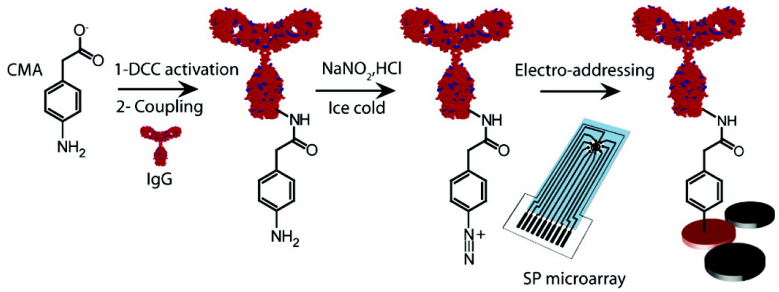
Article

# Diazonium–Protein Adducts for Graphite Electrode Microarrays Modification: Direct and Addressed Electrochemical Immobilization

Benjamin P. Corgier, Christophe A. Marquette, and Loc J. Blum

*J. Am. Chem. Soc.*, **2005**, 127 (51), 18328-18332• DOI: 10.1021/ja056946w • Publication Date (Web): 02 December 2005






## More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 22 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 12/02/2005

# Diazonium−Protein Adducts for Graphite Electrode Microarrays Modification: Direct and Addressed Electrochemical Immobilization

Benjamin P. Corgier, Christophe A. Marquette,* and Loïc J. Blum

*Contribution from the Laboratoire de Génie Enzymatique et Biomoléculaire, UMR 5013 EMB2, CNRS Université Claude Bernard Lyon 1, Bât CPE, 43, boulevard du 11 Novembre 1918, 69622 Villeurbanne, Cedex, France*

Received October 11, 2005; E-mail: christophe.marquette@univ-lyon1.fr

***Abstract:*** Diazonium cation electrodeposition was investigated for the direct and electro-addressed immobilization of proteins. For the first time, this reaction was triggered directly onto diazonium-modified proteins. Screen-printed (SP) graphite electrode microarrays were studied as active support for this immobilization. A 10-microelectrode (eight working electrodes, 0.2 $mm^2$ each; one reference; and one auxiliary) setup was used to study the addressing possibilities of the method. These electrode microarrays were shown to be able to covalently graft diazonium cations through electrochemical reduction. Cyclic voltammetry and X-ray photoelectron spectroscopy were used to characterize the electrochemical grafting onto our SP graphite surface and suggested that a diazonium monolayer was deposited. Rabbit and human immunoglobulins (IgGs) were then chemically coupled to an aniline derivative (4-carboxymethylaniline), followed by diazotation to form an aryl diazonium function available for the electrodeposition. These modified proteins were both successfully electro-addressed at the surface of the graphite electrodes without cross-talk or interference. The immuno-biochip obtained using this novel approach enabled the specific detection of anti-rabbit IgG antibodies with a detection limit of 50 fmol of protein. A promising strategy to immobilize markedly different biological entities was then presented, providing an excellent spatial specificity of the electro-addressing.

## Introduction

Chemical modification of surfaces has proven to be one of the best strategies to ensure the covalent immobilization of a large scale of either organic or biological molecules. Indeed, following the extended use of molecule adsorption onto activated polystyrene surfaces (96-well plate material), the coming of microarray developments has generated a dramatic increase in surface chemistry research programs. Thus, silane on glass[1−5] or silicon[6,7] and thiol on gold[8−11] were the first to be brought to the forefront because of their well-known chemistry. However, these techniques, when used for microarray developments, always require a physical addressing of the molecules of interest following the surface modification, i.e., spotting of nanodrops or photolithography technologies.

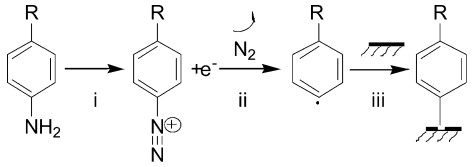
Other solutions to obtain the precise localization of immobilized molecules on a surface include the use of active materials such as electrode arrays acting as catalyst of the immobilization reaction. This is mainly obtained through the electropolymerization of insoluble polymers (polypyrrole,[12−14] polyaniline[15,16] and derivatives), entrapping or supporting the molecule of interest.

