN infections [15]. Amperometric measurements were performed with a model model 2612 Keithley programmable electrometer at room temperature (25ºC).

## 3. RESULTS AND DISCUSSION

The surface of LDPE-G-HEMA brushes was examined in detail to investigate the properties of the polyHEMA brushes. Fig. 1 (b) shows the scanning electron microscopy (SEM) images of the polyHEMA brushes on the LDPE surface. From these images, it appears that the polyHEMA chains became wrinkled and folded during the sample preparation. However, it is also clear that the polyHEMA chains fully coat the underlying LDPE surface homogeneously.

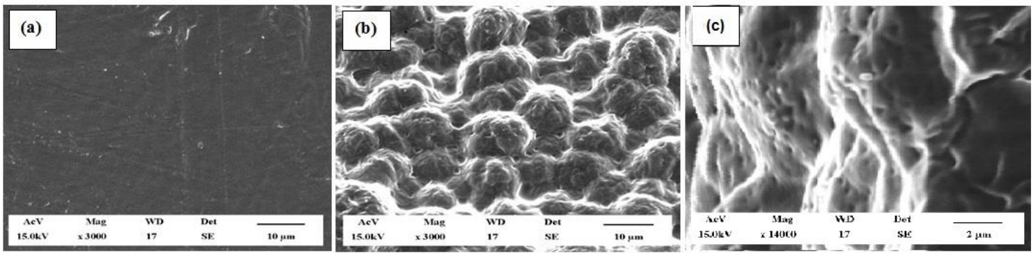



**Figure 1: SEM micrograph showing LDPE (a) and LDPE-G-HEMA (b,c) surfaces. Grafting yield (G): 20%.**

The effect of polyHEMA grafting on LDPE surface wettability are shown in Fig.2. The contact angles of the grafted sample gradually decrease from 66.43º ± 0.01 in the absence of polyHEMA to 63.89° ± 0.02 in the presence of polyHEMA at LDPE surface. This indicates that the wettability gradually increased after polyHEMA brushes grafting on LDPE surface.

