Cell Immobilization

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Electrically Addressable Cell Immobilization Using Phenylboronic Acid Diazonium Salts**

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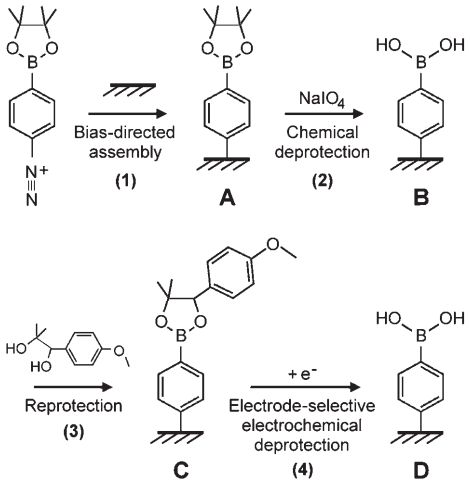
Recently there has been much interest in the development of cell arrays for such areas as drug screening,[1] gene expression profiling,[2] stem-cell differentiation,[3] understanding higher-level organization of tissues and organisms,[4] and other developing fields involving the functions of biological systems. Cell-based arrays have the potential to lead to a new generation of powerful sensing devices, as living cells contain specific biological and chemical receptors with processes that respond to minute concentrations of molecules. The ability to readily control the spatial organization and interactions between populations of cells would also prove valuable for research involving cell–cell or host–pathogen interactions and cell signaling pathways.

Some common cell attachment and detachment protocols utilize native poly- and oligosaccharides that are present in the outer cellular wall or membrane, which can bind to many sugar-specific proteins and antibodies. So-called artificial lectins, such as boronic acid, can form esters with diols to generate five- or six-membered cyclic complexes that can also be exploited to capture cells.[5] The boronic acid–saccharide interaction is particularly attractive for a number of reasons. In the physiological pH range of 6.8–7.5, boronic acid provides a stable boronate anion that reacts with 1,2- or 1,3-diols to form reversible complexes.[6] The formation of these complexes is highly dependent on the nature of a given saccharide, and has been exploited in numerous applications including affinity chromatography purification,[7] detection of glycoproteins,[8] as a stationary phase for separation of sugars in liquid chromatography,[9] capillary electrophoresis,[10] the development of aqueous sugar sensors,[11] and the orientation or reversible immobilization of glycoproteins in cellulose beads.[12] Herein, we demonstrate that phenylboronic acid diazonium salts can be used to activate individual electrodes for facile and reversible eukaryotic cell immobilization (yeast and macrophage). As far as we are aware, this is the first report of an electrically addressable cell immobilization method.

The use of aryl diazonium salts for electrode modification has been widely reported. The advantages of using diazonium chemistry include a highly stable covalent bond, ease of preparation, and the ability to selectively modify conducting and semiconducting surfaces with the application of a potential bias.[8b,13] Diazonium-modified electrodes have been used to immobilize many biomolecules including DNA,[14] proteins,[15] and peptides.[16] We have used the direct immobilization of diazonium-modified proteins to detect H_2O_2[17] and cytokines,[18] as well as to construct sensing platforms for multianalyte immunosensors[19] and to enable the simultaneous electrochemical detection of DNA and protein on the same electrode array.[20]

The method for the functionalization of an electrode surface with phenylboronic acid pinacol ester diazonium salt is presented in Scheme 1. The molecule is synthesized with a



Scheme 1. Phenylboronic acid functionalization: 1) electroreduction of the diazonium salt at a conductive substrate to form a covalently modified phenylboronic acid pinacol ester surface A; 2) chemical deprotection yielding a phenylboronic acid surface B; 3) reprotection with MPMP-diol to form surface C; 4) oxidative deprotection again to yield a phenylboronic acid surface D.

pinacol ester blocking group on the boronic acid (one step from the commercially available *para*-aminophenylboronic acid pinacol ester) and is deposited on the electrode by cyclic voltammetry (step 1), thus forming a pinacol ester-blocked phenylborate-modified electrode surface A (Scheme 1). Both the molecular synthesis and deposition protocol are described in the Supporting Information. The blocking group can then be chemically deprotected by the oxidant $NaIO_4$ (step 2),[21] to form a phenylboronic acid-modified surface B (Scheme 1).