A step further could be achieved by the use of aryl diazonium cations to perform a direct electrochemical grafting at the surface of a conducting electrode[17−19] (Scheme 1). Diazonium-based procedures offer the advantages of simplicity, efficiency, and speed of the chemistry involved: (i) the diazotation reaction of an aniline derivative leads easily to the formation of an aryl

(1) Kallury, K. M. R.; Krull, U. J.; Thompson, M. *Anal. Chem.* **1988**, *60*, 169−172.
(2) Maskos, U.; Southern, E. M. *Nucleic Acids Res.* **1992**, *20* (7), 1679−1684.
(3) Gray, D. E.; Case-Green, S. C.; Fell, T. S.; Dobson, P. J.; Southern, E. M. *Langmuir* **1997**, *13* (10), 2833−2842.
(4) Okamoto, T.; Suzuki, T.; Yamamoto, N. *Nat. Biotechnol.* **2000**, *18* (4), 438−441.
(5) Chrisey, L. A.; Lee, G. U.; O'Ferrall, C. E. *Nucleic Acids Res.* **1996**, *24* (15), 3031−3039.
(6) Macanovic, A.; Marquette, C.; Polychronakos, C.; Lawrence, M. F. *Nucleic Acids Res.* **2004**, *32* (2), e20−e27.
(7) Marquette, C. A.; Lawrence, I.; Polychronakos, C.; Lawrence, M. F. *Talanta* **2002**, *56*, 763−768.
(8) Patolsky, F.; Weizmann, Y.; Willner, I. *J. Am. Chem. Soc.* **2002**, *124* (5), 770−772.
(9) Trau, D.; Lee, T. M. H.; Lao, A. I. K.; Lenigk, R.; Hsing, I. M.; Ip, N. Y.; Carles, M. C.; Sucher, N. J. *Anal. Chem.* **2002**, *74* (13), 3168−3173.
(10) Wegner, G. J.; Lee, H. J.; Corn, R. M. *Anal. Chem.* **2002**, *74* (20), 5161−5168.
(11) Long, Y. T.; Li, C. Z.; Sutherland, T. C.; Kraatz, H. B.; Lee, J. S. *Anal. Chem.* **2004**, *76* (14), 4059−4065.
(12) Coche-Guerente, L.; Cosnier, S.; Innocent, C.; Mailley, P.; Moutet, J. C.; Morelis, R. M.; Leca, B.; Coulet, P. R. *Electroanalysis* **1993**, (*5*), 647−652.
(13) Liljegren, G.; Nyholm, L. *Analyst* **2003**, *128* (3), 232−236.
(14) Marquette, C. A.; Laurenceau, E.; Mallet, F.; Chaix, C.; Blum, L. J.; Mandrand, B. *Anal. Biochem.* **2005**, *340* (1), 14−23.
(15) Guadarrama, A.; Rodríguez-Mendez, M. L.; Sanz, C.; Ríos, J. L.; De Saja, J. A. *Anal. Chim. Acta* **2001**, *432* (2), 283−292.
(16) Cao, T.; Chen, J.; Cao, W.; Wei, F.; Liao, W.; Zhao, X.; Jiao, X. *Langmuir* **2003**, *19* (20), 8127−8129.
(17) Downard, A. J. *Electroanalysis* **2000**, *12* (14), 1085−1096.
(18) Barbier, B.; Pinson, J.; Desarmot, G.; Sanchez, M. *J. Electrochem. Soc.* **1990**, *137* (6), 1757−1764.
(19) Delamar, M.; Hitmi, R.; Pinson, J.; Savéant, J. M. *J. Am. Chem. Soc.* **1992**, *114* (14), 5883−5884.


**10.1021/ja056946w CCC: $30.25** 

***Scheme 1.*** (i) Diazotation of Aniline Derivative, (ii) Electroreduction of Diazonium Cation, and (iii) Covalent Linkage to the Electrode Surface




diazonium, (ii) the electrochemical reduction of this latter species generates an aryl radical, which attacks the surface and forms an X−C bond (where X is the electrode material, namely, Au, C, Cu, Si).

This technique of derivatization has already been demonstrated on a wide range of conducting materials such as carbon,[19−28] carbon nanotubes,[29] silicon,[30−33] metals,[34−38] and diamond.[39] In most cases, the electrochemical addressing was performed in organic solvent (acetonitrile), and the modified electrode was subsequently grafted with an interesting molecule (in some cases a protein).

An innovative approach is presented herein based on the modification of proteins with aniline derivatives ($NH_2$−Ar−R) and the subsequent diazotation and electro-addressing of the modified proteins at a graphite electrode surface (Figure 1). This approach benefits the possibility of diazonium reduction in acidic aqueous medium[28,40−42] and enables, to our knowledge, for the first time, a true addressed electrochemical covalent grafting of proteins at an electrode surface. The conducting material used is a 10-electrode microarray, developed and characterized previously by our group[14,43] and obtained through a screen-printing (SP) technique. This technology is presented as a promising tool for achieving marketable biosensors, taking advantage of low-cost and mass-production possibilities.[44,45]

Herein, a biological model is presented based on rabbit immunoglobulin (IgG) first functionalized with 4-carboxymethylaniline (CMA) and then diazotated and electro-addressed on target electrodes of the microarray (Figure 1). X-ray photoelectron spectroscopy (XPS) and cyclic voltammetry were used to characterize the diazonium reduction and grafting on this electrode material.

