

ciation leads to the presence of free assay reagents that may interfere with assays that employ the adsorbed assay reagents. This dissociation may be greatly slowed by cross-linking the adsorbed assay reagents so that the immobilized species are greater in molecular weight and have more points of contact with the surface. Accordingly, in the immobilization methods described above, the second binding partner is, preferably, cross-linked to minimize dissociation of the reagent during surface preparation and/or storage. The cross-linking may be carried out via covalent cross-linking using standard chemical cross-linking agents. Alternatively, the cross-linking is carried out using specific binding interactions. In a preferred embodiment of the invention, the second binding partner is polyvalent (i.e., has multiple binding sites for the first binding partner) and is cross-linked by combining it with a cross-linking reagent that is either a polyvalent first binding partner or a molecule which comprises multiple first binding partners. In this embodiment, the amount of the cross-linking agent is selected so as to provide a beneficial amount of cross-links without saturating all the available binding sites on the second binding partners. The cross-links may be formed after the second binding partner is immobilized but are, preferably, formed in solution prior to immobilization. Advantageously, we have found that this cross-linking procedure not only acts to form a more stable surface but also increases the number of available binding sites on the surface (i.e., the binding capacity of the surface) by allowing the immobilization of more than a packed monolayer of the second binding partner (e.g., by extension of the polymerized second binding partner into solution).

[0166] By way of example, avidin (a tetrameric binding protein having four binding sites for biotin) is cross-linked to form poly-avidin by the addition of a small quantity of biotin-labeled cross-linking agent (for example, a protein such as BSA) having multiple biotin labels per protein molecule. Poly-avidin is then immobilized and used as a capture surface for immobilizing a biotin-labeled assay reagent, e.g., using the immobilization methods described above. The amount of biotin-protein is selected to allow cross-linking while leaving sufficient biotin binding sites available so that the immobilized poly-avidin can be used to capture a biotin-labeled first binding reagent (e.g., a biotin-labeled antibody). Preferably, the biotin-labeled cross-linking agent comprises at least two, more preferably, at least four, or more preferably, at least eight biotins per molecule. Preferably, the number of molar equivalents of cross-linking agent per mole of avidin is between 0.01 and 4, more preferably, between 0.01 and 1, even more preferably between 0.01 and 0.25, even more preferably between 0.05 and 0.25 and most preferably between 0.05 and 0.10. The concentration of avidin used for immobilization was preferably between 50-1000 ug/mL, more preferably between 100-800 ug/mL and most preferably around 400 ug/mL. By analogy, avidin may be replaced in these methods by other poly-valent biotin-specific receptors such as streptavidin.

[0167] Experiments were conducted to demonstrate the benefit of using poly-avidin capture layers on carbon ink electrodes and/or the two-step immobilization procedures of the invention. These experiments used screen printed carbon ink electrodes that were patterned on a plastic substrate. The working electrodes had an exposed circular area of about 3 mm² that was defined by a patterned dielectric layer that was screen printed over the carbon ink electrodes. The substrate

also comprised at least one additional carbon ink electrode for use as a counter electrode. Reagents were immobilized by depositing (using a Bio-Dot dispenser) small volumes (200-300 nL) of a solution comprising the reagent onto the exposed electrode area (the solution being confined to the exposed electrode area by the dielectric layer) and allowing the solution to dry on the electrode. Poly-avidin was prepared by combining the appropriate amounts of avidin and biotin-BSA and incubating for 15 minutes. After the immobilization and/or washing steps (as described below), the substrate was either mated with a multi-well plate top so as to form the bottom surface of a well of multi-well plate or it was mated using a gasket made of double stick tape to a plastic sheet so as to form the bottom surface of a flow cell of an assay cartridge. The electrode surfaces were contacted with a buffered solution comprising tripropylamine (MSD Assay Buffer, MSD) by adding the buffer to a well of a multi-well plate or by introducing the buffer into the flow cell. ECL was induced by applying a voltage between the working and counter electrode (a ramp of 2-5 V over 3 seconds). ECL was measured by taking an image of the substrate using a cooled CCD camera.

[0168] Electrodes were coated with either avidin (by treating with 200 nL of a 75 ug/mL solution of avidin) or with poly-avidin (by treating with 200 nL of a solution containing 75 ug/mL avidin and 3.1 ug/mL biotin-labeled BSA and allowing the solutions to dry overnight; the BSA being labeled with a 4-fold excess of biotin-LC-sulfo NHS ester and having an expected ratio of biotins per BSA of roughly 2-3). The substrates were washed with water and the electrodes were then treated with 300 nL of a solution containing 100 ug/mL of an biotin-labeled anti-TSH antibody. The electrodes were washed with water, assembled into a cartridge into which was introduced a solution containing 20 uIU/mL of TSH and 12 ug/mL of an anti-TSH antibody that was labeled with a Sulfo-TAG NHS ester (MSD), an electrochemiluminescent label. The cartridge was incubated for 8 minutes to allow the binding reactions to occur, the substrate was then washed by passing MSD Assay Buffer into the flow cell and ECL was measured. The average emitted electrochemiluminescence intensity from the poly-avidin treated electrode (1652 units) was approximately three times that from the avidin treated electrode (602 units). Without being bound by theory, it is believed that the higher signal on the poly-avidin electrode represents an increased number of binding sites on the poly-avidin treated electrode and/or a reduction in the amount of avidin that washes off the poly-avidin electrode and adsorbs on other surfaces of the cartridge (thus competing with binding sites on the electrode).

[0169] In a similar experiment, the direct adsorption of anti-TSH antibody (by treatment of the electrode with a 100 ug/mL solution of an anti-TSH antibody) was compared to immobilization via a poly-avidin layer (as described above except that the poly-avidin solution contained 400 ug/mL avidin and 25 ug/mL biotin-BSA and the biotin-labeled anti-TSH was at a concentration of 100 ug/mL). The results showed that signal obtained using immobilization via poly-avidin (**2207**) was roughly twice that obtained using direct adsorption (**1264**). In addition, two step immobilization protocol was found to provide more precise results; the coefficients of variation (CVs) were three times lower when the two step method was employed.