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This surface can be reblocked with a 1-(4-methoxyphenyl)-2-methylpropane-1,2-diol (MPMP-diol) reprotection unit (step 3) to give surface C (Scheme 1). *Para*-methoxybenzyl groups can be used to provide a removable protecting group through oxidation. This allows for electro-addressable deprotection (step 4), again yielding a phenylboronic acid surface D (Scheme 1), but only at the oxidatively treated electrode. The MPMP-diol protecting group described here has been previously used to yield an oxidatively removable protecting group for boronic acids.[22] Presumably, the electrochemical deprotection serves to oxidize the MPMP-diol to 2-hydroxy-1-(4-methoxyphenyl)-2-methylpropan-1-one which cannot bind effectively to the boronic acid, thereby shifting the equilibrium to the free boronic acid.

Grazing-angle FTIR, ellipsometry, and contact angle measurements were made to understand and verify the chemistry of the assembled films at each stage of their chemical manipulation (Figure 1). Film thicknesses corresponding to approximately 1.7 equivalent monolayers[23] (17.5 ± 3.8 Å) of the phenylboronic acid pinacol ester (Scheme 1, surface A) were assembled on gold from the diazonium precursor by cyclic voltammetry. As expected, the contact angle for water on this surface was greater than 90° (Figure 1 A). The FTIR spectrum reveals CH modes at approximately 3000 and 1090 cm^{-1}, weak aromatic modes at 1609 cm^{-1}, and a clear B–O bending mode at 1363 cm^{-1}.[24] The spectrum also reveals a broad, weak O–H stretch at 3500 cm^{-1}, presumably arising from some inadvertent hydrolysis.

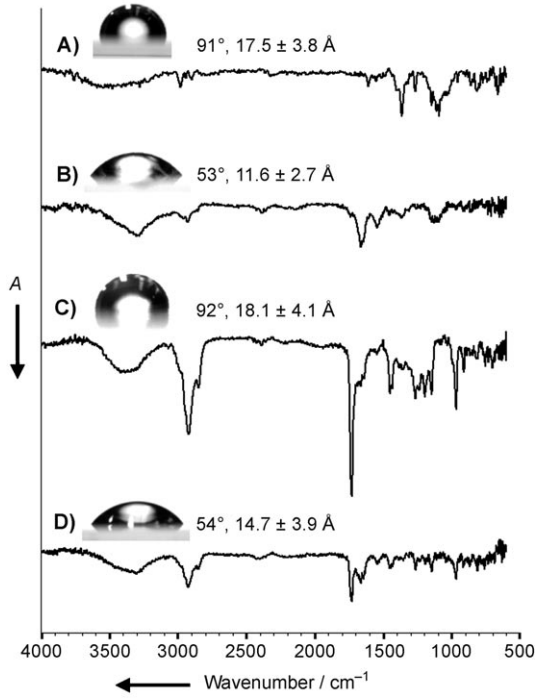

Figure 1. Grazing-angle FTIR spectroscopy, contact angle, and ellipsometry thickness measurements for gold electrodes prepared according to Scheme 1. A) Phenylboronic acid pinacol ester surface, B) chemically deprotected boronic acid surface, C) MPMP-diol reprotected surface, and D) electrochemically deprotected boronic acid surface. Standard deviations were calculated from eight or more independent measurements on each of three electrodes sampled.

After deprotection by periodate, the contact angle of the film dropped to 53°, as expected for a film with increased hydrophilicity (Figure 1 B). The surface thickness also fell by 5.9 Å, which is near the 3.3 Å length of the pinacol ester blocking group. The FTIR spectrum indicates a large increase in the OH stretch relative to the CH modes at 3000 cm^{-1}. The deprotection also enhances the phenyl ring modes at 1661 cm^{-1}. This mode seems to be suppressed by substitution on the boronic acid; it vanishes during reblocking of the boronic acid by the MPMP-diol.

Reblocking of the free boronic acid by the MPMP-diol (Figure 1 C) is more complicated. Presumably, as the reblocking was performed in anhydrous toluene the product should be trigonal borate. However, inadvertent hydrolysis and steric crowding may result in incomplete borate ester formation. Nonetheless, the contact angle of the reblocked film increased to over 90° and the film thickness increased in elipsometric measurements, as expected for the replacement of the pinacol by the larger MPMP-diol. Grazing-angle FTIR spectroscopy indicates a much weaker OH stretch relative to the CH modes at about 3000 cm^{-1}. Again, the substitution of the boronic acid for an ester decreases the strength of the aromatic ring modes. The peak at 1729 cm^{-1} suggests the presence of a ketone. We believe that this might be the result of some oxidation of the MPMP-diol by trapped periodate (or possibly from the formation of some perborate) and subsequent sequestration in the film.