Validation of the quality of the addressed immobilization was performed through the recognition of the immobilized proteins by peroxidase-labeled anti-IgG antibodies and the measurement, with a CCD camera imaging system, of the chemiluminescent signals emitted.

## Experimental Section

**Reagents.** 4-Aminophenylacetic acid (4-carboxymethylaniline; CMA), 4-bromobenzenediazonium tetrafluoroborate (BrDz), bovine serum albumin (BSA), immunoglobulin from human serum (human IgG), luminol (3-aminophthalhydrazide), *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), and peroxidase-labeled polyclonal anti-rabbit IgG antibodies developed in goat and polyoxyethylenesorbitan monolaureate (tween 20) were purchased from Sigma (Lyon, France). Sodium nitrite and Veronal (diethylmalonylurea sodium) were purchased from Prolabo (Fontenay Sous Bois, France). Immunoglobulins from rabbit serum (rabbit IgG) were obtained from Life Line Lab (Pomezia, Italy). Peroxidase-labeled polyclonal anti-human IgG (H + L) antibodies developed in goat were purchased from Jackson ImmunoResearch (West Grove, PA). All buffers and aqueous solutions were made with distilled demineralized (dd) water.

**Electrode Microarray Preparation.** A DEK 248 screen-printing machine (DEK, Lyon, France) was used to produce the graphite electrode microarrays. A polyester monofilament fiber screen (DEK, Dorset, U.K.) characterized by a mesh size of 260 counts per inch and a thickness of 13 $\mu$m was used to print the graphite ink (Electrodag 423 SS, Acheson, Erstein, France) onto a polyester flexible foil. After being printed, the polyester foils supporting 16 electrode arrays were baked for 10 min at 100 °C to cure the thermoplastic carbon ink.

A second layer, composed of insulating polymer (MINICO M 7000, Acheson, Erstein, France) was then printed onto the microarrays to define a window (easily covered with a 35-$\mu$L drop of solution) delimiting the active area composed of eight 0.2-mm$^2$ working electrodes, one ring-shaped reference electrode, and one central auxiliary electrode (Figure SI 1, Supporting Information).

**X-ray Photoelectron Spectroscopy (XPS).** XPS spectra were realized on a 1-mm analysis window with an ESCALAB instrument (VG Scientific) using an Al anode (K$\alpha$ X-rays at 1486.6 eV) and an analysis energy of 50 eV. Larger screen-printed electrodes (25 mm$^2$), prepared as described above, were used to achieve convenient XPS experiments. The atom percentages were determined from the peak areas, the sensitivity factors given by the VG software (inelastic mean free path and transmission function), and the cross section for X-ray excitation as calculated by Scofield.[46]

**Protein Modifications.** A two-step reaction protocol was elaborated to modify the proteins. First, 10 mg of 4-carboxymethylaniline (CMA) was activated in the presence of 20.6 mg of DCC and 11.5 mg of NHS in 1 mL of DMSO for 30 min under stirring. Then, 20 $\mu$L of the activated CMA was added to 500 $\mu$L of a 5 mg/mL protein solution in 0.1 M carbonate buffer, pH 11. The protocols were identical for both

