Title: SENSOR COASTING LAYER, DEVICE AND METHOD

Abstract: The present invention provides a sensor coating layer comprising: (a) a layer of a polymer deposited on a transducer; and (b) a layer of a peptide deposited on said layer of a polymer. The present invention also provides a sensor device comprising (a) a substrate upon which a transducer is formed; and (b) a sensor coating layer of the invention. The present invention also provides a method of preparing a sensor coating layer of the invention, comprising: (a) depositing a layer of a polymer on a transducer; and (b) depositing a layer of a peptide on said layer of a polymer. The present invention also provides a method of preparing a sensor device of the invention and a method for the detection of a protease using a sensing device of the invention.
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SENSOR COATING LAYER, DEVICE AND METHOD

Field of the Invention
The present invention relates to a sensor coating layer capable of changing its properties due to the interaction with an analyte solution, a sensor device comprising such a sensor coating layer and methods of preparing the sensor coating layer and sensor device. The present invention also relates to the use of the sensor device in a method for the sensitive detection of a protease.

Background to the Invention
Enzyme electrodes have been described previously, for example, in WO 87/07295 and WO 89/03871 which refer to enzyme electrodes capable of responding amperometrically to the catalytic activity of the enzyme in the presence of its respective substrates, wherein the enzyme is immobilised or adsorbed onto the surface of an electrically conductive support member. WO 00/75360 and GB-A-2350677 also describe the detection of enzymes using a protein cross-linked hydrogel.

Synthetic polymer hydrogels have been extensively studied for various applications. Modifications with the inclusion of biomaterials such as peptides or polysaccharides convert the hydrogel into bioactive materials suitable for modulation of cellular functions such as cell adhesion, migration and proliferation. Incorporation of enzymatically degradable linkages in the side chains or cross-links renders the hydrogel susceptible to degradation by proteolytic processes, making it useful for applications in tissue remodelling and regeneration, duplication of wound healing and drug delivery.

Development of polymer hydrogels containing enzymatically degradable bonds has been carried out for many years, and examples include studies on poly [N-(2-hydroxypropyl)-methacrylamide] (Strohalm, J. & Kopecek, J. Angew. Makromol. Chem., 70, 109-118 (1978)). Enzymatically degradable hydrogels based on this polymer have been prepared by incorporating oligopeptide sequences containing 2-4 amino acid residues in the crosslink. Such polymers were intended to be used in drug

Copolymerized oligopeptides with poly (ethylene glycol), PEG, have also been made into various architectures. Telechelic BAB block copolymers of PEG and small peptides having sequences cleavable by collagenase and plasmin were synthesized (West, J.L. & Hubbell, J.A. Macromolecules, 32, 241-244 (1998)). The hydrogel materials produced upon crosslinking were targeted for use in wound healing and tissue engineering. Biospecific cell adhesion properties of hydrogels were achieved by grafting cell adhesive peptides such as RGD into hydrogels containing peptide units that are degraded by collagenase and elastase (Mann et al Biomaterials, 22, 3045-3051 (2001)). Collagenase and elastase are enzymes involved in cell migration.

Further hydrogels were also developed that can assist tissue regeneration by mimicking matrix metalloproteinase (MMP) mediated invasion of the extracellular matrix (ECM). Linear oligopeptide substrates for MMPs were crosslinked with multiarm end functionalized PEG macromers (Lutolf, M. P. & Hubbell, J. A. Biomacromolecules, 4, 713-722 (2003)). Integrin-binding domains were attached in a pendant fashion to the hydrogel providing molecular signals for cell adhesion. The kinetic parameters for the substrate hydrolysis were determined through Michaelis-Menten analysis. Substrate degradation was followed by fluorescamin reaction. In a subsequent work, the rate of enzymatic gel degradation was monitored fluorimetrically by quantifying the amount of released dansylated oligopeptide from a didansyl-L-lysine-tagged protease-sensitive oligopeptide (Seliktar et al J. Biomed. Mater. Res. A. 2004, 68, 706-716 (2004)).
The concept of generic sensor materials for the detection of proteases based on peptide crosslinked hydrogels has more recently been described in WO 2008/047095. Generic and tunable sensor materials for the detection of proteases based on the thin film degradation of peptide cross-linked dextran hydrogels have been developed (Stair et al, Biosensors and Bioelectronics, 25, 2113-2118 (2009)). However, there is a need in the art for sensor materials which produce a rapid response to proteases.

Summary of the Invention
The present inventors have surprisingly found that producing peptide cross-linked hydrogel films by depositing a polymer layer and a peptide layer in two consecutive steps produces highly sensitive sensor coatings for the detection of proteases. This is in contrast to the previously known films which are obtained by a single deposition step where polymer and cross-linker are mixed and deposited together.

In a first aspect, the present invention provides a sensor coating layer comprising:

(a) a layer of a polymer deposited on a transducer; and
(b) a layer of a peptide deposited on said layer of a polymer.

Detailed Description of the Invention
The sensor coating layer of the present invention is capable of changing its properties on interaction with an analyte. Typically, the sensor coating layer is a hydrogel cross-linked with a peptide comprising up to 20 amino acids. The analyte is typically a protease. The protease degrades the peptide in the sensor coating layer, thus degrading the sensor coating layer. This degradation of the sensor coating layer can be monitored in various different ways.

The sensor coating layer of the first aspect of the present invention comprises: (a) a layer of a polymer deposited on a transducer; and (b) a layer of a peptide deposited on said layer of a polymer.
The sensor coating layer of the first aspect of the present invention can also be described as comprising: (a) a layer of polymer deposited on a transducer; and (b) a layer of peptide deposited on said layer of a polymer.

The polymer can be any convenient polymeric material which permits incorporation of short peptide sequences as described herein. The polymer can be synthetic or naturally-occurring. A single polymer or different polymers can be used. The polymer is typically a water soluble polymer.

Suitable examples of polymers for use in the invention include polysaccharides, for example dextran, starch, glucomannan, pectin, cellulose and its derivatives (such as cellulose acetate, carboxymethyl cellulose, and methyl cellulose), chitosan, lipopolysaccharides, sodium alginate, xanthan gum, carrageenan, hyaluronic acid and alginic acid (and chemically modified versions thereof).

Typically, when the polymer is a polysaccharide it is an oxidised polysaccharide. Typically, the oxidised polysaccharide is oxidised dextran.

Oxidised polysaccharides for use in the present invention can be modified with amines, for example benzyl amine. This improves the sensing properties of the sensor coating layer. Oxidised polysaccharides can also be modified with other nucleophiles such as alcohols, thiols and organometallics.

Other suitable examples of polymers for use in the invention include but are not limited to polyethylene glycol (PEG), polyvinylpyrrolidone, acrylamide, and polymers formed from acrylic and methacrylic monomers, polyvinylalcohol, polyethylene glycol acrylate, ethylene glycol methyl ether acrylate and dendrimers. Typically, when the polymer is polyethylene glycol it is a modified polyethylene glycol but unmodified polyethylene glycol can also be used. The PEG can be modified with suitable chemical functionality to confer electrophilic character upon it, for example using succinimidyl carbonate. This means that the PEG is susceptible to nucleophilic attack, for example by diamines.
The dendrimers are typically commercially available amino-terminated dendrimers, and are typically cross-linked using a diacrylated peptide via Michael addition reactions. Poly(ethylene oxide) is typically cross-linked using the unmodified peptide. The poly(ethylene oxide) is typically activated, for example, by activation of functional groups to render them electrophilic, for example, by conversion of alcohol groups to tosylate, mesylate, halide etc. Such electrophilic functional groups will then react with the amine functional groups of the cross-linking peptides.

The layer of polymer is typically a thin film and may range from monolayers to several hundred nm thick. Preferably, the polymer layer is from about 2 to 2000 nm thick, for example from 3 to 1000 nm, from 4 to 500 nm, suitably from 5 to 300 nm or from 7 to 200 nm thick. More preferably, the polymer layer is 10 to 100 nm thick.

The polymer layer can be deposited on the transducer by any suitable method. Typically, the polymer layer is deposited on the transducer by spin-coating, drop-coating, dip-coating, spray coating, casting, screen printing or inkjet printing.

Typically, the polymer layer is deposited on the transducer using a solution of the polymer in an appropriate solvent (for example dimethylformamide, acetonitrile, water, alcohols such as isopropanol, ethanol or methanol, chloroform or acetone or mixtures thereof). Typically, the polymer layer is deposited on the transducer using a solution of the polymer in water. The solution of the polymer may also contain one or more additives, for example a surfactant such as Tween 20 or buffer salts such as a phosphate buffer. In one embodiment, the solvent is an alcohol, such as isopropanol, and comprises a buffer. In one embodiment, the polymer layer is deposited on the transducer in an aqueous solution that also contains buffer salts and/or alcohol.

As used herein, a "transducer" is a means of translating a chemical signal into a signal that can be processed by electronic equipment. Such a signal is therefore representative of an analyte concentration. It can therefore be seen that a transducer for use in the present invention is a sensor or detector.
Transducers for use in accordance with the present invention include electrodes, optical transducers such as surface plasmon resonance substrates, holographic transducers, and weight sensitive transducers such as quartz crystal microbalance or surface acoustic wave devices. Transducers for use in accordance with the invention are typically non-porous. By a "non-porous" transducer is meant a transducer that liquids (such as solvents and/or reagents) cannot flow through.