Electrochemical deblocking of the film decreases the contact angle to nearly that of the chemically deblocked film (Figure 1 D). Likewise, ellipsometry reveals that the film decreased in thickness, but not quite to the thickness of the original phenylboronic acid film (Scheme 1, surface B), which again may suggest that some of the oxidation products are trapped in the film. The FTIR spectrum reveals the aromatic mode at 1660 cm^{-1}, which seems to reflect that the presence of the free boronic acid is restored. The OH peak again increased in intensity.

The affinity for yeast-cell adhesion was determined for each of the four surfaces A–D (Scheme 1) after conditioning for 1 hour in Tris-HCl (100 mM, pH 8.5) and exposure to 1.5×10^7 cells per mL for 2 min (Figure 2 A). Microscope images of each prepared electrode show that cells adhere to the deblocked boronic acid surfaces (b and d, Figure 2 A) while very few cells bind nonspecifically to the two blocked surfaces (a and c, Figure 2 A). These findings show activity consistent with the protocol proposed in Scheme 1, and are in agreement with the contact angle and FTIR data. Adhesion of the yeast cells was stable overnight.

Competitive binding of sugars was examined as a possible method for cell detachment. Fructose has a particularly high affinity for aryl boronic acids in the pH range 7–9. A prepared electrode that was electrochemically deblocked, exposed to yeast cells, and then incubated for 30 min with fructose (20 mM) and Tris-HCl (100 mM, pH 8.5) showed an almost complete removal of cells (Figure 2 B, images a and b). Bound fructose was then removed by treatment with phosphate

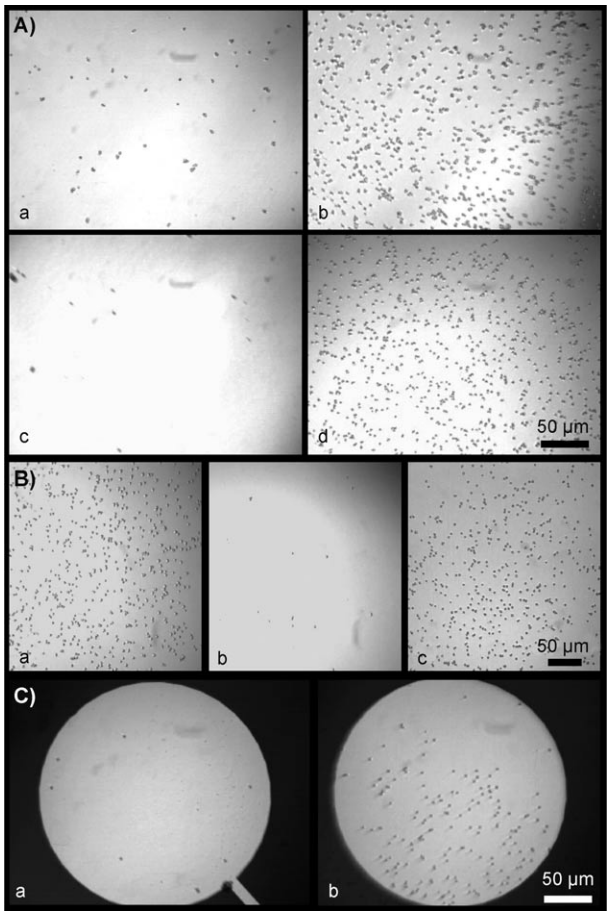

Figure 2. Microscope images of gold electrode surfaces exposed to yeast cells. A) Yeast-cell adhesion affinity: a) phenylboronic acid pinacol ester surface; b) chemically deprotected boronic acid surface; c) MPMP-diol reprotected surface; d) electrochemically deprotected boronic acid surface. B) On-demand release of yeast cells and surface regeneration: a) electrochemically deprotected boronic acid surface treated with yeast cells; b) after treatment for 30 min with fructose solution (20 mM); c) after regeneration at low pH, buffer reconditioning, and treatment with yeast cells. C) Selective patterning of closely spaced electrode arrays: a) MPMP-diol reprotected and b) electrochemically deprotected boronic acid individually addressable electrodes after simultaneous treatment with yeast cells.

solution of pH 3. After reconditioning with Tris-HCl (100 mM, pH 8.5) and exposure to yeast cells, the apparent activity of the boronic acid groups seems unaffected, as shown by the subsequent reattachment of cells in image c of Figure 2B. The ability to release the attached cells by fructose treatment and to regenerate the surface for subsequent cellular adhesion shows the promise of this surface as a reusable platform for cell capture with on-demand release.

