

region of a surface (preferably, a carbon ink electrode surface) with a solution comprising a second binding partner so as to form an adsorbed capture layer (or, alternatively, a covalently bound layer) of said second binding partner (preferably, avidin) within the predefined region of said surface; (ii) treating the capture layer in the pre-defined region with a solution comprising the assay reagent, wherein the assay reagent is linked to or comprises a first binding partner (preferably, an assay reagent that is labeled with biotin) that binds the second binding partner. Preferably, a micro-dispensing technique is used to pattern the second binding partner and/or the assay reagent into the pre-defined region (more preferably both are patterned). More preferably, the pre-defined region is defined by a boundary (preferably defined by a dielectric layer patterned on the surface) adapted to confine small volumes of fluid to the pre-defined region.

[0158] The treating steps may comprise allowing the solutions to dry on the pre-determined regions. Between binding the second binding partner and binding the assay reagent, it may be advantageous to wash the surface with one or more wash solutions to remove excess unbound second binding partner. The wash solutions, preferably, comprise surfactant and/or blocking agents. After immobilizing the assay reagent, it may be advantageous to wash the surface with one or more wash solutions to remove unbound assay reagent. The wash solutions, preferably, comprise surfactants, blocking agents and/or protein stabilizers such as sugars. Useful blocking agents include standard blocking agents of the art (BSA, casein, etc.) but also include blocking reagents comprising the first binding partner (for example, free biotin) so as to block free binding sites on the immobilized layer of the second binding reagent. The wash steps may employ the wash techniques of the invention that employ concentric tubes for adding and removing wash solution. The surfaces are optionally dried after preparation for long term storage.

[0159] Preferably, the amounts of the second binding reagent and assay reagent applied to the pre-defined region are equal to or less than that required to saturate the surface. By choosing amounts roughly equal to the amounts required to saturate the surface, it may be possible to minimize both the amount of excess unbound reagent and the amount of unbound sites and thus avoid the need for washing or blocking steps. In an alternative embodiment, the amount of the assay reagent is kept below the amount of available binding sites in the capture layer to ensure that the binding capacity is determined by the amount of assay reagent added and not by amount of immobilized second binding partner (thus reducing the effect of variability in the efficiency of, e.g., the adsorption of the second binding partner).

[0160] The method may be applied to forming a plurality of assay domains comprising assay reagents immobilized in a plurality of pre-defined regions. In this case, the method is simply repeated for each of the pre-defined regions. Preferably, at least two of the assay domains comprise assay reagents that differ in selectivity for analytes of interest. When forming a plurality of assay domains, it is particularly advantageous to block the final product with a blocking reagent comprising the first binding partner (but not the analyte specific components of the assay reagent) to block excess binding sites on immobilized second binding partners; this procedure prevents assay cross-talk due to excess assay reagent on one pre-defined region diffusing and binding, via first binding partner-second binding partner interactions, to a different assay domain. For example, after using the two step

procedure of binding avidin and then a biotin-labeled antibody, the surface may be blocked with free biotin. Alternatively, after using a two step procedure of binding Protein A (or other Fc binding receptor) and then an antibody against an analyte of interest, the surface may be blocked by using a different antibody or, more preferably, an Fc fragment of an antibody.

[0161] It has been observed that in some cases assay reagents adsorbed on a surface such as a carbon ink may, over time, slowly dissociate from the surface. This dissociation 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).

[0162] 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 prefer-