A suitable method of detecting signal output which measures changes in the polymer layer is electrochemical impedance spectroscopy or impedance measurements at a single frequency. In one such embodiment, the transducer is an electrode coated by the sensor coating layer in which the electrode is part of an electrode pair. The counter electrode is uncoated and placed adjacent or opposite to the coated electrode and measurements of the impedance of a surrounding electrolyte solution and the sensor coating layer can be taken. In an alternative arrangement, the sensor coating layer can be applied to interdigitated electrodes, in which case the impedance measurement depends on the impedance of the sensor coating layer and the electrode/sensor coating layer interface. Interdigitated electrodes may be advantageous since sensor coatings with an impedance significantly larger or significantly smaller than that of the surrounding electrolyte can be detected.

Other electrochemical methods such as amperometry, cyclic voltammetry and potentiometry can also be used to detect signal output.

The transducer can be a single electrode but is typically a plurality of electrodes, that is to say more than one electrode. Typically, the transducer is a pair of electrodes, i.e. two electrodes. However, more electrodes can be used, such as 3, 4, 5, 6, 7, 8, 9 or 10 electrodes, or a plurality of pairs of electrodes can be used, for example 2, 3, 4 or 5 pairs of electrodes, giving 4, 6, 8 or 10 electrodes in total.

Typically, the electrodes are interdigitated electrodes. The electrode or electrodes are typically non-porous.
Typically, the electrodes for use in the present invention contain a metal or carbon. The metal is typically a noble metal. Noble metals include metals such as gold, silver and platinum, or alloys thereof, which display resistance to corrosion or oxidation. Preferably the electrode is gold. Typically, the gold is deposited by thermal evaporation. For example, 50 run chromium and 150 nm gold can be deposited onto insulator (silicon nitride) coated silicon, onto alumina substrates or onto glass slides using thermal evaporation. Alternatively, screen printed gold electrodes on alumina can be used. The thickness of the gold coating may vary considerably, but is usually between 10 and 1000 nm, for example between 15 and 700 nm, for example between 17 and 500 nm, for example between 20 and 100 nm. Preferably, the thickness of the gold coating is between 45 and 80 nm.

Typically, the transducer is a plurality of electrodes deposited onto a substrate. The substrate is typically a ceramic material, such as alumina, or a plastic material.

In one embodiment, the electrodes are modified with a self assembled monolayer. A self assembled monolayer (SAM) is an organized layer of amphiphilic molecules in which one end of the molecule, the "head group", shows an affinity for a substrate. The amphiphilic molecules which form the SAM also consist of a "tail" with a functional group at the terminal end. For example, the head group can be a thiol group, which binds with the surface of our electrodes, for example a gold surface. In one embodiment, the electrodes are modified using aminoethanethiol. Other suitable head groups include a silyl group. The functional group at the terminal end can for example be an -OH group, i.e. a hydroxyl terminal group, or an -NH₂ group, i.e. an amino terminal group. Typically, the monolayer comprises amino terminal groups or hydroxyl terminal groups. Other suitable terminal groups could be a carboxylic acid group.

The sensor coating layer of the first aspect of the invention comprises a layer of a peptide deposited on the layer of a polymer. The sensor coating layer of the first
aspect of the invention can also be described as comprising a layer of peptide deposited on the layer of polymer.

The layer of a peptide may comprise one type of peptide or a plurality of different peptides.

The peptide or peptides in the peptide layer are capable of cross-linking the polymer. In some embodiments, the peptide contains a lysine residue at the C-terminus to enable the peptide to cross-link the polymer via its amine terminal group. The peptides may be optionally amidated (NH₂) at the C-terminus and may also comprise succinate residues at the N-terminus. Other C-terminus and N-terminus linker groups may also be included as desired.

In some embodiments, the peptide is unmodified.

The peptide typically comprises up to 20 amino acid residues. Suitably, the length of the peptide may be up to 19 residues, 18 residues, 17 residues, 16 residues, 15 residues, 14 residues, 13 residues, 12 residues, 11 residues, 10 residues, 9 residues, 8 residues, 7 residues, 6 residues, 5 residues, 4 residues or up to 3 residues. For example, the peptide may be between 3 to 9 amino acid residues in length, for example 7, 8 or 9 amino acid residues in length.

The peptide may be composed of any one of the following naturally occurring amino acid residues in any combination or number as may be required, for example for a protease enzyme to cleave the sequence:

Alanine, Arginine, Asparagine, Aspartic acid, Cysteine, Glutamine, Glutamic acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, Valine.

The above amino acids may be represented by the short 3-letter code as:
Ala, Arg, Asn, Asp, Cys, Gin, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val

or alternatively, by the one-letter code as:


Where Asx or B is used to denote Asparagine or Aspartic acid, Glx or Z is used to denote Glutamine or Glutamic acid, and X is any amino acid residue.

The sequence of the peptide chain may be chosen to reflect the specificity of a protease enzyme to be detected or it may be a variant of the natural cleavage site and/or binding site.

An example of a variant sequence is a sequence in which one or more residues have been altered or modified, or a sequence in which there has been a substitution of one or more amino acids with one or more other amino acids. The skilled person is aware that various amino acids have similar properties. One or more such amino acids of a substance can often be substituted by one or more other such amino acids without eliminating a desired activity of that substance.

Thus the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids which can often be substituted for one another include: phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulphur containing side chains).
Substitutions of this nature are often referred to as "conservative" or "semi-conservative" amino acid substitutions. Amino acid deletions or insertions may also be made relative to the amino acid sequence of the peptide cleavage site and/or binding sequence of the protease referred to above. Thus, for example, amino acids which do not have a substantial effect on the activity of the protease, or at least which do not eliminate such activity, may be deleted.

Amino acid insertions relative to the sequence of the peptide can also be made. This may be done to alter the properties of the peptide (e.g. to enhance binding or specificity of cleavage).

Amino acid changes relative to the sequence given above can be made using any suitable technique e.g. by using site-directed mutagenesis for recombinantly expressed peptides.

It should be appreciated that amino acid substitutions or insertions within the scope of the present invention can be made using naturally occurring or non-naturally occurring amino acids. Whether or not natural or synthetic amino acids are used, either D- or L-amino acids may be used. Seleno analogues of cysteine or methionine may be used.

The layer of peptide is typically a thin film and may range from monolayers to several hundred nm thick. Preferably, the peptide layer is from about 2 to 2000 nm thick, for example from 3 to 1000 nm, from 4 to 500 nm, suitably from 5 to 300 nm or from 7 to 200 nm thick. More preferably, the peptide layer is 10 to 100 nm thick.

The peptide layer can be deposited on the polymer layer by any suitable method. Typically, the peptide layer is deposited on the polymer layer by spin-coating, drop-coating, dip-coating, spray coating, casting, screen printing or inkjet printing.

Typically, the peptide layer is deposited on the polymer layer (on the transducer) using a solution of the peptide in an appropriate solvent (for example dimethylformamide, acetonitrile, water, alcohols such as isopropanol, ethanol or
methanol, chloroform or acetone or mixtures thereof). Typically, the peptide layer is deposited on the polymer layer using a solution of the peptide in water. The peptide layer is thus typically deposited from a water-based solution. The solution of the peptide may also contain one or more additives, for example a surfactant such as Tween 20 or buffer salts such as a phosphate buffer. In one embodiment, the solvent is an alcohol, such as methanol, and comprises a buffer. In one embodiment, the peptide layer is deposited on the transducer in an aqueous solution that also contains buffer salts and/or alcohol.

In one embodiment, the layer of a peptide does not include any other components apart from those present in the solution in which the peptide is deposited. For example, the layer of peptide may also comprise a residue of salts. In some embodiments, the layer of peptide is a layer of peptide alone. In this embodiment, the sensor coating layer comprises (a) a layer of a polymer deposited on a transducer; and (b) a layer consisting of a peptide deposited on said layer of a polymer.

In one specific embodiment, the sensor coating layer of the first aspect of the invention consists of: (a) a layer of a polymer deposited on a transducer; and (b) a layer of a peptide deposited on said layer of a polymer. In other words, in one embodiment the sensor coating layer contains no other components or layers in addition to the layer of polymer and the layer of peptide. In an alternative embodiment, the sensor coating layer contains no other components or layers in addition to the layer of polymer and the layer of peptide apart from those present in the solutions in which the polymer and/or peptide is deposited. For example, the layer of polymer and/or peptide may also comprise a residue of salts.

As set out above, the sensor coating layer of the first aspect of the invention is capable of changing its properties on interaction with an analyte.

The analyte is typically an analyte solution. In one embodiment, the analyte solution contains a charge transfer reagent. Typically, the charge transfer reagent is ferricyanide or ferrocyanide or a mixture of both ferricyanide and ferrocyanide.
The analyte is typically an enzyme capable of cleaving the peptide in the peptide layer. Thus, the analyte is typically a protease, such as a protease including but not limited to serine proteases, matrix metalloproteinases, and gingipain proteases.


