

redox mediator is applied to the surface only after the test sample reaches the detection zone **31**. Some examples of suitable redox mediators that may be used in the present invention include, but are not limited to, oxygen, ferrocene derivatives, quinones, ascorbic acids, redox polymers with metal complexes, glucose, redox hydrogel polymers, organometallic complexes based upon osmium, ruthenium, iron, etc., and so forth. Particular examples of suitable redox mediators include ferricyanide, 2,5-dichloro-1,4-benzoquinone, 2,6-dichloro-1,4-benzoquinone, 2,6-dimethyl-1,4-benzoquinone, phenazine ethosulfate, phenazine methosulfate, phenylenediamine, 1-methoxy-phenazine methosulfate, and 3,3',5,5' tetramethyl benzidine (TMB). Substrates may also be used in conjunction with a soluble redox mediator present in solution. In such instances, the solution-based substrate may be simply placed on the surface of the applicable electrode. Some commercially available examples of such solution-based substrates include 1-Step turbo TMB (Pierce Chemical Co., Rockford, Ill.) and K-Blue Substrate Ready-to-Use (Neogen Corp., Lexington, K.Y.). For instance, "K-Blue Substrate" is a chromogenic substrate for horseradish peroxidase that contains 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Other suitable redox mediators are described in U.S. Pat. No. 6,281,006 to Heller, et al.; U.S. Pat. No. 5,508,171 to Walling, et al.; U.S. Pat. No. 6,080,391 to Tsuchiva, et al.; and U.S. Pat. No. 6,461,496 to Feldman, et al., which are incorporated herein in their entirety by reference thereto for all purposes. As will be readily recognized by those skilled in the art, many other different reaction mechanisms may be used in the present invention to achieve the electrolysis of an analyte through a reaction pathway incorporating a redox mediator.

[0028] As indicated above, the substrate **80** may optionally include a calibration working electrode **44**. When utilized, the calibration working electrode **44** may enhance the accuracy of the analyte concentration determination. For instance, a current will generally be generated at the calibration working electrode **44** that corresponds to intrinsic background interference stemming from the counter and reference electrodes, as well as the working electrodes themselves. Once determined, the value of this intrinsic background current may be used to calibrate the measured current value at the detection working electrode **42** to obtain a more accurate reading. The calibration working electrode **44** may generally be formed as described above with respect to the detection working electrode **42**. In fact, because the calibration working electrode **44** is configured to calibrate the detection working electrode **42**, it is generally desired that such electrodes are formed in approximately the same manner, from the same materials, and to have the same shape and/or size.

[0029] The detection and calibration working electrodes **42** and **44** are also generally applied with the same surface treatments to improve the calibration accuracy. However, one primary difference between the detection working electrode **42** and the calibration working electrode **44** is that the electrode **44** does not typically contain a specific binding capture ligand for the analyte of interest. This allows most, if not all, of the analyte to bind to the electrode **42**, thereby enabling the electrode **42** to be used primarily for detection and the electrode **44** to be used primarily for calibration.

[0030] For example, the use of this calibration electrode **44** would help determine if non-specific binding was occurring on the electrode surfaces. In some instances, non-specific binding of the redox label or other current-generating compounds to the capture ligand present on the detection working electrode **42** may create inaccuracies in the measured current. Contrary to the specific binding ligands, the non-specific binding ligands do not have a high specificity for the analyte of interest. In fact, the non-specific binding capture ligand typically has no specificity for the analyte of interest at concentrations as high as about 10<sup>-2</sup> moles of the analyte per liter of test sample (moles/liter), and in some embodiments, as high as about 10<sup>-3</sup> moles/liter. The non-specific binding ligands may form bonds with various immunoreactive compounds. These immunoreactive compounds may have a redox center or may have inadvertently been provided with a redox center through attachment of a redox compound (e.g., enzyme). Without the calibration working electrode **44**, these immunoreactive compounds would thus generate a low level of current detected from the detection working electrode **42**, which causes error in the resulting analyte concentration calculated from the generated current. This error may be substantial, particularly when the test sample contains a low analyte concentration.

[0031] To minimize any undesired binding (including non-specific binding as described above) on the surfaces of the working electrodes **42** and **44**, a blocking agent may be applied thereto. The term "blocking agent" means a reagent that adheres to the electrode surface so that it "blocks" or prevents certain materials from binding to the surface. Blocking agents may include, but are not limited to,  $\beta$ -casein, Hammerstern-grade casein, albumins such as bovine serum albumin, gelatin, pluronic or other surfactants, polyethylene glycol, polyvinyl pyrrolidone or sulfur derivatives of the above compounds, a surfactant such as Tween 20, 30, 40 or Triton X-100, a polymer such as polyvinyl alcohol, and any other blocking material known to those of ordinary skill in the art. This includes commercial blends, such as SuperBlock® or SEA BLOCK (Pierce Chemical Co., Rockford, Ill.) or Heterophilic Blocking Reagent (Scantibodies, Santee, Calif.). Depending on the conductive materials used for preparing the working electrodes, the blocking agents may be formulated to adapt to the electrode surface properties. In some embodiments, a cocktail containing multiple blocking agents may be applied onto an electrode and incubated for 5 to 30 minutes, and any excess solution may be removed and the resulting electrode thoroughly dried.

[0032] Referring to FIG. 2, one embodiment of a second substrate **40** is illustrated that may be used in the assay device of the present invention. The second substrate **40** may be formed from the same type of materials as the first substrate **80**, or from different materials. The substrate **40** has a surface **27** on which is disposed one or more auxiliary electrodes, such as a reference electrode **46** and a counter electrode **48**. If desired, the reference and counter electrodes **46** and **48** may be combined into a single "pseudo" electrode. This may be particularly beneficial when the solution resistance is negligible or the generated current is relatively small. Moreover, it should be understood that separate counter and working electrodes may be provided for each working electrode **42** and **44**. The reference and counter electrodes **46** and **48** may be formed in a manner such as described above, or using any other method known to those