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Electrospray Induced Surface Activation of Polystyrene Microbeads for Diagnostic Applications

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Electrospray is generally regarded as a "soft" technique due to an absence of any observable molecular fragmentation or destruction. This study reports on a novel and easy way to induce surface activation on the surface of polystyrene microbeads through electrospray deposition into a grounded aqueous electrolyte solution bath. This process, nicknamed EISA, which stands for Electrospray Induced Surface Activation, proposes that when a highly-charged microbead formed by the electrospray process sinks into the aqueous electrolyte solution, it behaves like a highly charged spherical capacitor that discharges in the conductive liquid. The energy released leads to a breakup of the polystyrene surface bonds and water oxidation with oxygen. Further reactions produce a carboxylated surface that was confirmed by X-ray photoelectron spectroscopy (XPS) and protein coupling. An immunoassay based on these modified microbeads was also developed and presented for use in Syphilis detection, demonstrating a reliable signal-to-noise ratio between positive and negative results.

Introduction

Polystyrene (PS) microbeads were first reported in the early 1990s, as a product of suspension/dispersion polymerization.[1,2] Around this same time, their use in biomedical applications such as immunoassays, cell separation and in site-specific drug delivery was first proposed.[3,4] However, the first report pertaining to the morphological characterization of electrosprayed polystyrene microbeads did not appear until as late as 2006.[5] Currently, microbeads are widely used for a variety of different laboratory procedures and protocols, due to their availability with different functional groups and codification with fluorescent dyes or quantum dots.[6-12] They are also available with application-specific coatings, or embedded with magnetic nanoparticles that allow their manipulation during specific cross-linking processes and procedures.[7,13,14] Surface functionalization of PS microbeads is usually achieved through grafting or copolymerization techniques, which can be used to add carboxyl, amino, hydroxyl and/or thiol groups.[15-17] Alternative surface modification techniques are also available in the form of UV irradiation and plasma treatment.[18,19] Manso et al.[20] have also reported the plasma-discharge-assisted deposition of monomers in a fluidized bed, which permits surface functionalization of polymeric beads with carboxylic groups. Further processing allows chemical attachment of biological molecules to those carboxyl (-COOH) sites for specific functions, such as the attachment of EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and S-NHS (sulfo-N-hydroxysuccinimide) to enhance coupling efficiency in carbodiimide coupling reactions.[21] In this process, a semi-stable NHS or Sulfo-NHS ester is formed that can react with primary amines (-NH2) to form amides. This process is being broadly used to cross-link proteins to micro well plates for diagnostic purposes, such as in ELISA (enzyme-linked immunosorbent assay) tests.

Although currently considered a practical tool for nanotechnology,[22] the electrospray (ES) technique originated with the studies of Lord Rayleigh in 1882 into ejection instabilities in charged liquids.[23] Later, practical applications of this process were patented by J. F. Cooley and W.J. Morton,[24,25] with supplementary explanation of this phenomenon provided by J. Zeleny in 1914;[26] however, it was not until 1964 that the physical principles of electrified liquid capillaries were firmly established by Taylor.[27] In 1989, J. B. Fenn first proposed the use of ES ionization for the mass spectrometry of large biomolecules due to the technique's relatively "soft" characteristics. [28] In doing so, he demonstrated the ability of ES to produce multiple intact ions, without any evidence of the molecules being fragmented. In recent years, more intensive studies of electrospray droplet impact (EDI) on PS and PMMA surfaces has demonstrated that these polymer surfaces do not suffer from any discernible chemical modification or grafting when etched by EDI.[29,30]