The best known examples of serine proteases include trypsin, chymotrypsin, cathepsin G, subtilisin and elastase (for example, human neutrophil elastase (HNE)).
Chymotrypsin is responsible for cleaving peptide bonds flanked with bulky hydrophobic amino acid residues. Preferred residues include phenylalanine, tryptophan, and tyrosine, which fit into a hydrophobic pocket in the protein folds of the enzyme. Trypsin is responsible for cleaving peptide bonds flanked with positively charged amino acid residues. The hydrophobic pocket in the enzyme has an aspartic acid residue at the back of the pocket. This can then interact with positively charged residues such as arginine and lysine. Elastase is responsible for cleaving peptide bonds flanked with small neutral amino acid residues, such as alanine, glycine and valine. The hydrophobic pocket is lined with valine and threonine thus it can accommodate these smaller amino acid residues.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases. Other family members are ADAMs, Serralysins, Astacins. The MMPs belong to a larger family of proteases, the Metzincin superfamily. Collectively such enzymes are capable of degrading all kinds of extracellular matrix proteins, but also can process a number of bioactive molecules. They are known to be involved in cleavage of cell surface receptors, release of apoptotic ligands, and chemokine in/activation. MMPs are indicated in a number of disease states including autoimmune diseases such as arthritis and MS, transplant rejection, arterial stiffness and cancer.

The main MMPs known to date include the enzymes specific for collagen known as "Collagenases". These MMPs are capable of degrading triple-helical fibrillar collagens into distinctive 3/4 and 1/4 fragments. These collagens are the major components of bone and cartilage, and MMPs are the only known mammalian enzymes capable of degrading them. The collagenases are: MMP-1, MMP-8, MMP-13, and MMP-18. MMP-14 (MT1-MMP) has also been shown to cleave fibrillar collagen, and there is evidence that MMP-2 is capable of collagenolysis. Another group of MMPs is the "Stromelysins" which display a broad ability to cleave extracellular matrix proteins but are unable to cleave the triple-helical fibrillar collagens. The group includes: MMP-3, MMP-10 and MMP-11. MMP-11 shows more similarity to the MT-MMPs, is convertase-activatable and is secreted therefore usually associated to convertase-activatable MMPs.
Other MMPs include Metalloelastase (MMP-12), MMP-19, Enamelysin (MMP-20), MMP-27 (MMP-22, C-MMP), the "Matrylysins" which include Matrylysin (MMP-7), Matrylysin-2 (MMP-26), and the "Gelatinases". The main substrates of the gelatinase MMPs are type IV collagen and gelatin, and these enzymes are distinguished by the presence of an additional domain inserted into the catalytic domain. This gelatin-binding region is positioned immediately before the zinc binding motif, and forms a separate folding unit which does not disrupt the structure of the catalytic domain. The two members of this sub-group are: MMP-2 (expressed in most tissues) and MMP-9 (predominantly found in neutrophils).

There are also the "Convertase-activatable MMPs" and the secreted MMPs including Stromelysin (MMP-11), MMP-21 (X-MMP), Epilysin (MMP-28). The Membrane Bound MMPs include: the type-II transmembrane cysteine array MMP-23; the glycosyl phosphatidylinositol-attached MMPs 17 and 25 (MT4-MMP and MT6-MMP respectively), and the type-I transmembrane MMPs 14, 15, 16, 24 (MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP respectively). All 6 MT-MMPs have a furin cleavage site in the pro-peptide, which is a feature also shared by MMP-11. Other known MMPs include MMP-23A, MMP-23B.

Gingipain proteases include arg-gingipain and lys-gingipain.

The peptide in the peptide layer is therefore a target for a particular protease. Preferred sequences include, but are not limited to:

Ala-Ala-Pro-Val-Ala-Ala-Lys (AAPVAAK)

Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Lys (APEEIMDRK)

Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Glu (APEEIMDRQ)

Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg (APEEIMDR)
Ala-Ala-Pro-Val (AAPV)

Ala-Ala-Pro-Phe (AAPF)

Ala-Ala-Pro-Phe-Phe-Lys (AAPFFK)

Gly-Gly-Arg (GGR)

Gly-Pro-Gln-Gly-Ile-Trp-Gly-Gln (GPQGIWGQ)

Gly-Pro-Gln-Gly-Ile-Trp-Gly-Gln-Lys (GPQGIWGQK)

Phe-Ala-Ala-Phe-Phe (FAAFF)

Val-Arg-Ser-Ser-Ser-Arg-Thr-Lys (VRSSSRTK)

Ile-Glu-Gly-Arg-Thr-Ala-Trh-Lys (IEGRTATK)

Leu-Asp-Arg-Arg-Gly-Ile-Gln-Lys (LDRRGIQK)

Gly-Pro-Gln-Gly-Ile-Phe-Gly-Gln-Lys (GPQGIFGQK)

The peptides Ala-Ala-Pro-Val-Ala-Ala-Lys (AAPVAAK), Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Lys (APEEIMDRK), Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg-Glu (APEEIMDRQ), Ala-Pro-Glu-Glu-Ile-Met-Asp-Arg (APEEIMDR) and Ala-Ala-Pro-Val (AAPV) are all preferentially cleaved by human neutrophil elastase (UNE).

Ala-Ala-Pro-Phe (AAPF) and Ala-Ala-Pro-Phe-Phe-Lys (AAPFFK) are preferentially degraded by cathepsin-G, Gly-Gly-Arg (GGR) is cleaved by arg-gingipain, Gly-Pro-Gln-Gly-Ile-Trp-Gly-Gln (GPQGIWGQ) and Gly-Pro-Gln-Gly-Ile-Trp-Gly-Gln-Lys (GPQGIWGQK) are preferred substrates for MMP8 and Phe-Ala-Ala-Phe-Phe-
(FAAFF) is a preferred substrate for subtilisin. Val-Arg-Ser-Ser-Ser-Arg-Thr-Lys (VRSSSRTK) is specifically cleaved by arg-gingipain and trypsin.

Ile-Glu-Gly-Arg-Thr-Ala-Thr-Lys (IEGRTATK) is derived from a cleavage site in prothrombin. Lysine was added to the end of the natural cleavage site. Leu-Asp-Arg-Gly-Ile-Gln-Lys (LDRRGIQK) is derived from the Arg506 cleavage site of activated factor V, which is the natural substrate of activated protein C (APC). Again, lysine was added to the end of the natural cleavage site. Gly-Pro-Gln-Gly-Ile-Phe-Gly-Gln-Lys (GPQGIFGQK) is cleaved by MMP-2.

The sensor coating layer according to the first aspect of the invention degrades rapidly under the catalytic action of a specific protease directed to the peptide used in the peptide layer. The term degradation is used in its conventional sense, i.e., a chemical reaction in which a compound is converted, or decomposes in some way, to give a simpler compound, for example, by dissolution. In the context of the present invention, such degradation of the cross-linked polymer describes the process of dissolving or reducing the cross-link density of the polymer.

In a second aspect, the present invention provides a sensor device comprising:

(a) a substrate upon which a transducer is formed; and

(b) a sensor coating layer according to the first aspect of the invention.

The sensor device of the second aspect of the invention can also be described as a biosensor device. In one embodiment, the sensor device consists of (a) a substrate upon which a transducer is formed; and (b) a sensor coating layer according to the first aspect of the invention.

In one embodiment, the substrate is an insulating plate.

In one embodiment, the transducer is a plurality of electrodes.
Typically, the sensor device further comprises a capillary fill channel. Typically, the analyte sample is applied to the sensor device by means of the capillary fill channel.

In one embodiment, an insulator, typically a patterned insulator, is deposited onto the insulating plate prior to step (b). Typically, the insulator is an epoxy resin, such as an SU-8 photoresist, which is typically patterned by photolithography. SU-8 is an epoxy-based negative photoresist.

In a third aspect, the present invention provides a method of preparing a sensor coating layer according to the first aspect of the invention, comprising:

(a) depositing a layer of a polymer on a transducer; and
(b) depositing a layer of a peptide on said layer of a polymer.

The method of the third aspect of the invention can also be described as comprising the following steps:

(a) depositing a layer of polymer on a transducer; and
(b) depositing a layer of peptide on said layer of a polymer.

Step (b) of the method of the third aspect of the invention involves depositing a layer of peptide on said layer of polymer. Typically, the peptide is not deposited in a mixture with a polymer. Step (b) typically involves depositing a layer of peptide alone (by itself) onto said layer of polymer, or only with its solvent (including any additives). The layer of peptide is typically not deposited in a mixture (with other reactive components) or as a conjugate.

Typically, the layer of polymer is allowed to dry prior to step (b). For example, the polymer can be left to dry at any suitable temperature for any suitable length of time. In one specific embodiment, the polymer is left to dry for 24 hours at 30°C.

In one embodiment, the method of the third aspect of the invention consists of: (a) depositing a layer of a polymer on a transducer; and (b) depositing a layer of a peptide on said layer of a polymer, optionally with a drying step in between. In other words,
the method of the third aspect of the invention contains no steps other than the steps of depositing the layer of polymer on the transducer, an optional drying step and depositing the layer of peptide on the layer of polymer.

It is noted that the process of depositing the polymer and peptide layer is distinct from other processes such as adsorbing the polymer and/or peptide layer on a surface.

Once step (b) has been carried out, the sensor coating layer can be left for a suitable period of time, for example overnight or for up to 3 days, to allow the crosslinking reaction to proceed. This can be done at any suitable temperature, for example at room temperature, or up to 60°C, for example at around 30°C, 40°C or 50°C.

In a fourth aspect, the present invention provides a method of preparing a sensor device according to the second aspect of the invention, comprising:

(a) providing a substrate upon which a transducer is formed; and
(b) carrying out the method of the third aspect of the invention.

The sensor coating layer of the first aspect of the present invention is suitable for use in the methods described in WO 2008/047095.