(20) Combellas, C.; Kanoufi, F.; Pinson, J.; Podvorica, F. I. *Langmuir* **2005**, *21* (1), 280−286.
(21) Liu, S.; Tang, Z.; Shi, Z.; Niu, L.; Wang, E.; Dong, S. *Langmuir* **1999**, *15* (21), 7268−7275.
(22) Kuo, T. C.; McCreery, R. L.; Swain, G. M. *Electrochem. Solid-State Lett.* **1999**, *2* (6), 288−290.
(23) Kariuki, J. K.; McDermott, M. T. *Langmuir* **1999**, *15* (19), 6534−6540.
(24) Bahr, J. L.; Tour, J. M. *Chem. Mater.* **2001**, *13* (11), 3823−3824.
(25) Liu, Y. C.; McCreery, R. L. *J. Am. Chem. Soc.* **1995**, *117* (45), 11254−11259.
(26) Saby, C.; Ortiz, B.; Champagne, G. Y.; Bélanger, D. *Langmuir* **1997**, *13* (25), 6805−6813.
(27) Allongue, P.; Delamar, M.; Desbat, B.; Fagebaume, O.; Hitmi, R.; Pinson, J.; Savéant, J. M. *J. Am. Chem. Soc.* **1997**, *119* (1), 201−207.
(28) Delamar, M.; Désarmot, G.; Fagebaume, O.; Hitmi, R.; Pinson, J.; Savéant, J. M. *Carbon* **1997**, *35* (6), 801−807.
(29) Lee, C. S.; Baker, S. E.; Marcus, M. S.; Yang, W.; Eriksson, M. A.; Hamers, R. J. *Nano Lett.* **2004**, *4* (9), 1713−1716.
(30) De Villeneuve, C. H.; Pinson, J.; Bernard, M. C.; Allongue, P. *J. Phys. Chem. B* **1997**, *101* (14), 2415−2420.
(31) Allongue, P.; Henry De Villeneuve, C.; Pinson, J.; Ozanam, F.; Chazalviel, J. N.; Wallart, X. *Electrochim. Acta* **1998**, *43* (19−20), 2791−2798.
(32) Allongue, P.; Henry de Villeneuve, C.; Cherouvrier, G.; Cortès, R.; Bernard, M. C. *J. Electroanal. Chem.* **2003**, *161*, 550−551.
(33) Adenier, A.; Bernard, M. C.; Chehimi, M. M.; Cabet-Deliry, E.; Desbat, B.; Fagebaume, O.; Pinson, J.; Podvorica, F. *J. Am. Chem. Soc.* **2001**, *123* (19), 4541−4549.
(34) Pinson, J.; Podvorica, F. *Chem. Soc. Rev.* **2005**, *34* (5), 429−439.
(35) Chaussé, A.; Chehimi, M. M.; Karsi, N.; Pinson, J.; Podvorica, F.; Vautrin-Ul, C. *Chem. Mater.* **2002**, *14* (1), 392−400.
(36) Bernard, M. C.; Chaussé, A.; Cabet-Deliry, E.; Chehimi, M. M.; Pinson, J.; Podvorica, F.; Vautrin-Ul, C. *Chem. Mater.* **2003**, *15* (18), 3450−3462.
(37) Adenier, A.; Cabet-Deliry, E.; Lalot, T.; Pinson, J.; Podvorica, F. *Chem. Mater.* **2002**, *14* (11), 4576−4585.
(38) Laforgue, A.; Addou, T.; Bélanger, D. *Langmuir* **2005**, *21* (15), 6855−6865.
(39) Wang, J.; Firestone, M. A.; Auciello, O.; Carlisle, J. A. *Langmuir* **2004**, *20* (26), 11450−11456.
(40) Heald, C. G. R.; Wildgoose, G. G.; Jiang, L.; Jones, T. G. J.; Compton, R. G. *ChemPhysChem* **2004**, *5* (11), 1794−1799.
(41) Ruffien, A.; Dequaire, M.; Brossier, P. *Chem. Commun.* **2003**, *9* (7), 912−913.
(42) Dequaire, M.; Degrand, C.; Limoges, B. *J. Am. Chem. Soc.* **1999**, *121* (29), 6946−6947.
(43) Corgier, B. P.; Marquette, C. A.; Blum, L. J. *Anal. Chim. Acta* **2005**, *538* (1−2), 1−7.
(44) Hart, J. P.; Wring, S. A. *Trends Anal. Chem.* **1997**, *16* (2), 89−103.
(45) Albareda-Sirvent, M.; Merkoçi, A.; Alegret, S. *Sens. Actuators B: Chem.* **2000**, *69* (1), 153−163.
(46) Scofield, J. H. *J. Electron Spectrosc. Relat. Phenom.* **1976**, *8* (2), 129−137.