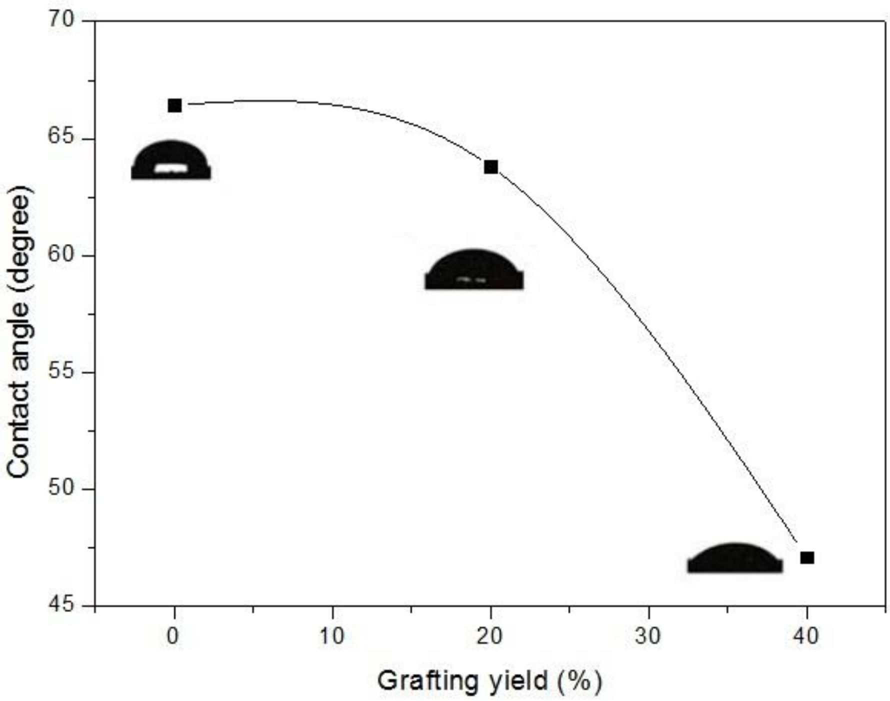



**Figure 2: S Surface contact angles of the LDPE after polyHEMA grafting.**

Further evidence for the successful grafting of the polyHEMA brushes onto LDPE surface was obtained from analyzing FTIR experiments (Figure 3). FTIR Absorption spectra of the HEMA monomer, ungrafted LDPE and LDPE-G-HEMA films are depicted for comparison in Figure 3. The FTIR spectrum of the LDPE substrate revealed three bands at 2915 $cm^{-1}$, 1471 $cm^{-1}$ and 717 $cm^{-1}$, which are due to the asymmetric and the symmetric $CH_2$ vibrations of the alkyl chain [16]. A strong absorption at 1715 cm-1 (C=O stretching) and the broad multibands at about 1000 to 1300 (sharply at 1150 due to coupling of C-O- stretching and -OH inplane bending) are showed in the spectrum of the LDPE-G-HEMA. This was evident from the LDPE surface that was covered with grafted poly(HEMA) [17].

For electrochemical characterization of IgG the polyHEMA electrodes were doped with ferrocyanide ($[Fe(CN)_6]^{-4}$)/ferricyanide ($[Fe(CN)_6]^{-3}$) redox reaction couple. The system $[Fe(CN)_6]^{3-/4-}$redox reaction couple is commonly used in electrochemistry as a mediator shuttling electrons between electroactive species dissolved in a solution and a working electrode [18-19].

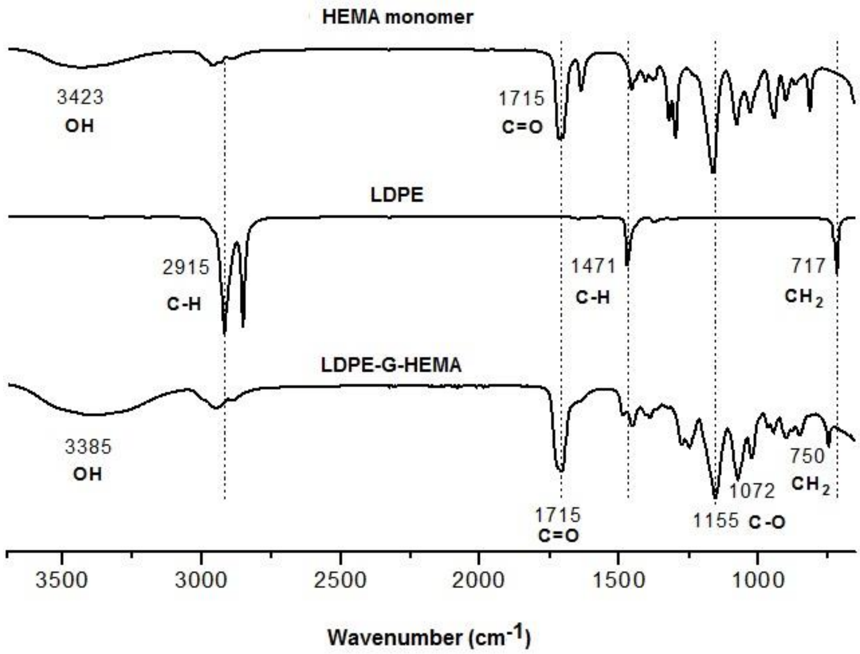



**Figure 3: FTIR Spectra of HEMA monomer, ungrafted LDPE and LDPE-G-HEMA films. Grafting yield (G): 20%.**

In this work biosensing electrodes to detection DEN virus were prepared by the direct radiation graft copolymerization of aqueous HEMA monomer onto LDPE films. Grafted LDPE films having grafting yields of 20% and 40% were obtained and used for this study. Figure 4 shows the electrical behavior of the LDPE-G-HEMA electrodes before (Fig. 4(A)) and after (Fig. 4(B) immersion in 0.1 M PBS electrolyte solution (pH 7.0) containing 0.1 mM of ferrocyanide ($[Fe(CN)_6]^{-4}$)/ferricyanide ($[Fe(CN)_6]^{-3}$) solution.