Accordingly, in a fifth aspect, the present invention provides a method for the detection of a protease, comprising:

(a) contacting a sample to be assayed with a sensing device according to the second aspect of the invention; and
(b) measuring a signal output of said sensing device.

The sensing device of the second aspect of the invention is useful in the diagnosis of periodontitis. Periodontal diseases are a group of related inflammatory conditions affecting the supporting tissues of teeth. Inflammation of the gums (gingivae) is caused by the local accumulation of an oral biofilm, dental plaque. In its most severe form the disease can lead to destruction of the tooth supporting tissues, including resorption of alveolar bone and loss of teeth. There are several distinct clinical forms
of periodontitis but chronic periodontitis, most frequently found in adults, is the most common. Disease progression is usually site specific but is not uniform and it is difficult to distinguish clinically those sites that are progressing from those that are inflamed but not progressing. As a result a considerable level of unnecessary treatment may occur of periodontal sites that are not progressing. The sensing device of the second aspect of the invention can be used in a non-invasive technique for more accurately identifying periods of acute inflammatory destruction.

Both early in the pathogenesis of periodontitis and as acute exacerbations during its chronic course, neutrophils (PMNL) migrate to the site and there is a fluid exudate (gingival crevicular fluid (GCF)) into the "pocket" between the tooth and the gingival tissue. Analysis of this fluid provides a convenient, non-invasive means of monitoring host or bacterial molecules. Human neutrophil elastase (HNE), cathepsin G, and MMP8 have been identified as suitable markers for disease progression and therefore as target proteases for this detection technology.

The sensing device of the second aspect of the invention is therefore useful for the analysis of GCF to detect enzymes such as HNE, cathepsin G, and MMP8.

In one embodiment, the present invention provides a sensing layer capable of changing its properties due to the interaction with an analyte solution comprising:

(a) a polymer layer deposited on a transducer; and
(b) a peptide layer deposited on top of the polymer layer, wherein the peptide(s) is (are) capable of cross-linking the polymer.

In one embodiment, the present invention provides a biosensor device for the detection of analyte, comprising:

(i) an insulating plate upon which a plurality of electrodes are formed;
(ii) a coating of a polymer over the electrodes; and
(iii) a layer of peptide on the polymer; wherein the peptide is capable of forming cross-links with the polymer,

such that in the presence of an analyte, the properties of the cross-linked
polymer sensing film are altered which can be followed by an electrochemical measurement.

The present invention has the advantage that the sensor coating layer produced by depositing a polymer first and subsequent deposition of the peptide cross-linker produces highly sensitive sensor coatings for the detection of proteases. It is assumed that the two-step coating method produces a highly cross-linked surface layer on the polymer, such as dextran. Once the cross-links in the surface layer have been broken by a protease such as HNE, the underlying non-cross-linked polymer such as dextran should dissolve rapidly resulting in a large sensor response. An added advantage of the method of the invention is that it allows printing of films by inkjet printing and other methods such as screen-printing and drop coating preventing the cross-linking reaction from occurring in the cartridge.

It is not intuitive that two-step coated films should be more sensitive than films obtained by a single step deposition where polymer and cross-linker are mixed and deposited together. Indeed, there are a number of problems that should be expected when depositing a polymer (such as a water soluble polymer) in a solvent and then subsequently depositing a peptide cross-linker also from a solution such as a water based solution:

1. The solution of the cross-linker would be expected to partially or completely dissolve the underlying polymer (such as a water soluble polymer) before drying (degree of dissolution depends on the deposition technique) resulting in an uneven concentration distribution causing uneven, badly reproducible films. Surprisingly, the present inventors found that this was not the case. As shown in the Examples herein, films coated in this manner were highly reproducible and showed high sensitivity. In one of the Examples high-molecular weight dextran was used. It is assumed that these films dissolved only slowly in the cross-linker solution thereby avoiding or at least reducing the above problem.
2. Using a two-step method, it would be expected that the surface of the polymer film
is highly cross-linked while the lower part of the film that is in contact with the sensor
substrate is not. One would expect that such a film would not be stable in a solution
such as an aqueous solution rendering the films useless as sensor coatings. However,
the Examples herein demonstrate that the films are very stable in buffer solutions not
containing any analyte.

Preferred features for the second and subsequent aspects of the invention are as for the
first aspect *mutatis mutandis.*

The present invention will now be further described by way of reference to the
following Examples which are present for the purposes of illustration only. In the
Examples, reference is made to a number of Figures in which:

Figure 1 shows the change of resonant frequency of a QCM crystal coated
with 25% AAPVAAK cross-linked dextran hydrogel before and after
exposure to various HNE activities (left graph) and data from the linear
portion of the degradation curve normalized to time of initial degradation
(right graph). Films were exposed to 0 U ml⁻¹ HNE (a), 2.5 U ml⁻¹ HNE (b), 5
U ml⁻¹ HNE (c), 10 U ml⁻¹ HNE (d), 15 U ml⁻¹ HNE (e), 20 U ml⁻¹ HNE (f),
and 30 U ml⁻¹ HNE (g). Mass loss begins after -15 min for 2.5 U ml⁻¹ HNE,
~8 min for 5 U ml⁻¹ HNE, ~4 min for 10 U ml⁻¹ HNE, ~3 min for 15 U ml⁻¹
HNE, ~3 min for 20 U ml⁻¹ HNE, and -1.5 min for 30 U ml⁻¹ HNE
(reproduced from Stair et al, Biosensors and Bioelectronics, 25, 2113-
2118(2009)).

Figure 2 shows impedance change measured before and after addition of 10
U/mL HNE to a pH 7.4 phosphate buffer containing a charge transfer reagent
(5mM [Fe(CN)₆]³⁻ and 5mM [Fe(CN)₆]⁴⁺). The graph shows the response of an
AAPVAAK cross-linked hydrogel film produced by a single step coating
method (squares) and the response of an AAPVAAK cross-linked hydrogel
film produced by a two step coating method (circles).
Figure 3 shows a comparison of two-step coated dextran film degradation by HNE in buffer solutions of different BSA concentrations.

Figure 4 shows (a) the degradation of both unmodified and benzyl amine modified dextran films in the presence of cathepsin G and (b) the degradation of benzyl amine modified dextran films in the absence and presence of 2% BSA.

Figure 5 shows the degradation of a dextran film prepared by ink-jet printing on an interdigitated electrode.

Figure 6 is a micrograph of drop coated oxidised dextran and drop coated peptide on an interdigitated electrode.

Figure 7 shows the change of the electrical impedance at 100 Hz before and after the addition of (a) HNE, (b) cathepsin G and (c) MMP8. Each film was cross-linked with a different peptide containing one or more cleavage sites for the individual enzyme to be detected.

Figure 8 shows schematics of (a) array of 5 pairs of screen printed gold electrodes, (b) array with patterned SU-8 2000.5

Figure 9 shows AAPVAAK cross-linked hydrogel on an unmodified sample, measured with pH=7.4 charge transfer buffer at 1 kHz, 10 U/mL HNE was added at 773 s.

Figure 10 shows AAPVAAK cross-linked hydrogel on 4-mercapto-l-butanol treated samples, measured with pH=7.4 charge transfer buffer at 1 kHz, HNE was added at 844 s.
Figure 11 is a schematic representation of the formation of a functionalised hydrogel from succinimide-terminated PEG and peptide.

Figure 12 (a) is a micrograph of two-step inkjet printed PEG hydrogel films and (b) shows change of impedance with time before and after the addition of 10 U/mL HNE to a pH 7.4 phosphate buffer containing a charge transfer reagent (5mM [Fe(CN)₆]³⁻ and 5mM [Fe(CN)₆]⁴⁺).

Figure 13 is a schematic of a capillary fill device.

Figure 14 shows impedance spectra of AAPVAAK cross-linked hydrogel films prepared by a one-step coating method before (A) and after (B) the addition of 10 U ml⁻¹ HNE to pH 7.5 charge transfer buffer.

Figure 15 shows impedance change measured with AAPVAAK cross-linked hydrogel coated IDEs on silicon before and after addition of different concentrations of HNE (0.1 U ml⁻¹ (A), 1 U ml⁻¹ (B) and 10 U ml⁻¹ (C)) to a pH 7.5 charge transfer buffer containing (a) 0% BSA, (b) 2% BSA. HNE was added at t = 5 min.

Figure 16 shows impedance change measured with an AAPVAAK cross-linked hydrogel coated on IDEs on 96% alumina modified with 4-mercapto-l-butanol as monolayer (A) and 2-aminoethanethiol as monolayer (B) before and after addition of 10 U ml⁻¹ HNE to pH 7.5 charge transfer buffer. HNE was added at t = 6 min.

Figure 17 (a) shows impedance change measured with AAPFFK cross-linked hydrogel coated IDEs on alumina before and after addition of different activities of cathepsin G to pH 7.5 charge transfer buffer: 0 mU ml⁻¹ (A), 5 mU ml⁻¹ (B), 10 mU ml⁻¹ (C), 25 mU ml⁻¹ (D), 50 mU ml⁻¹ (E); (b) is a calibration curve showing the rates of impedance change for the first 100 s after the addition of cathepsin G versus cathepsin G activity. Points represent the average of two individual measurements at each activity.
Figure 18 (a) shows impedance change measured with GPQGIWGQK cross-linked hydrogel coated IDEs on alumina before and after addition of different activities of MMP8 to a pH 7.5 charge transfer buffer: 0 mU ml⁻¹ (A), 0.05 mU ml⁻¹ (B), 0.1 mU ml⁻¹ (C), 0.2 mU ml⁻¹ (D), 0.4 mU ml⁻¹ (E), 0.8 mU ml⁻¹ (F); (b) calibration curve showing the rates of impedance change for the first 100 s after the addition of MMP8 versus MMP8 activity. Points represent the average of two individual measurements at each activity.