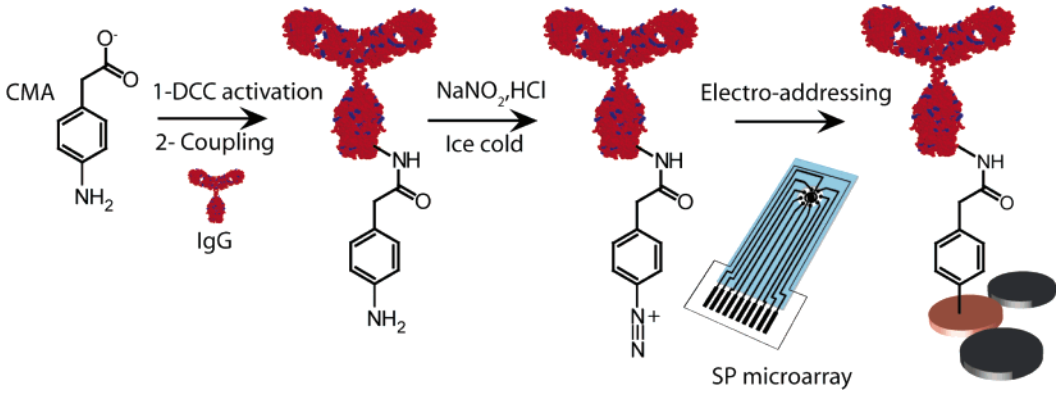



***Figure 1.*** Strategy for direct electro-addressing of modified antibody onto SP graphite electrode surface. CMA, 4-carboxymethylaniline; DDC, *N,N′*-dicyclohexylcarbodiimide.

rabbit and human IgG modification. This coupling solution was left for 2 h under stirring at room temperature before being purified on a P-10 Sephadex G-25M 5 mL column (Supelco, Bellefonte, PA). The desalted modified protein obtained was then concentrated to 10 mg/mL under centrifugation using MicroconYM-3 (Millipore, Billerica, MA). The obtained retentate was recovered in 100 $\mu$L of dd water and stored at +4 °C.

**Diazotation of 4-Carboxymethylaniline.** The aniline derivative CMA, either free or coupled to protein, was diazotated in an aqueous solution of 20 mM HCl and 20 mM $NaNO_2$ for 10 min under stirring in ice-cold water. The diazonium solution formed was then immediately used to perform electro-addressing.

**Electrodeposition of Diazonium Derivatives.** Electro-addressing was achieved by directly depositing on the SP microarray surface a 35-$\mu$L drop of either 5 mM free diazonium solution or 0.1 mg/mL modified proteins. Therefore, three cyclic voltammograms were obtained from 0 to −1000 mV, on the chosen electrodes of the microarray at a scan rate of 200 mV/s. The cyclic voltammograms were obtained with a Voltalab PGZ 100 potentiostat (Radiometer Analytical). A specially designed 10-path connector was used to control the potential application on the electrodes of the SP microarray. An external platinum counter electrode and a ring-shaped carbon reference electrode of the screen-printed chip were used to achieve a three-electrode setup. After deposition, the carbon electrodes were fully rinsed with distilled water and sonicated to remove unbound molecules.

**Protein Detection Procedure.** Following electro-addressing, sonication, and washing, the SP microarrays were saturated for 20 min at 37 °C with VBSTA (30 mM Veronal, 0.2 M NaCl, pH 8.5, with addition of 1% Tween and 1 g/L BSA). Peroxidase-labeled anti-rabbit or anti-human IgG antibodies at various concentrations in VBSTA were incubated for 1 h at 37 °C on the microarrays and then washed with VBS (30 mM Veronal, 0.2 M NaCl, pH 8.5) for 20 min. Finally, the SP microarrays were covered with a 35-$\mu$L drop of VBS containing in addition 200 $\mu$M luminol, 20 $\mu$M *p*-iodophenol, and 500 $\mu$M hydrogen peroxide, and introduced into a charge-coupled device (CCD) cooled camera light measurement system (Las-1000 Plus, Intelligent Dark Box II, Fujifilm, Tokyo, Japan). The emitted light was then collected and integrated for 10 min. The numeric micrographs obtained were quantified with a Fujifilm image analysis program (Image Gauge 3.122).

## Results and Discussion

Screen-printed (SP) microarrays, previously described and characterized,[14,43] were used as robust and single-use supports to study the possibilities of electrochemically addressing diazonium cations and diazonium-cation-modified proteins. First, the reduction and deposition of aryl diazonium cations at the surface of our SP graphite microarrays was investigated to validate the possibility of electro-addressing these molecules.

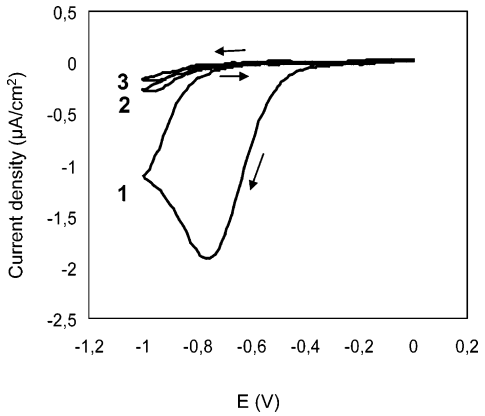



***Figure 2.*** Cyclic voltammograms of 4-bromobenzenediazonium (5 mM) in 0.1 M HCl and 0.1 M KCl. Potential scanned from 0 to −1000 mV vs graphite pseudoreference electrode. Three cycles performed at a scan rate of 200 mV/s.