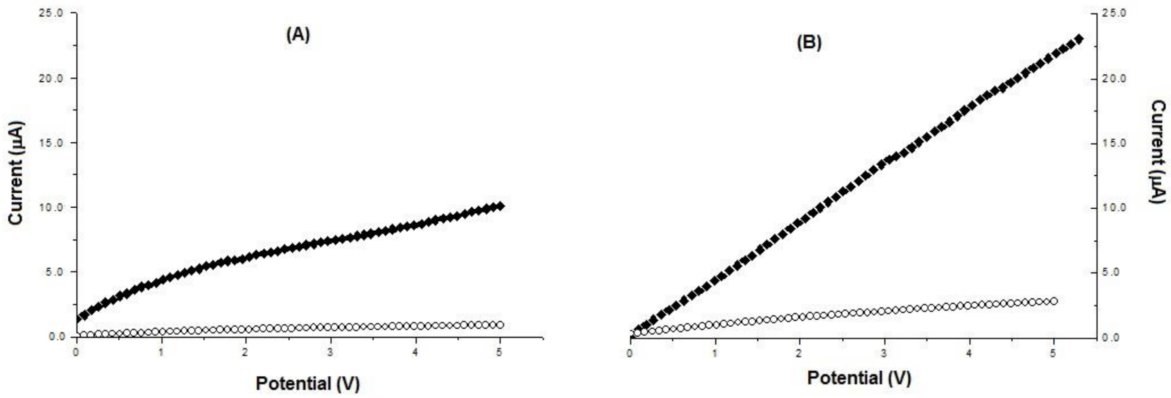



**Figure 4: Amperometric responses obtained for LDPE-G-HEMA electrodes before (A) and after immersion in 0.1 mM $[Fe(CN)_6]^{3-/4-}$ solution. The symbols (○) and (■) represents grafting yields (G) of 20% and 40%, respectively.**

The DC electrical conductivity of grafted (sample of 20%G and 40%G) was measured at room temperature (25 ºC). Fig. 4(A) shows clearly that increasing % grafting, electrical conductivity simultaneously increased. It can be seen that prepared electrodes of LDPE-G-HEMA with G of 40% possess electrical conductivity approximately 3.0 times higher than that of films with G of 20% at room temperature (25ºC). Fig. 4(B) shows the electrical conductivity of LDPE-G-HEMA electrodes with G of 20% and 40%, respectively, after immersion in $Fe(CN)_6]^{-4}/[Fe(CN)_6]^{-3}$ solution. It can also be seen that after immersion in $Fe(CN)_6]^{-4}/[Fe(CN)_6]^{-3}$ solution the electrical conductivity was significantly higher for LDPE-G-HEMA electrodes with G of 40% relatively to electrodes of G of 20% (Fig. 4(B)). The increase of electrical conductivity after grafting may be due to the free mobility of hydroxyl (OH) groups of the grafted polyHEMA brushes at LDPE surface.

The amperometric responses of the LDPE-G-HEMA electrodes after being incubated in the solution with different IgG concentration were shown in Fig. 5. As the IgG concentration is increased from 2 to 32 mg/mL, the current increased correspondingly for the both G of 20% and 40%, respectively. There is a good relationship between the increase in current and the concentration of the IgG in the concentration range studied in this case.

Fig. 6 shows that are a good linear relationship between the peak current and the concentration of the IgG in the concentration range studied in this work. Each data in Fig. 6 is an average value of four measurements. However, as the concentration of the IgG is larger than 15mg/mL, such a linear relationship no long exists and saturation in current was observed, as the concentration of the IgG is further increased. This may be due to the fact that most of the immobilized anti-IgG molecules have been complexed with the IgG molecules, as the concentration of the IgG reaches 15 mg/mL. There are very few unoccupied sites for the bonding of the additional IgG molecules.

The LDPE-G-HEMA electrodes with G of 40% exhibit superior sensitivity to IgG relatively to LDPE-G-HEMA electrodes with G of 20% (Fig. 6) and shows better performance for use as biosensors for DEN diagnosis. From the experimental results, the detection limit was estimated to be 3.5 µA/mg/mL, and the linear range was from 2 to 15 mg/mL.