Figure 19 shows impedance change after filling capillary fill device on IDEs coated with an AAPVAAK cross-linked dextran hydrogel with pH 7.5 charge transfer buffer containing different activities of HNE: 0 U ml⁻¹ (A), 5 U ml⁻¹ (B), 10 U ml⁻¹ (C) and 30 U ml⁻¹ (D).

Figure 20 shows the relative impedance change of a sensor coated with a LDRRGIQK cross-linked dextran hydrogel before and after the addition of 0.1 U/ml APC to TRIS buffer, pH 7.4 at 27°C.

Figure 21 shows the relative impedance change of a sensor coated with a GPQGIFGQK cross-linked dextran hydrogel before and after the addition of 1500 ng/ml MMP-2 to PBS, pH 7.4 at 27°C.

Figure 22 is a schematic of (a) array of interdigitated electrodes and (b) electrode array with SU-8 insulator.

Figure 23 shows the relative impedance change of AAPVAAK cross-linked dextran film before and after the addition of 10 U/ml HNE to PBS, pH 7.4. The impedance was measured at 1 kHz at 30°C.

Figure 24 is a schematic of a sensor array (12 x 20 mm) with 5 interdigitated electrodes coated with peptide cross-linked dextran hydrogels and a capillary fill channel. The fill volume of the capillary fill device is 1.4 µl.
Figure 25 shows relative impedance changes after the addition of buffer and enzyme solution to a capillary fill device. Drop coated dextran and peptide films on thin screen-printed electrodes with amino-terminated monolayer measured in pH 7.4 PBS at 1 kHz. (a) films cross-linked with AAPVAAK, (b) films cross-linked with AAPFFK.

Figure 26 shows sample response of capillary fill sensor array to 2mU/ml cathepsin G. Each sensor coating was cross-linked with a different peptide. Sensor 1 was cross-linked with AAPVAAK - sensitive to HNE, sensor 2 was cross-linked with AAPFFK - sensitive to cathepsin G, sensor 3 was cross-linked with VRSSSRTK - sensitive to arg-gingipain, and sensor 4 was cross-linked with GPQGIWGQK - sensitive to MMP-8.

Examples

Comparative Example - single-step coating method of peptide cross-linked hydrogels

Generic and tunable sensor materials for the detection of proteases based on the thin film degradation of peptide cross-linked dextran hydrogels have been developed (Jacqueline L. Stair, Michael Watkinson, Steffi Krause, "Sensor materials for the detection of proteases", Biosensors and Bioelectronics, 25 (2009) 2113-2118).

Hydrogel cross-links were formed via simple imine linkages between aldehyde groups in oxidized dextran and a peptide sequence susceptible to protease cleavage. Dextran with molecular weight two million was oxidized by excess sodium periodate to produce aldehyde functionalized dextran, which was then coated together with peptide AAPVAAK on the surface of the sensor substrates. The coated electrodes were left in an incubator at 25°C for two days to make sure the reaction was complete. Degradation of the hydrogel films was monitored in this study using quartz crystal microbalance (QCM). The sensor material was developed using the protease/peptide
pair of human neutrophil elastase (HNE) and Ala-Ala-Pro-Val-Ala-Ala-Lys (AAPVAAK). A direct relationship between the hydrogel degradation rate and protease activity was observed; HNE activities from 2.5 U ml⁻¹ to 30 U ml⁻¹ were detected. A second protease/peptide pair of cathepsin G and Ala-Ala-Pro-Phe-Phe-Lys (AAPFFK) was tested where 25% AAPFFK cross-linked hydrogels demonstrated a rapid response at 100 mU ml⁻¹. Swapping the protease/peptide pairs to HNE/AAPFFK and cathepsin G/AAPVAAK showed low levels of cross-sensitivity further demonstrating the specificity of film degradation.

Figure 1 shows the change of the resonance frequency of a hydrogel coated quartz crystal with time before and after addition of the enzyme human neutrophil elastase.

A drawback of the hydrogel films described is that there is a long delay between the addition of the enzyme and the change in the sensor signal, particularly at low concentrations. This renders the sensor materials unsuitable for applications where a rapid response is required, e.g. monitoring periodontal disease, where a sensor response within 3 minutes is desirable.

**Example 1 - Two-step coating method of peptide cross-linked hydrogels**

1. Peptide cross-linked dextran hydrogels

The sensor response of peptide cross-linked hydrogels to various proteases has been monitored using impedance measurements using a protease solution in a pH 7.4 phosphate buffer containing a charge transfer reagent (5mM [Fe(CN)₆]³⁻ and 5mM [Fe(CN)₆]⁴⁺). However, the films still showed the same delay in sensor response upon addition of enzyme to the solution (Figure 2, squares).

A two-step coating method was developed depositing the oxidised dextran and the peptide in two consecutive steps. In the two-step coating strategy, a dextran film was prepared by spin coating oxidised dextran solution first. After leaving the dextran layer to dry overnight, the peptide solution was coated on the surface of dextran. The electrode was left overnight again for the crosslinking reaction to proceed.
Surprisingly, addition of HNE to a pH 7.4 phosphate buffer containing a charge transfer reagent (5mM [Fe(CN)₆]³⁻ and 5mM [Fe(CN)₆]⁴⁺) resulted in a much more rapid impedance response from the film degradation (Figure 2, circles) than the films produced by a single step coating method.

Further results with two-step coated peptide cross-linked dextran hydrogel films

Figure 3 shows the sensor response to HNE in the presence of different concentrations of BSA to show the effects of non-specific binding. The impedance change in the absence of BSA was over 20% in 3 minutes. The addition of BSA resulted in a longer degradation time. The impedance change in 3 minutes was 11% and 6% in the presence of 1% and 2% BSA, respectively. This shows that a significant sensor response can be obtained at relatively high protein background concentrations.

The same synthetic strategies have been applied to prepare hydrogel films for detecting the enzyme cathepsin G by replacing the peptide chain used with a sequence that is cleaved by cathepsin G (AAPFFK). The clinically relevant concentration range of cathepsin G that will need to be detected for monitoring periodontal disease is approximately 1000 times lower than that of HNE. Dextran hydrogel films show a favourable response even at low cathepsin G concentration, as shown in Figure 4.

Modifying the dextran films by incorporating benzyl amine was found to further improve the impedance response to cathepsin G. At a low cathepsin G concentration of 2.4 mU/ml, the impedance change reaches around 9% in three minutes with the benzyl amine modification and only around 2% without this modification (see Figure 4a). The modified dextran film also showed a favourable impedance response in the presence of 2% BSA (see Figure 4b), indicating that non-specific binding of BSA does not significantly reduce the degradation rate.

Deposition techniques

The two-step coated hydrogel films have proven to be robust in that hydrogel films that show a good sensor response can be produced by different coating techniques.
Apart from spin-coating, inkjet printing and drop coating have resulted in good quality hydrogel films.

(i) **Inkjet printing**

A dextran layer was spin coated onto the electrode and a solution of peptide in pH 8 buffer was ink-jet printed onto this layer. The printed peptide solution did not spread well on the dextran surface. The addition of a small amount of Tween-20 (0.005%) to the peptide solution significantly improved the solution spreading. Continuous films of high quality were produced using a drop spacing of 18 μm. Ink-jet printed films prepared on interdigitated electrodes were used as opposing electrodes to detect HNE with a platinum counter electrode. A favourable impedance response to HNE was obtained (see Figure 5).

(ii) **Drop coating**

Dextran hydrogel films have been prepared successfully by drop coating oxidised dextran onto a sensor substrate and subsequent drop coating of a peptide solution onto the dextran film. A film image of a drop coated film on an interdigitated electrode is shown in Figure 6.

Drop coated films have been used to successfully detect HNE, cathepsin G and MMP8 (Figure 7).

**Hydrogel deposition into a well formed by SU8 on screen printed gold electrodes**

An array of 5 pairs of interdigitated gold electrodes was screen printed on a 96% alumina substrate by Gwent Electronic Materials Ltd. (UK) (Figure 8 a). SU-8 was patterned on the surface of the electrode array to separate the electrodes during peptide deposition and insulate the tracks in the array (Figure 8 b).

Samples were prepared as follows:

a) Screen printed electrodes were cleaned in piranha solution ($\text{H}_2\text{SO}_4$:$\text{H}_2\text{O}$, 3:1) for 1 min 30 s and then left on a hotplate at a temperature of 200°C for 1 hour,

b) Spin coating of SU-8 2000.5
The spin coater was ramped up to 500 rpm in 5 s with an acceleration rate of 100 rpm/s, and then kept at 500 rpm for 10 s.

Spin coater was ramped from 500 rpm to 3000 rpm with an acceleration rate of 300 rpm/s, and kept at 300 rpm for 30 s.

c) The sample was left on a hotplate at temperature of 95°C for 2 min.

d) Sample was exposed with a UV lamp for 30 s.

e) Post exposure bake: The sample was left on a hotplate at a temperature of 95°C for 2 min.

f) The UV-exposed sample was developed in EC solvent for 15 s, and then rinsed with isopropanol.

g) The sample was blown dry by a stream of nitrogen gas.