4-Bromobenzenediazonium (BrDz) was used to study this electroreduction process at our SP graphite electrode surface. This derivative was chosen for its bromide residue, which is easily evidenced through XPS measurements. Optimized deposition of BrDz was shown to be obtained using three potential scans from 0 to −1000 mV in aqueous solutions of 0.1 M HCl and 0.1 M KCl.

The three consecutive cyclic voltammograms obtained are presented in Figure 2. They are characterized by a first cycle exhibiting a well-defined, reproducible, and irreversible reduction peak at −750 mV. This feature corresponds to the typical electroreduction wave of the diazonium function, leading to the elimination of a nitrogen molecule and the production of an aryl radical (Scheme 1, steps i and ii). This radical was previously shown to attack the surface and to form a covalent bond between the aryl group and the electrode material (Scheme 1, step iii).[39,42,47]

More information could be obtained from Figure 2 about the deposited layer. Very low currents were observed during the second and third voltammetric cycles, evidencing surface saturation and suggesting that a monolayer of fixed molecules was achieved. Indeed, very fast deposition processes (30 s), as used herein, usually lead to the grafting of only one molecular layer.[48,49]

(47) Vakurov, A.; Simpson, C. E.; Daly, C. L.; Gibson, T. D.; Millner, P. A. *Biosens. Bioelectron.* **2004**, *20* (6), 1118−1125.
(48) Anariba, F.; DuVall, S. H.; McCreery, R. L. *Anal. Chem.* **2003**, *75* (15), 3837.

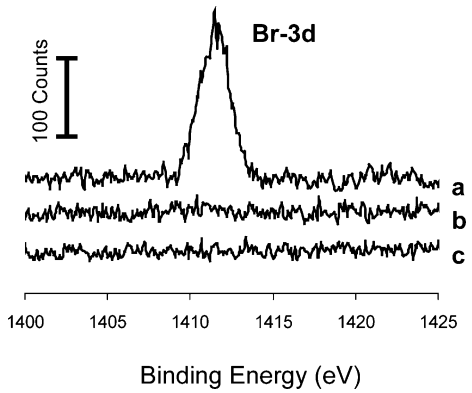



***Figure 3.*** X-ray photoelectron spectra of (a) electro-addressed BrDz, (b) control experiment without BrDz electroreduction, and (c) bare SP graphite electrode.

XPS experiments were performed on our SP graphite surface to demonstrate the electro-addressed deposition of the bromo aryl. Three SP graphite electrodes—either unmodified, modified with adsorbed BrDz, or modified with electrodeposited BrDz—were scanned at 50 eV, particularly focusing the analysis on the binding energies corresponding to the Br 3d and the N 1s electrons.

The electrodeposited electrode (Figure 3, curve a) exhibited a peak at 1412 eV, indicating the presence of bromide atoms at the surface with a relative composition of 2.6%. Conversely, no signal from the presence of Br could be found either on the electrode modified by dipping in a BrDz solution (Figure 3, curve b) or on the bare unmodified electrode (Figure 3, curve c).

Exclusively bromo aryl seems then to be present at the surface of the graphite electrode, following the electroreduction of BrDz. Moreover, no detectable signal was obtained for the N 1s decomposition at the electrodeposited electrode (data not shown), confirming the elimination of nitrogen and the agreement of the present reaction with the commonly accepted mechanism presented in Scheme 1.

The use of this electrochemical deposition to address proteins at the surface of our SP microarrays requires, first, the use of an aniline derivative that can be easily linked to the proteins and, second, the achievement of its diazotation under gentle conditions. 4-Carboxymethylaniline (CMA) was found to be such a potential candidate as it contains a carboxylic acid function that is easily activated and grafted through carbodiimide reaction.

Diazotation reaction conditions of the CMA aniline residue, adapted from ref 50, were optimized to fit with the pH and salt concentration requirements for biocompatibility. The cyclic voltammograms obtained during the electrodeposition of freshly diazotated CMA were found to be characteristic of an aryl diazonium grafting (Figure SI 2, Supporting Information), with behaviors similar to those obtained during BrDz deposition onto the SP graphite electrodes. Thus, a clear reduction wave was observed in the presence of diazotated CMA, whereas no current at all could be obtained from the nondiazotated aniline derivative.

The diazotation and electrodeposition of CMA was then shown to be easily obtained, and subsequently, its coupling to rabbit IgG was elaborated to produce proteins ready to be addressed (CMA−rabbit IgG). The rabbit IgG primary sequence suggests that up to 80 primary amines (essentially lysine) are available for grafting. Attempting as much as possible to keep the protein in its native conformation after immobilization, CMA was reacted with IgG molecules using stoichiometric reagent conditions (moles of CMA vs moles of primary amine).