The utility of this technique to selectively immobilize cells in an array format is presented in Figure 2C. Two closely spaced, individually addressable 250-µm gold disk electrodes were modified up to step 3, surface C (Scheme 1). One electrode was oxidatively treated at 0.6 V for 1 min in phosphate buffer (50 mM, pH 7.4) to deprotect the boronic acid group, and both were exposed to the same yeast-cell solution. As can be seen in the microscope images, the blocked electrode (a, Figure 2C) shows that few cells adhere to the surface whereas the electrochemically deprotected electrode (b, Figure 2C) shows excellent cell adhesion properties. We expect that replacement of the methoxy group of the MPMP-diol with a short ethoxylate chain will improve the selectivity by decreasing the effects of nonspecific binding.

Ethoxylated surfaces are well known to decrease the formation of biological films. This electrochemical activation of a boronic acid surface would allow a great deal of control over the spatial confinement of cells in devices that contain a high density of working electrodes. This system may also facilitate more complex cellular studies by providing a simple method to pattern differing cell types on closely spaced electrode arrays. Our future studies will determine the effect of modifying the phenyl diazonium salt with selective ligands as a method of selecting differing cell types from one another. In addition, the modification of electrodes near the captured cells with chemically or biologically sensitive groups can be used to monitor the cells' environment or response to stimuli in real time.

Finally, we demonstrated the compatibility of this technique with more sensitive and relevant mammalian cells. Murine macrophage cells were immobilized on a phenylboronic acid-modified gold electrode (Figure 3A) through

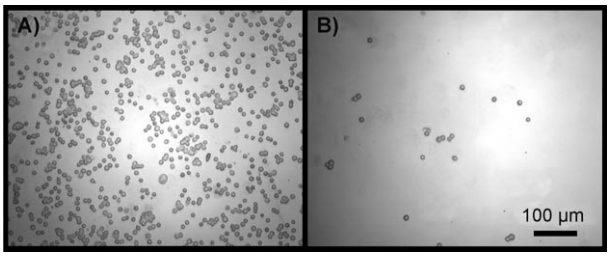

Figure 3. Microscope images of gold electrode surfaces exposed to mammalian macrophage cells. A) Capture of macrophage cells on a phenylboronic acid surface and B) on-demand release after treatment for 30 min with fructose solution (20 mM).

the same protocol as that described for yeast immobilization. Treatment of the surface in fructose solution was again successful in releasing the captured cells (Figure 3B). Additionally, captured cell viability was monitored (in 1X phosphate-buffered saline solution, pH 7.4, 37 °C) 30, 60, and 120 min after immobilization. These time frames are relevant to many cell–cell and cell-signaling interaction studies. After 30 min, roughly $3 \pm 1\%$ of the immobilized cells had died. After 60 min, an additional $3 \pm 2\%$ of cells died and after 120 min, approximately $13 \pm 4\%$ of cells had died. Death is most likely caused by the lack of defined nutrients in the buffer solution, and could be minimized by determining which components of the medium can be added to the buffer without affecting cellular immobilization to the boronic acid-modified surface. Still, nearly 80% of the immobilized cells remained viable over 2 h in buffer, which demonstrates the utility of this method for cell-based studies.

In summary, we have demonstrated for the first time an electroselective cell immobilization method. This procedure is compatible with both fungus and mammalian cells and provides a simple method for on-demand release of captured cells. The majority of captured mammalian cells remain viable over timescales relevant to many studies that utilize surface-immobilized cells. Therefore, this platform shows great promise for use in single-cell or array-based studies including cell signaling, host–pathogen interactions, and other cellular function studies. Boronic acid arrays could also have further applications in the formation of carbohydrate arrays and dopamine detection.