Samples were then split into two groups. The first group was treated with 4-mercapto-1-butanol (10 mM/L) for 1 hour. The second group was left untreated.

Process for thiol treatment:
Samples were immersed in a 10 mM thiol solution in a H2O/CH3CH2OH mixture (H2O:CH3CH2OH, 1:4) for 1 hour. After the treatment, samples were rinsed with ultrapure water and blown dry by nitrogen.

The oxidised dextran was then spin-coated onto the sample surface at 3000 rpm for 25 s. Samples were dried overnight (12 hours) at 30°C. Finally a 40 mM solution of the peptide AAPVAAK was drop coated into the SU-8 wells. Samples were cured at 30°C for 24 hours.

The degradation of the films was monitored at 1 kHz. The results for hydrogels deposited onto unmodified electrodes (Figure 9) and electrodes with a self assembled monolayer of 4-mercapto-1-butanol (Figure 10) were compared. There is no significant difference in the behaviour of both types of sample. In both cases the impedance is stable in a charge transfer buffer solution and increases after the addition of HNE indicating that the presence of a self-assembled monolayer is not essential for the function of the sensor. Surprisingly, the impedance increased rather
than decreased upon the addition of HNE as normally observed for electrodes modified with a hydroxyl terminated monolayer. It is assumed that the dextran bonded to the SU-8 edge due to the reaction of the epoxy resin in SU-8 to the amine functional group in the peptide. Therefore, after the peptide was cleaved, the films swelled rather than dissolved in the solution.

2. Peptide cross-linked poly(ethylene glycol)

Poly(ethylene glycol) (PEG) is a common polymer used in biological applications because it is stable in common biological conditions and shows limited interaction with proteins. It is often used to coat particles and other formulations for intravenous use to prevent or restrict non-specific adsorption. Our approach uses commercially available branched poly(ethylene glycol) with each branch functionalised with N-hydroxysuccinimide ester (Figure 11). The peptide reacts with the succinimide groups to form a cross-linked hydrogel.

Films were produced by inkjet printing the functionalised PEG onto a gold electrode and subsequent inkjet printing of the peptide AAPVAAK onto the dried PEG films. A micrograph and the sensor response at 10 U/mL HNE are shown in Figure 12.

Example 2 - further experiments using two-step coating method of peptide cross-linked hydrogels

Experimental

Materials

Polished, gold-coated QCM crystals (10 MHz) were purchased from ICM (Oklahoma City, U.S.A.). Silicon with 20 run thermal oxide and 30 nm CVD nitride was purchased from Si-Mat (Landsberg, Germany). 96% alumina (0.5 mm thick) was purchased from Laser cutting-ceramic Ltd. (Sheffield, UK).

Dextran (Mr = 2 x 10^6 Da) was purchased from Fluka. Peptide sequences AAPVAAK, AAPFFK, GPQGIWGQK (95% purity) were purchased from
Genscript (New Jersey, USA). Each peptide sequence was dissolved in water to a concentration of 100 mg ml⁻¹ and filtered through a PL-HC03 MP solid phase extraction (SPE) tube purchased from Polymer Laboratories (Massachusetts, USA) to neutralise the TFA salts and freeze dried before use. Human neutrophil elastase (HNE) and cathepsin G were purchased from Elastin Products (Missouri, USA). Matrix metalloproteinase 8 (MMP 8) was purchased from Calbiochem (Darmstadt, Germany).

Photoresist S1813 and developer 351 were purchased from Chestech Ltd. (Rugby, UK). Other chemicals and reagents were obtained from commercial sources (Sigma-Aldrich, Fisher Scientific) and used without further purification. Ultra pure water was obtained from an ELGA Purelab Ultra system.

The following buffer solutions were prepared:

- pH 8.0 phosphate buffer (10 mM).
- pH 7.5 charge transfer buffer: pH 7.5 phosphate buffered saline (10 mM) containing 5 mM potassium ferricyanide, 5 mM of potassium ferrocyanide and 140 mM sodium chloride.
- pH 5 charge transfer buffer: pH 5 sodium acetate buffer (1 mM) containing 5 mM potassium ferricyanide, 5 mM of potassium ferrocyanide and 140 mM sodium chloride.

**Fabrication of electrodes**

Chromium/gold interdigitated electrodes were patterned onto silicon substrates with a SiO₂/Si₃N₄ insulator and 96% alumina substrates. S1813 photoresist was spin-coated using CHEMAT Technology spin coater kw-4A and exposed with a Karl Suss MicroTec MJB3 mask aligner following a standard procedure. The exposed photoresist was left in chlorobenzene for 10 minutes to harden the film surface. The photoresist was then developed. Some 30 nm chromium and 150 nm gold were deposited by thermal evaporation using an Edwards Coating System E306A, followed by lift-off in acetone. Interdigitated electrodes with 10 µm line width and 10 µm line spacing were patterned on the silicon substrates.
Interdigitated electrodes with 50 µm line width and 300 µm spacing were patterned on the alumina substrate.

**Oxidation of dextran**

Dextran was oxidised adapting a procedure described by Ruys et al. *(ACTA PHARM TECHNOL, 1983, 29, 105-1 12.)*

Sodium periodate (1.41 g, 6.6 mmol) was added cautiously to a stirred dextran (0.48 g, 3 mmol repeat units) solution in 7 ml de-ionized water. The mixture was left for 24 hours in the dark. The oxidized dextran solution was then dialyzed against 1 l water using Spectra/Por Float-a-Lyser (MWCO 500) from Spectrum Laboratories Inc for 2 days. (California, U.S.A.). The bulk water was changed three times during dialysis. After freeze drying, a white fluffy solid was obtained (0.32 g, 83%), which was stored at room temperature. The degree of oxidation of dextran was confirmed to be 100% by titration as described previously (Stair et al., supra), i.e. each glucose unit was modified with two aldehyde groups resulting in a decrease of molecular weight of the repeat units from 162 g mol⁻¹ to 130 g mol⁻¹ during oxidation. δH (270 MHz, D₂O, Me₄Si) 3.80 (b, CH₂) 4.02 (b, CH), 5.10 (b, CH), 5.39 (b, CHOH), 8.38 (s, CHO). IR: (cm⁻¹) 3375 (OH), 2934 (CH), 1637(CHO), 1342, 1103, 1021.

**Film preparation**

Hydrogel films were prepared following two different procedures. For comparison with previous results, gold coated quartz crystals were coated by spin-coating a solution of oxidised dextran and peptide following the procedure described by Stair et al. (supra). To overcome the problems encountered with these films, a new coating procedure was developed where oxidised dextran and peptide were deposited successively.

The interdigitated electrodes/quartz crystals were immersed in piranha solution (3:1 v/v concentrated H₂SO₄ and 30% H₂O₂) for one minute. After rinsing with water, the electrodes were transferred into a 10 mM solution of 4-mercapto-1-
butanol in 4:1 v/v ethanol and water and left for one hour to form a self-assembled monolayer. Afterwards, the electrodes were rinsed with water and dried with nitrogen. A solution of oxidized dextran (6.5 mg, 0.05 mmol repeat units) in pH 8.0 phosphate buffer (0.5 ml) was then spin-coated at 3000 rpm for 25 seconds onto the surface of the electrode. The coated dextran layer was left in a sample box at room temperature overnight to dry. A solution of AAPVAK (3.1 mg, 0.005 mmol) in pH 8.0 phosphate buffer (50 μl) was prepared. Some 200 μl of methanol was then added and mixed with a vortex mixer. The solution of AAPVAAK was then drop coated onto the surface of the dextran layer. The amount of AAPVAAK solution for drop coating was dependent on the area of the electrode. Specifically, 0.5 μl of AAPVAAK solution was drop coated onto a 3 mm x 3 mm electrode area. Film curing was finished by incubating the film at 30°C for 1 day. The thickness of the film on the silicon substrate was measured to be 200-300 nm thick using a Dektak3ST surface profiler. The thickness of the film on the alumina surface could not be measured due to the surface roughness of the substrate. The average grain size of alumina was estimated to be about 2 μm using an SEM.

AAPVAAK cross-linked hydrogel films were used for the detection of FINE, AAPFFK cross-linked films were used for the detection of cathepsin G. These peptide sequences were previously used by Stair et al. (supra). MMP8 activated by p-aminophenylmercuric acetate using organomercurial activation protocol was detected using GPQGIWGQK as the cross-linker. This peptide sequence was obtained by adding lysine to a peptide described that was shown to be a preferred substrate for MMP8 previously (Netzelarnett et al J. Biol. Chem., 1991, 266, 6747-6755) to obtain an amine functionality at the C terminal of the peptide. Cross-linking with AAPFFK and GPQGIWGQK was carried out using the same molar concentration as described for AAPVAAK above. Initial investigations were carried out using the AAPVAAK cross-linked hydrogel because of the low cost of HNE compared to the other enzymes.

**Film degradation experiments**

The hydrogel coated interdigitated electrodes were inserted into a customized cell
with an o-ring to insulate the contacts from the solution and a magnetic stirrer bar. Some 950 µl of pH 7.5 charge transfer buffer was then added to the cell at room temperature. The charge transfer buffer was constantly stirred and the impedance measured with time in a frequency range from 10 Hz to 50 kHz using an Autolab PGSTAT10 with FRA2 (Windsor Scientific, UK). All impedance measurements were carried out between the two electrodes of the interdigitated structure with an ac voltage amplitude of 10 mV and at a dc potential of 0 V. The impedance usually became stable after 15 minutes, and then 50 µl of different concentration enzyme solution in pH 5 charge transfer buffer was added into the pH 7.5 charge transfer buffer in the cell. After the addition of enzyme, an impedance change was generally observed in less than 30 seconds. A control experiment was carried out by replacing the enzyme solution with pH 5 charge transfer buffer.