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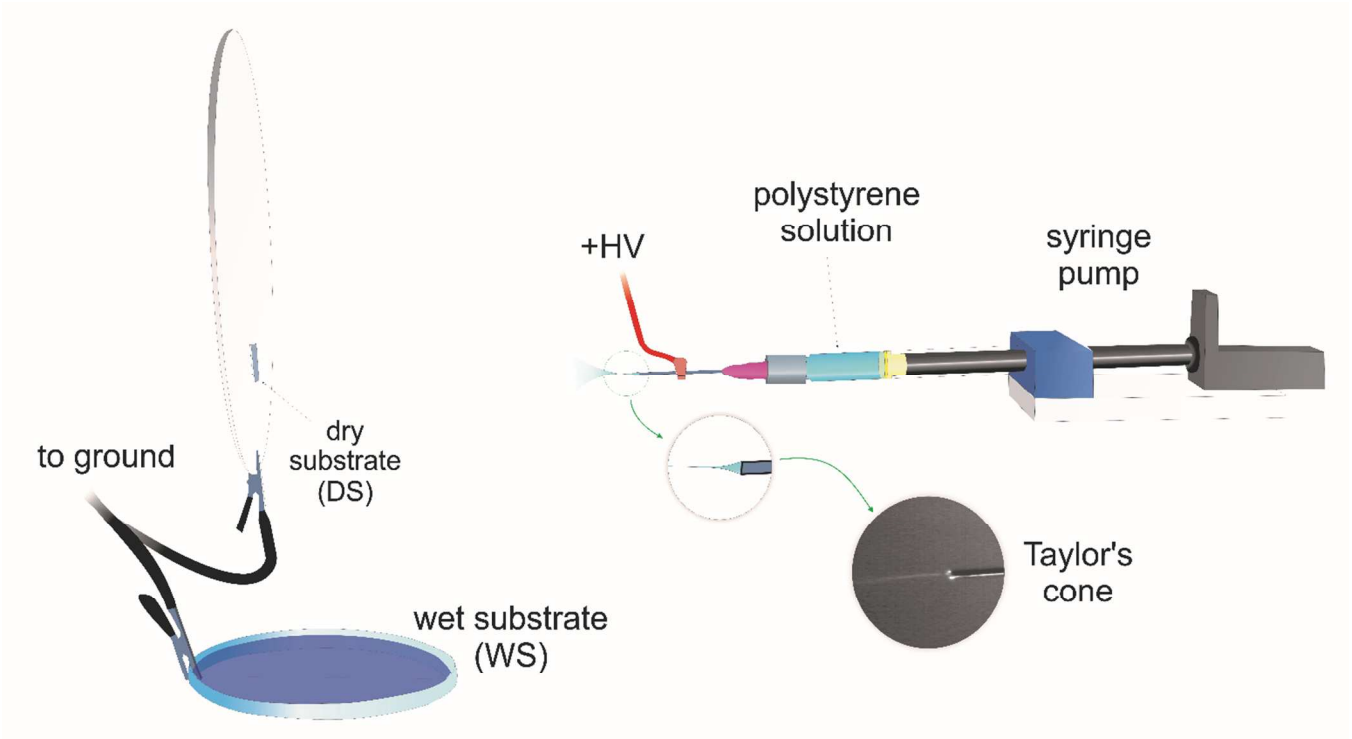

Figure 1 – Experimental electrospray setup showing two possible configurations: Deposition on a Dry Substrate (DS) and deposition on a Wet Substrate (WS). The insets present both a pictography and a photography of the Taylor cone which stability was used as a parameter during deposition.

This study expands on this earlier work in reporting a novel and simple method to achieve surface chemical modification and grafting of polystyrene microbeads by electrospraying them into a grounded aqueous electrolyte solution bath.

Experimental

Polystyrene (PS) Synthesis and Solution Preparation

Solutions were prepared by dissolving quantities of between 0.6 and 2.0 wt% Innova N1921, a commercially-sourced polystyrene, in chloroform PA ACS from Vetec Química Fina. The Innova PS selected has a Mn of between 175.000 and 185.000g/mol, and a polydispersity of between 1.6 and 1.8.

Electrospray (ES) Deposition

Microbeads of PS were produced using the ES setup depicted in Figure 1, which consists of a Harvard® 11 Plus syringe pump, a METEC® high voltage source (40kV), and two different substrates for particle collection. The polymeric solution was contained in a Hamilton Gastight® #1750 syringe, driven by a syringe pump through a rectified 24G¾ (0.55 x 20 mm) chirurgical stainless steel needle, connected to the high voltage source. The pumping rate was maintained at 12 µl/min, and the high voltage was adjusted until a stable Taylor cone-jet was obtained;[31] as monitored by a Photron Fastcam® PCI-R2 equipped with a macro lens. The voltage during deposition was kept within 5.0 + 0.9 kV, under standard room temperature and pressure conditions.

Depositions were performed onto either a dry substrate (DS) or wet substrate (WS) by means of two different experimental configurations. The dry configuration (DS) utilized conductive Flexitec, or fluorinated tin oxide (FTO), coated glass slides as a conductive surface, which was electrically connected to a grounded sample holder in order to reduce field distortions and therefore guarantee deposition of microbeads on the surface. In the wet configuration (WS), the substrate consisted of a 150 mm diameter Petri dish filled with a 0.2 M sodium bromide (NaBr) solution (bi-distilled water-ethanol 50 vol%), which was grounded through a submerged stainless steel mesh. Ethanol was added to this solution to reduce the surface tension, thus allowing the microbeads to sink and preventing the formation of a dry skin of beads on the liquid surface. Beads produced by this wet configuration were held in the WS until use, at which point they were filtered and washed six times with an excessive amount of bi-distilled water to remove any NaBr residues. For

both configurations, the distance between the needle tip and the substrate was set to 25 cm.