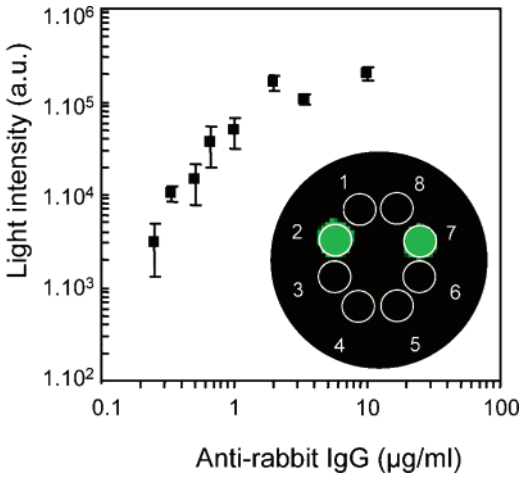



***Figure 4.*** Dose response curve of anti-rabbit IgG antibodies. Error bars are the standard deviation of four microarrays. Inset: chemiluminescent micrograph of a SP graphite microarray incubated with peroxidase-labeled anti-rabbit IgG antibodies (2 $\mu$g/mL). Electrodes 2 and 7 were electro-addressed with CMA−rabbit IgG.

The CMA−rabbit IgG conjugates obtained were diazotated according to the procedure optimized above with free CMA and electrodeposited onto the SP graphite microarray. Nevertheless, the low protein concentration used (0.1 mg/mL) did not enable the recording of any diazonium reduction peak as the corresponding maximum concentration of coupled diazonium was found to be 100 times lower than the CMA concentration used above (<50 $\mu$M).

An indirect demonstration was then performed to detect the electrodeposited rabbit IgG. Peroxidase-labeled anti-rabbit IgG antibodies were used to specifically detect the immobilized proteins through chemiluminescent imaging of the SP microarray surface. As the inset of Figure 4 shows, a clear distinction between the addressed electrodes (nos. 2 and 7) and the six unaddressed electrodes was obtained. Moreover, no nonspecific adsorption of the proteins onto either the graphite material or the SP support (PVC or insulating layer) was observed, demonstrating extremely interesting potentialities for analytical applications.

This possibility of using the addressed electrodeposited protein as an analytical tool was studied by applying to the SP microarrays a range of peroxidase-labeled anti-rabbit IgG antibody concentrations from 0.25 to 10 $\mu$g/mL and determining a dose−response curve (Figure 4). A classical ligand/receptor binding curve was obtained with a pseudolinear relation for antibody concentrations ranging from 0.25 to 2 $\mu$g/mL, followed by a plateau at higher concentrations. Assuming that the immobilization of the proteins occurred as a monolayer, a limited number of macromolecules are accessible for recognition, leading to saturation at high antibody concentrations. These performances compared well with those obtained using SP

(49) Brooksby, P. A.; Downard, A. J. *Langmuir* **2004**, *20* (12), 5038−5045.
(50) Bourdillon, C.; Delamar, M.; Demaille, C.; Hitmi, R.; Moiroux, J.; Pinson, J. *J. Electroanal. Chem.* **1992**, *336*, 113−123.