For measurements in the presence of 2% BSA, 250 µl of 8% BSA solution in pH 7.5 charge transfer buffer was added to 700 µl of pH 7.5 charge transfer buffer followed by the addition of 50 µl of enzyme solution in pH 5 charge transfer buffer after the 15 min stabilisation period.

**Measurements in small sample volumes**

Capillary fill devices were constructed on top of the hydrogel coated interdigitated electrodes (IDEs) on 96% alumina after coating the hydrogel as described above. A single sided adhesive tape was attached to the hydrogel coated electrode surface as an insulator that also formed a well around the electrode followed by a double sided adhesive as a spacer and a hydrophilic lid manufactured by Adhesives Research Ireland Ltd. A schematic of the device is shown in Figure 13. The volume of the capillary fill device was about 1.5 µl. After filling the device with test solution, it was sealed from both sides of the capillary channel using silicone grease to prevent evaporation of the solution. Impedance changes were monitored using an Autolab PGSTAT10 with FRA2 at 100 Hz.

**Standard characterisation techniques**

1H and 13C NMR spectra were recorded on a Jeol Ex270 instrument at room
temperature. Infrared spectra were recorded on a Bruker Tensor 37 FT-IR system with an ATR diamond press. Scanning electron microscope images were obtained from FEI Inspect F instrument with acceleration voltages of 10 keV. Optical microscope image was obtained from OLYMPUS BX60 microscope.

Results and Discussion
The impedance change during the enzyme induced degradation of the peptide cross-linked hydrogel films was initially investigated using gold-coated quartz crystals as substrates for the hydrogel to allow comparison with previous data obtained with quartz crystal microbalance measurements (Stair et al, supra). Impedance spectra were measured in a two electrode arrangement using a platinum electrode as the counter electrode. No impedance change was observed during the degradation of an AAPVAAK cross-linked hydrogel film by HNE in phosphate buffered saline. This was attributed to the hydrophilicity of the film resulting in a significant uptake of electrolyte and therefore a low film resistance. In the presence of a charge transfer reagent (ferricyanide/ferrocyanide), a significant change in impedance was observed at the low frequency end of the spectrum (Figure 14) due to the change in the charge transfer resistance during the degradation of the film. Hence subsequent degradation experiments were carried out at frequencies between 30 Hz and 100 Hz.

Two-step coating of hydrogel films
Hydrogel films produced using a single step method where a solution containing both oxidised dextran and the peptide was spin-coated onto a quartz crystal showed a relatively slow response preceded by an induction period when measured by QCM measurements (Stair et al, supra). A similar result was obtained when measuring the degradation of these hydrogel films by impedance measurements (Figure 2). The impedance was stable in pH 7.5 charge transfer buffer, it increased briefly upon addition of HNE and then decreased slowly over a long period of time.

For the production of the final clinical device, the hydrogel films will be deposited
using standard technologies such as drop coating or inkjet printing. As pre-mixing of the amine containing peptide and the oxidised dextran in solution prior to coating would lead to cross-linking, albeit at a slow rate, we did not anticipate being able to use this approach with any standard printing method. Hence a two-step coating method was developed in which the dextran and the peptide were deposited in two consecutive steps. Using this two step coating strategy, the cross-linking condensation reaction would only happen on the surface of the substrate instead of in nozzles or ink cartridges. Adopting this approach had the attendant advantage of producing a highly cross-linked surface layer on the dextran which provided a sufficiently rapid response to the enzyme. Presumably once the cross-links thus formed in the surface layer were broken by HNE, the underlying non-cross-linked dextran dissolved rapidly resulting in a large sensor response (Figure 2).

The effect of non-specific binding

Further development work was carried out using hydrogel coated IDEs on insulator/silicon and alumina substrates. Cr/Au IDEs were obtained by microfabrication. It is anticipated that the microfabricated electrodes will be replaced by screen printed electrodes on alumina in the future to provide low-cost mass-produced electrodes. Response curves to different activities of FINE using IDEs on a silicon substrate are shown in Figure 15(a). The impedance response is comparable to that observed with two opposing electrodes (Figure 2). The rate of degradation was found to be strongly activity dependent.

Samples of gingival crevicular fluid (GCF) are expected to contain protein concentrations between 2% and 4%. The effect of BSA on the rate of degradation was therefore investigated. Figure 15 shows the comparison of the impedance change before and after the addition of three different activities of HNE in the absence and in the presence of 2% BSA. In the absence of BSA (Figure 15a), the impedance change after the addition of 10 U ml$^{-1}$ was about 18% in 3 minutes. The addition of BSA resulted in slower degradation (Figure 15b). The impedance change in 3 minutes was 13% in the presence of 2% BSA. The reduction in the
degradation rate was mainly attributed to an increase in the viscosity of the solution. The response in the presence of BSA remained sufficient over the entire concentration range of HNE for obtaining adequate sensor signals in the 3 min timeframe required by the dental application.

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The effect of the monolayer on the mechanism of degradation

If not specified, the gold electrodes on all substrates were modified with a hydroxyl terminated monolayer prior to hydrogel deposition to aid the spreading of the solution on the surface. To study the effect of the self-assembled monolayer on the degradation of hydrogel films, the gold IDEs were modified using two different thiols - one with a hydroxyl terminal group, which provided a hydrophilic surface but did not react with the oxidised dextran - and a second one with an amine terminal group that provided a hydrophilic surface, which was also capable of reacting with the aldehyde groups of the oxidised dextran. The response curves to both types of hydrogel films are shown in Figure 16. Both films appeared stable in pH 7.5 charge transfer buffer. Upon addition of HNE, the hydrogel film on a hydroxyl terminated monolayer displayed a decrease in impedance caused by a dissolution of the film, while the hydrogel film formed on the amine terminated monolayer showed an increase in impedance indicative of a swelling rather than a dissolution process as the dextran film was covalently bound to the surface after the cleavage of cross-links due to the enzymatic reaction.

The use of an amine terminated monolayer may be preferable in applications where greater mechanical stability is required, e.g. if the sensor were required to be placed into a flowing medium, and where the release of degradation products of the films is undesirable, e.g. for in-vivo applications.

Sensor response to different proteases

Three host proteases, HNE, cathepsin G and MMP8 have been identified as suitable markers for monitoring periodontal disease. Oxidised dextran films were thus cross-linked with three different peptides that each contained cleavage sites for one of the three target enzymes. The activity dependent response of the sensor
materials to the different proteases was investigated. The response of AAPVAAK
5 cross-linked films to different activities of HNE is discussed above (Figure 15).
Figures 17a and 18a show the impedance change during degradation of dextran
hydrogel films cross-linked with AAPFFK and GPQGIWGQK under the same
10 experimental conditions. The film impedance for AAPFFK and GPQGIWGQK
cross-linked films increased over time in the charge transfer buffer. This can be
attributed to continued swelling of the films. Addition of the proteases caused the
films to degrade for all three hydrogel/enzyme systems. At high enzyme activities,
15 this resulted in a decrease of impedance; at low activities, this resulted in a
reduced rate of impedance increase. Calibration curves for cathepsin G (Figure
18b) and MMP8 (Figure 18b) were obtained by determining the rate of
degradation within the first 100 s of enzyme addition.

Measurement of protease activities in small volumes
A critical requirement of clinical measurements of GCF is the size of the sample
15 volumes of 1-5 μl. To enable measurements in such small sample volumes,
capillary fill devices were constructed on top of the hydrogel coated IDEs on
alumina substrates. In contrast to the results shown above, there was no
stabilisation period in PBS prior to the addition of enzyme solution, but the test
solutions were added to the device directly. Sample response curves for different
HNE activities are shown in Figure 19. The response to pH 7.5 charge transfer
20 buffer shows a small decrease in impedance followed by a slow increase in the
impedance with time. It is assumed that the decrease in impedance was caused by
the hydration of the hydrogel film. The continued swelling of the film resulted in
an increase of its thickness and therefore an increase in the impedance. The
addition of HNE to the capillary fill device caused a significant decrease of the
25 impedance, the rate and extent of which were strongly dependent on the HNE
activity. The response is deemed sufficiently large for the detection of HNE in
clinical samples, i.e. the capillary fill device will form the basis for a prototype of
the clinical device.

Conclusions
It has been shown that thin films of peptide cross-linked dextran hydrogel can be used to detect protease activities by monitoring the degradation of the films in the presence of the target protease and a charge transfer reagent using impedance measurements. The sensor materials are generic - specificity for a particular protease can be achieved by employing an appropriate peptide sequence as a cross-linker. To date, three markers for periodontal disease, HNE, cathepsin G and MMP8 have been detected using this sensor system. Preliminary results have shown that measurements in small sample volumes can be facilitated by using a sealed capillary fill device providing the basis for a prototype device for clinical applications.