Morphological Characterization

Morphological characterization of the microbeads was performed by scanning electron microscopy (SEM), using a JEOL Model JSM6360LV at 15 kV. All samples were sputter coated with gold to prevent charge buildup during secondary electron imaging.

Surface Characterization

Prior to analysis, all samples were placed on SiO_2 substrates and transferred to a vacuum desiccator to ensure dryness as well as to avoid further environment moisture adsorption. Chemical surface modification was then characterized using a VG Microtech ESCA3000 X-ray photoelectron spectrometer, operating at 15 kV, 15 mA and 10^{-9} mbar of vacuum.

Protein Linking Survey

A biological activity survey was conducted to confirm the coupling capabilities of the microbeads, for which two different reporter proteins were used.[33] The first of these was a goat antibody, anti-human Ig, conjugated to a fluorescent reporter Phycoerythrin (AbPE) (Moss Substrates, Pasadena, CA-USA). The other, was a recombinant auto-fluorescent green fluorescent protein (GFP) from the Aequorea Victoria jellyfish.[32]

Linking of these proteins was conducted in a Millipore model MABVN1250 96 microwell filter plate. Initially, only about 10^7 microbeads were washed with 18.2 MΩ.cm water and PBS, to which 80 µl of NaH_2PO_4 pH 7.6 buffer solution was added to adjust the pH for carbodiimide reaction. Solutions of N-hydroxysulfosuccinimide (S-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were prepared to a final concentration of 50 µg/µl by diluting with water, and 10 µl of each was added to the solution in the wells to enhance the protein coupling efficiency. The reaction was left to proceed for 20 minutes at 25 ºC, while being mixed in a horizontal shaker (Infors HT Multitron) at 300 rpm.

After washing each well with PBS Buffer, 100 µl of either GFP 250 µg/ml or AbPE 250 µg/ml was added to ensure saturation of all active sites on the microbeads. The conjugation reaction was then allowed to proceed for 2 hours at 37 ºC, with 300 rpm agitation. The remaining supernatant containing non-linked proteins was removed by successive washings with PBS Buffer, and the microbeads were then immobilized on a microscope slide using 2 % agarose and analyzed using a Leica SP5 confocal laser microscope.

Activation confirmation was provided by fluorescence images acquired using the excitation provided by a 488 nm Argonium Laser line at 10 % power and a 10 Hz scanning rate. Detection was performed from 500 to 530 nm for the GFP-linked microbeads, and from 560 to 630 nm for the AbPE microbeads. Bright field images of both GFP and AbPE microbeads were also obtained using the DIC (interferential contrast) imaging mode of the microscope.

In order to ensure a comparative test, this entire process was applied to microbeads produced by both the DS and WS methods described in Section 2.2.

Antigen Coupling to Microbeads

Recombinant antigens from Syphilis TP17 (#TRP-241) and Syphilis TP47 (#TRP-243) were acquired from Prospecbio (Rehovot, Israel), and in combination with microbeads and secondary antibodies, are able to perform detection of syphilis antibodies in serum samples.

The coupling of specific proteins to microbeads requires a surface chemical activation that allows for peptide ligation between the carboxyl groups of microbeads and the amine groups of the protein. The microbeads were therefore homogeneously mixed at room temperature using a vortex (Ika-Works Inc, USA) and an ultrasonic cleaner (Cole Parmer, Niles, IL). For coupling, approximately 10^6 microbeads were added into each well of a 96-well polystyrene microplate with a 1.2 µm filter (Millipore Inc., USA). The suspensions were then washed twice with 200 µl of a 100 mM sodium phosphate pH 6.3 activation buffer, which was subsequently removed using a 96-well purification module (QIAvac 96, Qiagen Inc., Germany) and a vacuum pump (MaxPump, Applied Biosystems, USA). The microbeads were then suspended using 80 µl of activation buffer, and activated by the addition of 10 µl of a 50 mg/ml solution of N-hydroxysulfo-succinimide (Thermo Scientific, USA) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Thermo Scientific, USA). The activated suspension was then incubated at 300 rpm for 20 minutes at 25 ºC, after which it was washed twice with 100 µl of coupling buffer (50 mM sodium phosphate pH 7.5). A 100 µl aliquot of a 25 µg/ml protein solution in a coupling buffer was then added to the activated microbeads suspension. Both the microbeads and antigen solution were then incubated at 300 rpm for 2 hours at 37 °C, after which the solution was washed three times using 100 µl of blocking buffer (phosphate buffered saline 1x, 250 mM Tris-HCl, 0.02 % tween, 1 % BSA, and 0.01 % sodium azide). The coupled microbeads were then transferred from the plate well to a 1.5 ml polypropylene tube using 200 µl of blocking buffer. They were then stored in darkness at 2–8 °C until required for use. Coupled microbeads in a 1:400 (401) concentration sample were counted using Coulter Z2 counter (Beckman Coulter, Fullerton, CA).