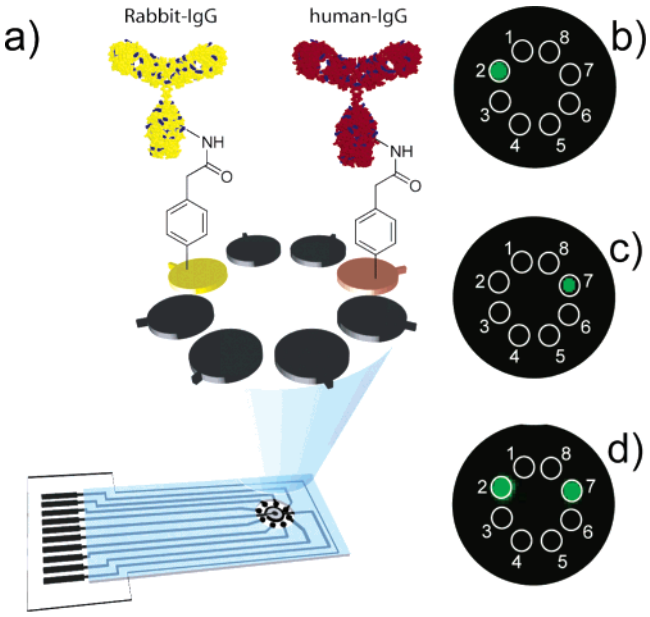



***Figure 5.*** (a) Illustration of the SP carbon electrode microarray supporting two distinct sensing layers covalently immobilized by electrochemical addressing. Chemiluminescent micrograph of an SP microarray modified with both rabbit and human CMA−IgG and incubated with peroxidase-labeled (b) anti-rabbit IgG antibodies (2 $\mu$g/mL), (c) anti-human antibodies (0.1 $\mu$g/mL), and (d) both anti-rabbit IgG antibodies (2 $\mu$g/mL) and anti-human antibodies (0.1 $\mu$g/mL). Electrode 2 was electro-addressed first with CMA−rabbit IgG, and then electrode 7 was electro-addressed with CMA−human IgG.

support and protein trapped in an electropolymer[14] or using a standard support such as coated glass slides.[51]

To be fully useful for analytical use, the present addressing system needs to be applied to the sequential immobilization of different proteins.

Two CMA proteins, rabbit and human IgGs, were then synthesized, diazotated, and sequentially electro-addressed to different electrodes of the SP microarray, as presented in Figure 5a.

Despite the fact that, in the addressing protocol, rabbit IgG was deposited first and was then in contact with the diazotated solution of CMA−human IgG, the reactivity of the immobilized rabbit IgG was preserved, and a fully acceptable chemiluminescent signal was obtained (Figure 5b, when compared to the dose−response curve presented above). As shown in Figure 5c, peroxidase-labeled anti-human IgG antibodies also react properly with the immobilized human IgG without cross-reactivity with the immobilized rabbit IgG. Moreover, the two sensing layers could be used concomitantly to detect anti-rabbit and anti-human IgG (Figure 5d).

Thus, the consecutive electro-addressing of two different proteins did not alter the specificity and the sensitivity of the detection, and no cross-talk between the two protein layers was observed, indicating that only the specificity of the antigen/antibody interaction was involved in the detection process.

To validate the possibility of producing eight electro-addressed sensing layers on the different electrodes of a microarray, eight different solutions of diazotated CMA−rabbit IgG were sequentially electrodeposited. Eight sensing layers composed of electro-addressed rabbit IgG were then obtained on a single SP microarray, with good reproducibility (data not shown), and characterized by a consistent chemiluminescent signal emitted from each electrode.

## Conclusion and Perspectives

Screen-printed (SP) graphite electrode microarrays demonstrated their ability to be modified with electro-addressed diazonium cations. Thus, an innovative approach was elaborated to electro-address proteins. Biomolecules were first modified with the aniline derivative 4-carboxymethylaniline (CMA) and then successfully electrodeposited—i.e., without parasitic reduction of the protein part—at the graphite surface through an optimized protocol compatible with biomolecules such as rabbit and human antibodies (IgG).

For the first time ever, such direct electro-addressing of multiple proteins was then described. The immuno-biochip obtained was shown to exhibit interesting detection properties and very low nonspecific binding.

The present system appeared to offer a promising strategy for strongly immobilizing different biological entities—proteins, nucleic acid strands, and peptides—with excellent spatial addressing of the interesting molecules onto electrode microarrays. Indeed, the CMA coupling procedure is compatible with the majority of the biomolecules that could be kept in their native configuration in rather acidic solutions. This is the case, for example, for DNA sequences, carbohydrate structures, peptides, and numerous proteins. Moreover, less drastic conditions could be examined for saving protein activity such as enzyme catalytic activity.

Interesting possibilities should also be obtained by concomitantly using the present modification system with poly(lysine)-tagged proteins. Thus, grafting CMA preferentially onto the polyamine tag[52] will lead to the electro-addressing of oriented proteins at the surface, enhancing the protein immobilization quality and thus the immuno-biochip performances.

**Acknowledgment.** The authors acknowledge P. Delichere from the Institut de Recherches sur la Catayse (Lyon, France) for his useful help in the recording and interpretation of XPS spectra.

**Supporting Information Available:** Diagram of the SP graphite electrode microarray and electrochemical (cyclic voltammetry) data for the electrodeposition of 4-carboxymethylaniline (CMA). This material is available free of charge via the Internet at http://pubs.acs.org.

JA056946W

(51) Cretich, M.; Pirri, G.; Damin, F.; Solinas, I.; Chiari, M. *Anal. Biochem.* **2004**, *332* (1), 67−74.

(52) Allard, L.; Cheynet, V.; Oriol, G.; Imbert-Laurenceau, E.; Mandrand, B.; Delair, T.; Mallet, F.; Gervasi, G. *Bioconj. Chem.* **2004**, *15*, 458−466.