Depending on the self-assembled monolayer used to modify the electrode surface, hydrogel films were either shown to dissolve or to swell upon interaction with the target protease causing either a decrease or an increase in the impedance. Dissolution was achieved using a chemically inert monolayer with hydroxyl terminal groups, while swelling was observed when using an amine terminated monolayer, which bound the oxidised dextran covalently to the sensor surface. As a fairly high-molecular weight dextran was utilised, it can be assumed that most of the dextran molecules present in the film were bound to the surface in this manner. A surface bound film may have advantages for applications where greater mechanical stability is required.

Measurements of the sensor response in the presence of BSA showed that background protein slowed down the sensor response. This was attributed to an increase in the viscosity of the solution and possibly to an interaction between the target protease and the dissolved protein.

**Example 3: Detection of activated protein C (APC)**

The potential to develop the protease sensor technology into a clinical tool for the bedside assay of coagulation factors was assessed.
Peptide sequences containing cleavage sites for coagulation factor X (FX) and activated protein C (APC) were identified from the natural substrates of these proteases. IEGRTATK was derived from prothrombin - a natural substrate of FX, LDRRGIQK was derived from the Arg506 cleavage site of activated factor V, which is the natural substrate of APC. Preliminary experiments have shown that hydrogels cross-linked with the above peptides do indeed respond to the target coagulation factors at physiologically relevant levels (see for example Figure 20).

Example 2: Detection of MMP-2

MMPs are indicated in a number of disease states including autoimmune diseases such as arthritis and MS, transplant rejection, arterial stiffness and cancer. Oxidised dextran was cross-linked with the peptide sequence GPQGIFGQK by drop-coating interdigitated electrodes first with oxidised dextran and then with the peptide. The resulting sensor coating was shown to be stable in buffer, but to increase its impedance upon the addition of MMP-2 (Figure 21). The sensor response obtained is comparable to that observed for other proteases in previous examples.

Example 3: Measurements without charge transfer buffer

Circular screen printed interdigitated gold electrodes of the design shown in Figure 22a were coated with SU-8 2005, exposed and developed to form wells around the electrodes (Figure 22b). The gold surface was modified using aminoethanethiol. A solution of oxidised dextran was drop-coated onto the electrodes and left to dry. A solution of the peptide AAPVAAK was then drop-coated onto the electrodes and left to cross-link with the oxidised dextran. Figure 23 shows that the impedance of the coated electrodes measured at 1 kHz was stable in PBS pH 7.4, but increased rapidly when exposed to 10 U/ml Human Neutrophil Elastase (HNE). This sensor signal was obtained in the absence of any charge transfer reagent. Evidently, the two-step coated films have impedance sufficiently greater than that of the surrounding electrolyte to make a significant contribution to the capacitance of the electrodes.
Enzyme response in capillary fill devices

The results presented above were obtained by measurements in an electrochemical cell with a large sample volume of 1 ml. A laboratory prototype of a sensor array and has been completed. Screen printed gold electrodes on alumina substrates were used as the sensor substrates. An SU-8 insulator layer was produced using photolithography. Protease sensitive hydrogels were obtained by drop coating and curing oxidised dextran and peptides under suitable conditions. To facilitate the measurement in small sample volumes, a capillary fill device was formed using commercially available spacer tapes and hydrophilic lids (Figure 24).

Samples of gingival crevicular fluid (GCF) were taken and deposited onto the fill hole of the capillary fill biosensor and measurements taken.

The response of drop-coated sensors was measured using a capillary fill device (Figure 24). Response curves for buffer and enzyme solutions are shown for hydrogels cross-linked with two different peptides, AAPVAAK (Figure 25a) and AAPFFK (Figure 25b). All sensors showed an increase in the impedance after exposure to solution. The rate of increase was considerably larger for solutions containing the target proteases, HNE (Figure 25a) and cathepsin G (Figure 25b).

Figure 26 shows a sample response of a sensor array to 2 mU/ml cathepsin G. Each electrode was coated with a different peptide cross-linker to achieve sensitivity to 4 different proteases. Sensor 2, coated with the cathepsin G specific peptide AAPFFK shows a greater initial increase in impedance than the other electrodes coated with different peptides.
CLAIMS

1. A sensor coating layer comprising:
   (a) a layer of a polymer deposited on a transducer; and
   (b) a layer of a peptide deposited on said layer of a polymer.

2. A sensor coating layer according to claim 1 wherein the polymer is an oxidised polysaccharide.

3. A sensor coating layer according to claim 2 wherein the oxidised polysaccharide is an oxidised dextran.

4. A sensor coating layer according to claim 1 or 2 wherein the oxidised polysaccharide is modified with an amine.

5. A sensor coating layer according to claim 4 wherein the amine is benzyl amine.

6. A sensor coating layer according to claim 1 wherein the polymer is a modified polyethylene glycol.

7. A sensor coating layer according to any one of the preceding claims wherein the polymer is deposited by spin-coating, drop-coating, dip coating, spray coating, casting, screen printing or inkjet printing.

8. A sensor coating layer according to any one of the preceding claims wherein the peptide is deposited by spin-coating, drop-coating, dip coating, spray coating, casting, screen printing or inkjet printing.

9. A sensor coating layer according to any one of the preceding claims wherein the transducer is a plurality of electrodes.
10. A sensor coating layer according to claim 9 wherein the electrodes contain a metal.

11. A sensor coating layer according to claim 10 wherein the metal is gold.

12. A sensor coating layer according to claim 9 wherein the electrodes contain carbon.

13. A sensor coating layer according to any one of claims 9 to 12 wherein the electrodes are deposited onto a substrate.

14. A sensor coating layer according to claim 13 wherein the substrate is a ceramic material.

15. A sensor coating layer according to claim 14 wherein the ceramic material is alumina.

16. A sensor coating layer according to claim 13 wherein the substrate is a plastic material.

17. A sensor coating layer according to any one of claims 9 to 16 wherein the electrodes are interdigitated electrodes.

18. A sensor coating layer according to any one of claims 9 to 17 wherein the electrodes are modified with a self assembled monolayer.

19. A sensor coating layer according to claim 18 wherein the monolayer comprises amino terminal groups.

20. A sensor coating layer according to claim 18 wherein the monolayer comprises hydroxyl terminal groups.
21. A sensor device comprising:
   (a) a substrate upon which a transducer is formed; and
   (b) a sensor coating layer according to any one of the preceding claims.

22. A sensor device according to claim 21 wherein the substrate is an insulating plate.

23. A sensor device according to claim 21 or 22 wherein the transducer is a plurality of electrodes.

24. A sensor device according to any one of claims 21 to 23 wherein the sensor device further comprises a capillary fill channel.

25. A sensor device according to any one of claims 21 to 24 wherein a patterned insulator is deposited onto the insulating plate prior to step (b).

26. A sensor device according to claim 25 wherein the insulator is an epoxy resin.

27. A sensor device according to claim 26 wherein the epoxy resin is an SU-8 photoresist patterned by photolithography.

28. A method of preparing a sensor coating layer according to any one of claims 1 to 20, comprising:
   (a) depositing a layer of a polymer on a transducer; and
   (b) depositing a layer of a peptide on said layer of a polymer.

29. A method of preparing a sensor device according to any one of claims 21 to 27, comprising:
   (a) providing a substrate upon which a transducer is formed; and
   (b) carrying out the method of claim 28.

30. A method for the detection of a protease, comprising:
(a) contacting a sample to be assayed with a sensing device according to any one of claims 21 to 27; and
(b) measuring a signal output of said sensing device.
FIG. 3
FIG. 4(a)

FIG. 4(b)
FIG. 5

10 U/mL HNE

Z / Ω

Time / min

0 10 20 30 40 50 60

2500 3000 3500 4000 4500 5000 5500

FIG. 6

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FIG. 7(a)

FIG. 7(b)
FIG. 7(c)
FIG. 8(a)

FIG. 8(b)
**FIG. 9**

**FIG. 10**
Fig. 11

Functionalized hydrogel

+ peptide

PEG
FIG. 16
FIG. 18(a)

FIG. 18(b)
**FIG. 19**

Graph showing the decrease of $\Delta Z$ in percentage over time $t$ (in minutes) for different curves labeled A to D.

**FIG. 20**

Graph showing the increase of $Z/Z_0$ over time $t$ (in seconds) with the notation of 0.1 U/ml APC.
**FIG. 23**

![Graph showing the change in Z/Z₀ with time](image)

**10 U/ml HNE**

![Diagram of a microfluidic device](image)

**FIG. 24**

**Screen printed gold electrodes**

**Alumina substrate**

**Capillary channel**

**Fill hole**

**Vent**
FIG. 25(a)

FIG. 25(b)
FIG. 26
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/00 C12Q1/37
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, INSPEC, BIOSIS, COMPENDEX, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>the whole document in particular: abstract figure 1 section &quot;4. Experimental&quot;; page 1630 ----- -----</td>
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<td>Y</td>
<td>wo 2008/047095 Al (QUEEN MARY &amp; WESTFIELD COLLEGE [GB]; KRAUSE STEFFI [GB]; KAMARUN DZARA) 24 April 2008 (2008-04-24) cited in the application on the whole document</td>
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<td>the whole document in particular: page 64; figure 3</td>
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<td>the whole document in particular: abstract 2. Experimental Section; page 6473 bridging paragraph; page 6472 - page 6473 page 6473; figure 1</td>
<td>2-6, 9-27, 29</td>
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<tr>
<td>Y</td>
<td>abstract par. &quot;Immobilization of melittin on a carboxymethylated dextran matrix&quot;; page 1432 - page 1433</td>
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paragraphs [0031], [0034], [0044] - [0051] | 18-29 |
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