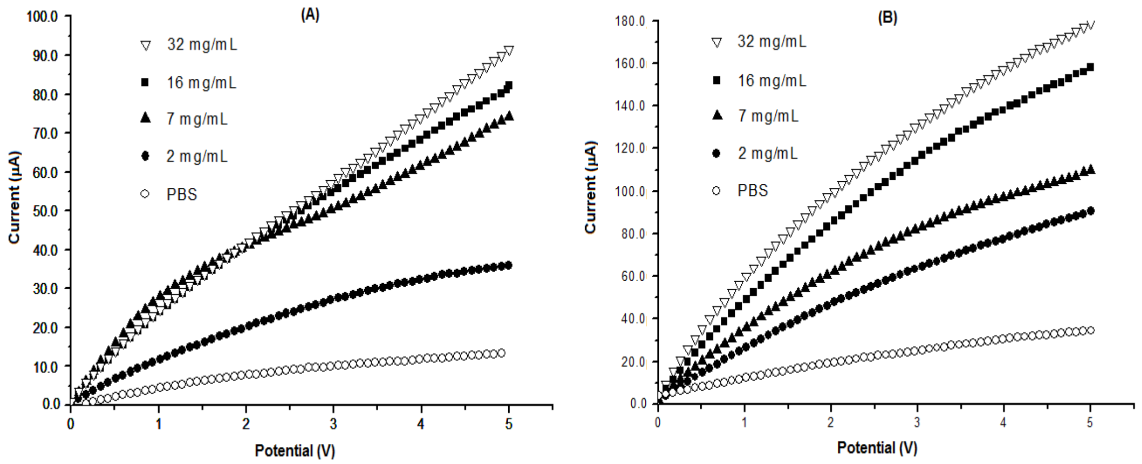



**Figure 5: Amperometric responses of LDPE-G-HEMA electrodes of G 20% (A) and 40% (B) after being incubated in different IgG concentration in 0.1 M pH 7.0 PBS solution at incubation temperature of 25ºC for 2 min.**

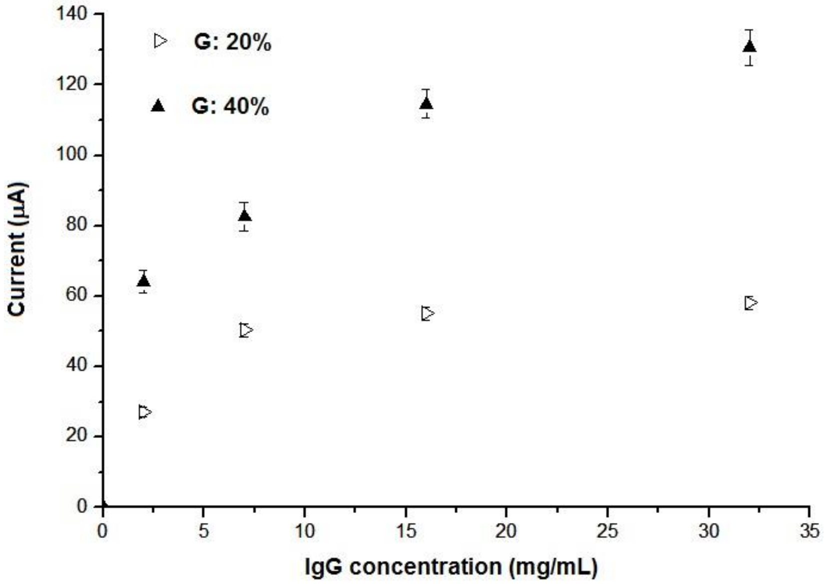



**Figure 6: Amperometric response of LDPE-G-HEMA electrodes at G of 20% and 40% to IgG concentration in 0.1 M pH 7.0 PBS solution at incubation temperature of 25ºC for 2 min. Applied potential of 3V.**

## 3. CONCLUSIONS

The surface of LDPE films were succefully grafted with polyHEMA brushes through simultaneous gamma rays irradiation. To confirm the surface structure of the grafted films, they have been examined by infrared spectrophotometry. After grafting, the spectra show the characteristics bands of polyHEMA. Hydrophilicity of LDPE-G-HEMA is improved, compared to that of ungrafted LDPE. This leads to the conclusion that this grafting as a thin layer is covalently bonded to the surface as is shown by SEM micrographs. The result presented here demonstrates the developed biosensor based on polyHEMA brushes is highly sensitive and stable in electrochemical measurement of IgG. The amperometric response was dependent significantly on the grafting yield and concentration of potassium ferricyanide. From the foregoing report, we predict the polyHEMA brushes with immobilized anti-IgG will have significant electro-analytical efficiency for DEN diagnosis. The sensitivity and the linear response range of the biosensor with G of 40% to IgG were better than that of G 20%.

## ACKNOWLEDGMENTS

This work was supported with research fund of CNPq, Capes and Finep. The authors wish to thank the Center for Radiation Technology (CTR) of IPEN-CNEN/SP by facilities for using the multipurpose reactor $^{60}Co$.

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