Immunoassay

As described in Section 2.6, well plates containing microbeads were all first washed with 200 µl of assay buffer in a vacuum device. A quantity of approximately 10^5 coupled microbeads was then added to a 50 µl sample diluted to 1:100 in assay buffer in each well. The plate was then incubated at 300 rpm for 15 minutes at 37 °C, after which it was washed twice with 200 µl of assay buffer. Next, 100 µl of goat anti-human IgG conjugated to Phycoerythrin (Moss Subtrates Inc., Maryland, USA) diluted in assay buffer was added and incubated at 300 rpm for 15 minutes at 37 °C, after which it was again washed with 200 µl of assay buffer. A further 200 µl of assay buffer was then added after washing to facilitate recovery of the microbeads. Results of the median fluorescence intensity (MFI) for each microbead sample were quantified using 96-well black polystyrene plates (Greiner Bio-One Inc.), using a Synergy H1 hybrid microplate reader (BioTek Instruments Inc., USA) set at 480 nm of excitation, 578 nm of emission and 100 nm of gain.

Assays were performed using serum samples from the AEQ serum panel of the Technology Institute for Immunobiologicals (Bio-Manguinhos, Rio de Janeiro, Brazil). This is a certified panel, which has its serology confirmed by commercial diagnosis kits. Receiver operating characteristic (ROC) and scatter plot analyses were also conducted to determine the assay cut-offs and diagnostic performance. The GraphPad Prism 6 software package (Graph Pad Software, Inc., San Diego, CA,

USA) was used to generate the graphs, after all background signals were subtracted from data.

Results and Discussions

Morphological Characterization

The morphology of both the DS and WS microbeads was observed to vary from almost perfect microspheres 10 µm in diameter at 0.6 wt%, to rough microbeads at 1.0 wt%; eventually forming raisin-like structures nearly 20 µm in size at 2.0 wt%. Figure 2 shows a SEM image of the 0.6 wt% microbeads produced by the DS electrospraying configuration, the morphology obtained with this concentration being selected for further testing.

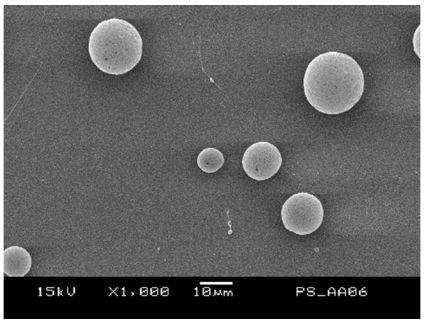

Figure 2 - SEM images of 0.6 wt% PS microbeads produced by the DS electrospraying configuration. The average diameter of these particles is 10 µm

Surface Characterization

A comparative XPS study of the microbeads produced by DS and WS electrospraying was made, with Figure 3 showing the C1s region of both samples. In the case of the DS microbeads, the spectrum obtained reveals a characteristic C1s peak corresponding to C-C and C-H bonds, and a shake-up peak originating from π-π* transitions in the aromatic ring.

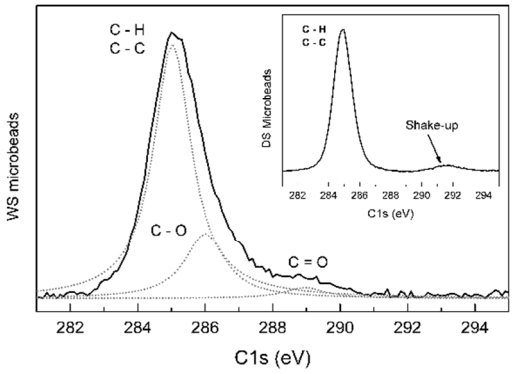

Figure 3 - XPS C1s spectra of WS microbeads and (inset) DS microbeads. The dotted lines present the fittings corresponding to the C-H/C-C peak, the C-O peak and the C=O peak.

Conversely, this shakeup peak is not visible in the spectrum of the WS sample; and instead, it is possible to observe both the carbon-oxygen double bond of the carbonyl group (C=O) peak, as well as the carbon-oxygen single bond (C-O) peak.
This provides strong evidence of a chemical reaction at the polystyrene surface, resulting from an attack on the polymer backbone leading to formation of hydroxyl, carbonyl, and carboxyl groups.
Although sodium bromide salt was used in the experiment to ionize the liquid collector plate solution and facilitate electrospray deposition, salt residues were not detected in the chemical analysis of any of the microbead samples.