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[1] D. R. Jung, R. Kapur, T. Adams, K. A. Giuliano, M. Mrksich, H. G. Craighead, D. L. Taylor, *Crit. Rev. Biotechnol.* **2001**, *21*, 111 – 154.
[2] J. J. Chen, *Pharmacogenomics* **2007**, *8*, 473 – 482.
[3] a) A. Khademhosseini, R. Langer, J. Borenstein, J. P. Vacanti, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 2480 – 2487; b) K. A. Moore, I. R. Lemischka, *Science* **2006**, *311*, 1880 – 1885.
[4] D. Di Carlo, L. P. Lee, *Anal. Chem.* **2006**, *78*, 7918 – 7925.
[5] A. E. Ivanov, H. A. Panahi, M. V. Kuzimenkova, L. Nilsson, B. Bergenståhl, H. S. Waqif, M. Jahanshahi, I. Y. Galaev, B. Mattiasson, *Chem. Eur. J.* **2006**, *12*, 7204 – 7214.
[6] S. Liu, B. Miller, A. Chen, *Electrochem. Commun.* **2005**, *7*, 1232 – 1236.
[7] a) Y. C. Li, E. L. Larsson, H. Jungvid, I. Y. Galaev, B. Mattiasson, *Chromatographia* **2001**, *54*, 213 – 217; b) F. Frantzen, K. Grimsrud, D. E. Heggli, E. Sundrehagen, *J. Chromatogr. B* **1995**, *670*, 37 – 45.
[8] a) H. Kitano, Y. Anraku, H. Shinohara, *Biomacromolecules* **2006**, *7*, 1065 – 1071; b) A. J. Downard, *Electroanalysis* **2000**, *12*, 1085 – 1096.
[9] C. G. Huber, G. K. Bonn in *Carbohydrate Analysis* (Ed.: Z. El Rassi), Elsevier, Amsterdam, **1995**, pp. 147 – 180 (*J. Chromatogr. Libr.* **1995**, *58*).
[10] A. Bossi, L. Castelletti, S. A. Piletsky, A. P. F. Turner, P. G. Righetti, *J. Chromatogr. A* **2004**, *1023*, 297 – 303.
[11] H. Fang, G. Kaur, B. Wang, *J. Fluoresc.* **2004**, *14*, 481 – 489.
[12] X. C. Liu, W. H. Scouten, *J. Mol. Recognit.* **1996**, *9*, 462 – 467.
[13] a) J. Pinson, F. Podrovica, *Chem. Soc. Rev.* **2005**, *34*, 429 – 439; b) D. V. Kosynkin, J. Yang, J. M. Tour, *Mater. Res. Soc. Symp. Proc.* **2001**, *660*, JJ3.5.1 – JJ3.5.5.
[14] a) A. Shabani, A. W. H. Mak, I. Gerges, L. A. Cuccia, M. F. Lawrence, *Talanta* **2006**, *70*, 615 – 623; b) C.-S. Lee, S. E. Baker, M. S. Marcus, W. Yang, M. A. Eriksson, R. J. Hamers, *Nano Lett.* **2004**, *4*, 1713 – 1716.
[15] a) G. Liu, J. J. Gooding, *Langmuir* **2006**, *22*, 7421 – 7430; b) M. Dequaire, C. Degrand, B. Limoges, *J. Am. Chem. Soc.* **1999**, *121*, 6946 – 6947; c) C. Bourdillon, M. Delamar, C. Demaille, R. Hitmi, J. Moiroux, J. Pinson, *J. Electroanal. Chem.* **1992**, *336*, 113 – 123.
[16] G. Liu, T. Böcking, J. J. Gooding, *J. Electroanal. Chem.* **2007**, *600*, 335 – 344.
[17] R. Polsky, J. C. Harper, S. M. Dirk, D. C. Arango, D. R. Wheeler, S. M. Brozik, *Langmuir* **2007**, *23*, 364 – 366.
[18] R. Polsky, J. C. Harper, D. R. Wheeler, S. M. Dirk, J. A. Rawlings, S. M. Brozik, *Chem. Commun.* **2007**, 2741 – 2743.
[19] R. Polsky, J. C. Harper, D. R. Wheeler, S. M. Dirk, D. C. Arango, S. M. Brozik, *Biosens. Bioelectron.* **2008**, *23*, 757 – 764.
[20] J. C. Harper, R. Polsky, D. R. Wheeler, S. M. Dirk, S. M. Brozik, *Langmuir* **2007**, *23*, 8285 – 8287.
[21] S. J. Coutts, J. Adams, D. Krolikowski, R. J. Snow, *Tetrahedron Lett.* **1994**, *35*, 5109 – 5112.
[22] J. Yan, S. Jin, B. Wang, *Tetrahedron Lett.* **2005**, *46*, 8503 – 8505.
[23] The calculated thickness of a phenylboronic acid pinacol ester monolayer with 70° tilt is approximately 10.0 Å.
[24] D. Barriet, C. M. Yam, O. E. Shmakova, A. C. Jamison, T. R. Lee, *Langmuir* **2007**, *23*, 8866 – 8875.