Protein Linking Survey

In order to confirm the presence of carboxylic surface activation, a fluorescent protein linking survey was conducted. Figure 4 shows a confocal laser microscopy image of a WS microbead coupled with GFP, in which it is possible to observe fluorescence on the exposed microbead surface. This suggests that the process only affects the external surface of the microbeads, with similar results obtained in the case of AbPE coupling on WS microbeads. This nonetheless confirms that carboxyl groups are active on the microbead surface, thus leading to carbodiimide coupling reactions. Fluorescence signals were not observed with DS microbeads subjected to the same procedure; indicating that they are unable to retain either GFP or AbPE, and therefore do not have carboxylated surfaces. This can be easily explained by the absence of carboxyl groups on the DS microbeads, which means they do not undergo any chemical reaction during the DS electrospray deposition process.

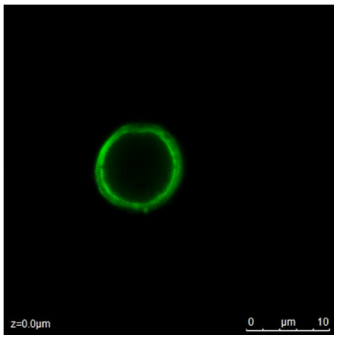

Figure 4 – Confocal cross-section of a WS deposited microbead coupled with green fluorescent protein (GFP) indicating a continuous activation at the surface.

Reaction Hypothesis

Based on the results of both the XPS and protein linking survey, it is assumed that the PS surface functionalization occurs by a chemical reaction that happens when the charged microbeads sink in the grounded aqueous electrolyte solution (WS configuration). This is depicted in Figure 5, in which the highly positively charged microbead actuates as an anode promoting the oxidation of water according to $2H_2O \rightarrow O_2 + 4\ H^+ + 4\ e^-$ (1,23V) and generating free oxygen at the beads surface, upon sinking into the ionized aqueous solution. The high electrical field developed in such a configuration should provide enough energy to induce electrochemical reactions that disrupt the typical PS surface-bonds. We believe that the positively charged PS chains are reduced according to: $(PS\bullet)^+ + e^- \rightarrow PS\bullet$. Then the free radical PS chains react with the produced oxygen leading to a polar PS surface with the presence of both double and single carbon-oxygen bonds. This ultimately leads to a carboxylated surface, which was confirmed by the results of XPS and protein coupling. Furthermore, this hypothesis is also consistent with previous reports regarding the plasma treatment of PS surfaces.[18,19]

Calculations were performed to estimate the energy existing on each microbead. They were based on the assumption that the whole charge from the electrical current of the process is equally distributed among all electrosprayed microbeads ejected over a given period of time, and that no charge losses occur during flight. As each bead sinks into the ionized liquid, a small spherical charged capacitor is formed in which the positively charged bead surface acts as a positive plate and polarized water molecules in solution serve as a negative plate. If we assume that this plate separation is the Helmholtz layer distance, as defined by the O^{-2} radii in the water molecule (0.14 nm), then it is possible to estimate the stored energy of the capacitor, and therefore the energy released upon discharge.

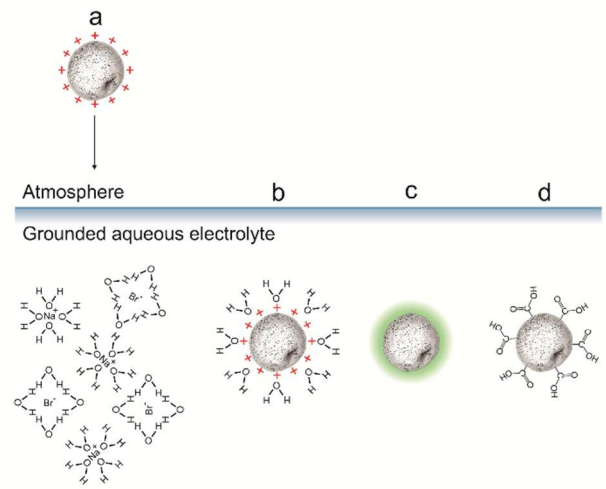

Figure 5 - Schematic depicting the proposed electrochemical reaction mechanism: (a) Highly-charged microbeads approach the aqueous electrolyte solution bath; (b) the microbeads sink rapidly being solvated by water molecules; (c) a water oxidation reaction generates free oxygen at the bead surface and disrupting the typical PS surface bonds; (d) oxygen is incorporated into the polymer chain as carboxyl groups.

From the electrospray regime of I. Marginean et al.,[34] at a pumping rate of 2 µl/min and voltage of 3.8 kV, a current of 150 nA was obtained. The increased flow rate, as well as the higher voltage used in the present study, ensures that the current levels are about one-order of magnitude higher. Assuming charge conservation, we can therefore estimate the individual charge of a single 10 µm bead, using a 1 µA current and a 0.6 wt% concentrated solution, to be approximately 310 pC. If each bead is fully submerged in the ionized WS, this leads to the formation of a spherical capacitor of about 20 pF (using vacuum permittivity). This, in turn, creates an estimated stored energy of 2.4 x 10^{-9} J or 1.5 x 10^{10} eV. Assuming this capacitor is fully discharged, it is more than capable of releasing enough energy to break 3.5 x 10^{9} H-C bonds or 4.2 x 10^{9} C-C bonds, and even the 2.4 x 10^{9} C=C bonds in polystyrene, as well as 3.2 x 10^{9} H-O bonds in water. If this energy is equally distributed to break carbon bonds and water molecules it is possible to roughly estimate a reaction on 32% of the available sites on the bead surface. Such high coverages were confirmed by fluorescence intensity measurements on WS obtained beads, with different solutions, when compared with standard commercial beads.[35]

This functionalization reaction does not occur during deposition on solid substrates, as the charge immobility on the PS microbead surface restricts discharge to the contact point. Consequently, full discharge can only take place very slowly to the atmosphere. This remaining charge is indirectly observable on the larger-sized dispersion depositions, as shown in Figure 6, in which the larger microbeads repel and scatter the smaller microbeads.

Immunoassay

The ultimate objective of this study was to evaluate the performance of antigens coupled to PS microbeads. The ideal antigen coupling conditions for the microbeads produced were determined on the basis of prior experience with the Luminex system (Luminex Corp., TX, USA).

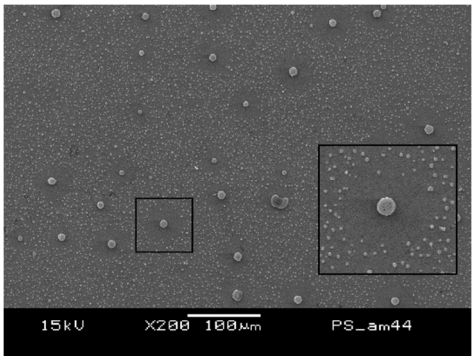

Figure 6 - Polystyrene beads deposited on a solid substrate evidencing the bead charging. Smaller beads were scattered by the charge of bigger beads. The insert to the right shows a magnified view of the selected area to the left of center.

To achieve this, two commercial recombinant antigens for syphilis disease were coupled to the microbeads; along with two concentrations of anti-human IgG conjugated to (PE) Phycoerythrin and three different quantities of microbeads to create a positive and negative pool of syphilis samples diluted to 1:200, as shown in Figure 7. The results of this immunoassay study demonstrate that 50,000 microbeads per well is sufficient to provide satisfactory disease detection. In comparison, anti-human IgG conjugated to (AbPE) Phycoerythrin at a 1:100 dilution presented a higher fluorescence signal than the same serum sample diluted to 1:1,000, thus increasing the signal to noise ratio by more than three times.

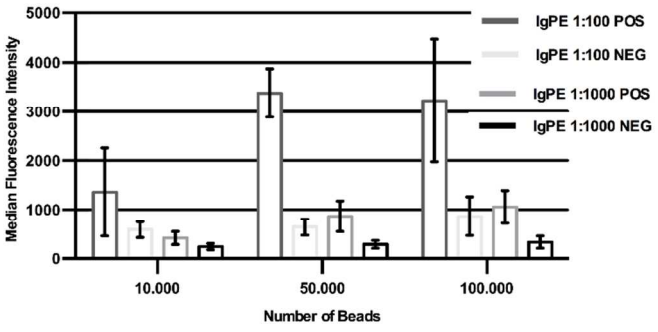

Figure 7 - Median fluorescence intensity (MFI) of the immunoassay between syphilis antigen TP17 coupled to microbeads and both positive and negative samples. Different amounts of microbeads per well were tested: 10,000; 50,000 and 100,000. Anti-human IgG conjugated to (PE) Phycoerythrin was evaluated at 1:100 and 1:1,000.

For a preliminary evaluation of the commercial recombinant antigens for syphilis coupled to microbeads, a 1:200 negative pool and 1:200 positive pool of sample were used to evaluate

the antigen-antibody reaction. On the basis of their recombinant membrane proteins, they were subsequently designated as "TP17" and "TP47", as shown in Figure 8. Note that the "TP17" antigen shows a signal-to-noise ratio increase of 783 times, whereas the "TP47" exhibits only a ten-fold increase and also demonstrates a reliable separation between the positive and negative samples.

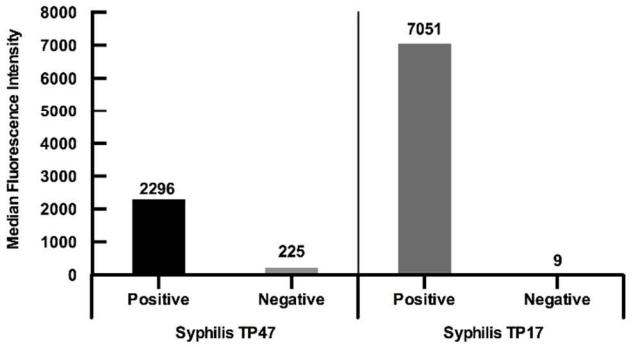

Figure 8 - Median fluorescence intensity (MFI) of positive and negative 1:200 samples in reaction with "TP17" and "TP47" antigens coupled to microbeads. A 1:100 AbPE antibody was used.

The area under the ROC (Receiver Operating Characteristic)[36] curve, which evaluates a diagnostic test's capacity for detecting a disease; and the scatter plots showing the dispersion of fluorescence in different samples, are shown in Figure 9.
During tests, eight different positive and negative samples were evaluated, obtaining 100 % sensitivity and 100 % specificity for both antigens. It is also worth noting that the difference in the signal range between these two antigens is quite significant.

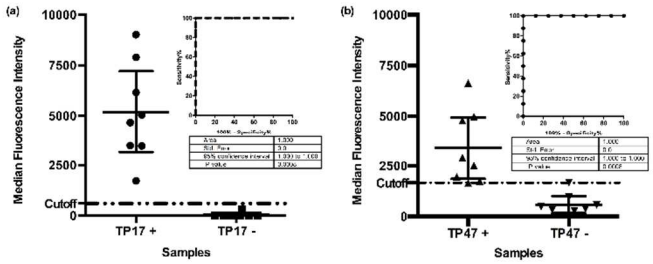

Figure 9 – Scatter plot graph for syphilis antigen "TP17" (a), and scatter plot for syphilis antigen "TP47" (b) with their respective area under the ROC curve plots in the insets.

The testing of syphilis TP47 and TP17 antigens enabled a comparison of their performance using identical samples from the AEQ panel, as shown in Figure 10. In this, the TP17 antigen can be seen to produce a better separation between the negative and positive samples, due to its higher signal-to-noise ratio.

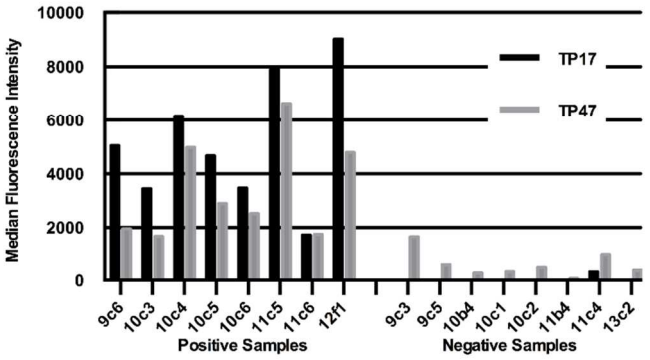

Figure 10 - Comparison of the median fluorescence intensities (MFI) of TP17 and TP47 antigens coupled to microbeads. Eight different 1:200 positive and negative samples show the difference in fluorescence range for each antigen.

Conclusions

This study reports on a novel and easy way to induce surface activation on polystyrene microbeads through electrospray deposition into a grounded aqueous electrolyte solution bath. This process nicknamed EISA which stands for Electrospray Induced Surface Activation, challenges the perception of electrospray as a "soft" technique whenever its deposition occurs under the very specific conditions described in this report.
Carboxylation of the microbead surface was confirmed by XPS, and by fluorescent protein linking (GFP and AbPE) through carbodiimide coupling reactions. The chemical differences between microbeads deposited onto a wet substrate (WS) and those deposited on a dry substrate (DS) arise due to a localized polystyrene chain oxidation on the microbead surface. The result of this process presents a great deal of similarity with oxygen plasma-treated polymer surfaces. An estimate of the stored energy in each microbead, assuming full discharge of a hypothetical spherical capacitor, indicates that there is enough energy to break about 10^9 typical polystyrene bonds.
Microbeads obtained by this new process, coupled with syphilis TP47 and TP17 antigens were shown to be able to distinguish between pools of positive and negative disease samples. These results are in accordance with the sample panel. Based on these results, microbeads coupled with proteins clearly show great potential for use in bioassay applications, such as immunodiagnostics.

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Notes and references

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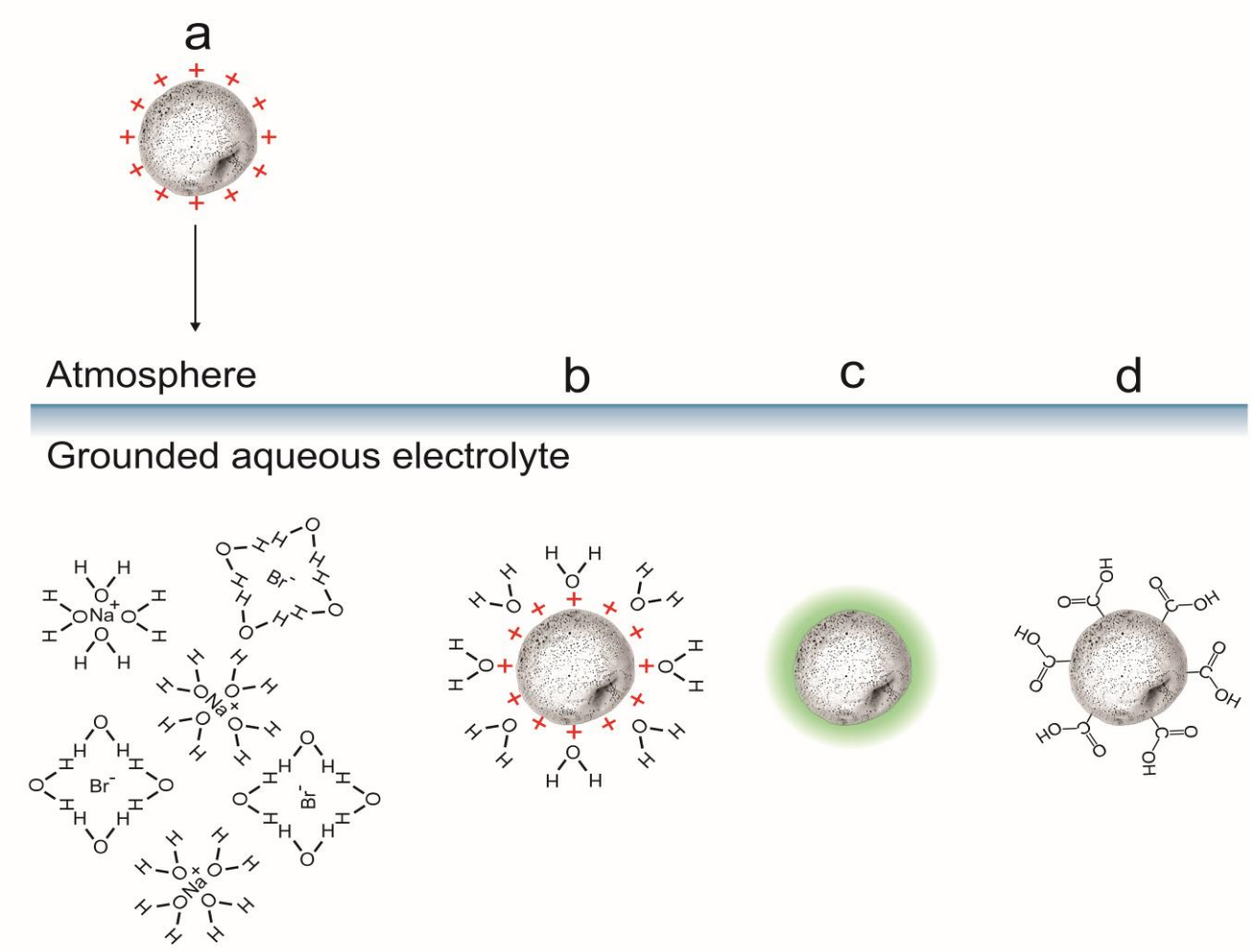

Schematic depicting the proposed electrochemical reaction mechanism: (a) Highly-charged microbeads approach the aqueous electrolyte solution bath; (b) the microbeads sink rapidly being solvated by water molecules; (c) a water oxidation reaction generates free oxygen at the bead surface and disrupting the typical PS surface bonds; (d) oxygen is incorporated into the polymer chain as carboxyl groups.