Disposable MMP-9 sensor based on the degradation of peptide cross-linked hydrogel films using electrochemical impedance spectroscopy

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Abstract

Matrix metalloproteinase-9 (MMP-9) plays an important role in both physiological and pathological processes. This enzyme is a peripheral biomarker of neuroinflammation in multiple sclerosis (MS), a chronic autoimmune disease of the central nervous system. Presently, expensive magnetic resonance imaging (MRI) studies are used to monitor subclinical disease activity in MS. An alternative to costly MRI scans could be the detection of MMP-9, using a low-cost, disposable sensor system for MMP-9 suitable for home-monitoring of inflammation. This would allow an early prediction of the failure of anti-inflammatory therapies and more timely clinical intervention to limit neuronal damage and prevent disability. Herein we present the development of a disposable sensor for fast and straightforward detection of MMP-9. Biosensors were produced by coating electrodes with oxidized dextran and subsequent cross-linking with peptides containing specific cleavage sites for MMP-9. Exposure of the films to the enzyme resulted in the degradation of the films, which was monitored using impedance measurements. Sensor response was rapid, a significant impedance change was usually observed within 5 min after the addition of MMP-9. Sensors showed a negligible response to matrix metalloproteinase-2 (MMP-2), a protease which may interfere with MMP-9 detection. The peptide sequence with the highest sensitivity and selectivity Leu-Gly-Arg-Met-Gly-Leu-Pro-Gly-Lys was selected to construct calibration curves. MMP-9 was successfully detected in a clinically relevant range from 50 to 400 ng/ml. Two different processes of hydrogel degradation were observed on electrode surfaces with different roughness, and both appeared suitable to monitor MMP-9 activity. The sensor

materials are generic and can be easily adopted to respond to other proteases by selecting peptide cross-linkers with suitable cleavage sites.

Keywords: MMP-9 sensor; multiple sclerosis; peptide cross-linked hydrogel; interdigitated gold electrodes; electrochemical impedance spectroscopy; film degradation

1 Introduction

Matrix metalloproteinase-9 (MMP-9) plays a critical role in pathological processes such as neuroinflammation in multiple sclerosis (MS) (Fainardi *et al*., 2006). This is a progressive autoimmune disease of the central nervous system affecting over 2.3 million people worldwide (Alonso and Hernan, 2008). In MS, inflammation is known to precede the onset of clinical attacks or relapses. Although there are therapies for suppressing inflammation, these are only partially effective (Comabella *et al*., 2009). At present, serial magnetic resonance imaging (MRI) studies are used to monitor subclinical disease activity in MS. This is not ideal as MRI is an expensive monitoring tool, and people with MS (pwMS) have to be examined in a hospital setting. Moreover, it would be beneficial to monitor this disease more frequently, as it is possible to miss the reoccurring, early phase of inflammation. MMP-9 is one of the best studied peripheral biomarkers of neuroinflammation. This biomarker correlates with MRI studies in relapsing-remitting multiple sclerosis (RR MS) (Waubant *et al*., 1999). MMP-9 is present in RR MS as an inactive form (92 kDa) and active form (82 kDa and 65 kDa) (Rossano *et al*., 2014). Numerous studies show elevated levels of MMP-9 in people with RR MS. Currently, biochemical assays such as enzyme-linked immunosorbent assay (ELISA) (Liuzzi *et al*., 2002; Avolio *et al*., 2003; Feinardi *et al*., 2006 Comabella *et al*., 2009; Benesova *et al*., 2009) and zymography (Rossano *et al*., 2014) are used for the detection of active and/or inactive forms of MMP-9. Avolio *et al.* (2003) detected active MMP-9 at a level of 647.2 ± 333.2 ng/ml (mean ± SD) for short disease duration, 529.1 ± 370.7 ng/ml for long disease duration, and 326.5 ± 140.3 ng/ml in control samples (Avolio *et al*., 2003). People with MS who responded to therapy showed decreased level of total MMP-9 (357.7 ± 272 ng/ml) compared to people who did not respond to treatment (442 ± 265.4 ng/ml) (Comabella *et al*., 2009).

The above mentioned assays for MMP-9 detection are sensitive; however, the procedures are time-consuming, require qualified personnel to perform the analysis and are costly. The

drawbacks of these assays can be avoided by employing electrochemical sensors. They may detect analytes rapidly and in real-time without many preparation steps that biochemical assays require. Moreover, electrochemical devices have potential to be miniaturized, are cheap and can be easily operated. A number of electrochemical sensors employing different recognition elements have been developed for the detection of MMP-9. Sensors can be divided into immunosensors, which were modified with antibodies specific to MMP-9, and activity sensors, which were coated with natural or synthetic substrates that were degraded by this enzyme (Shoji *et al*., 2011; Huska *et al*., 2008; Lee *et al*., 2009; Ciani *et al*., 2012). Ciani *et al*. (2012) developed an impedimetric immunosensor with a limit of detection of 1.1 nM (100 ng/ml). However, the sensor required incubation with the enzyme for 20 min followed by a 5 min washing step. Therefore, this approach may be suitable for constructing a point-of-care device, but not a meter for home-use. Furthermore, this immunosensor was used for detection of the inactive form of MMP-9. There is a need to develop a sensor for active MMP-9, since this form (65 kDa) may play a significant role in MS. Rossano *et al*. (2014) detected MMP-9 (65 kDa) only in people with RR MS, which suggests that this truncated form correlates with the inflammatory process and not with normal physiological processes occurring in the body. MMP-9 was detected by chronopotentiometric stripping analysis on a hanging mercury drop electrode in the absence or presence of collagen (Huska *et al*., 2008) in a range from 1 to 10 nM, and the detection limit was 100 pM. Unfortunately, there were several disadvantages of using this system. Natural substrates such as collagen have many cleavage sites, and sensors may be susceptible to interference from other proteases, which are present in blood specimens. Moreover, MMP-9 was not detected in real-time, namely the sensor required an enzyme accumulation step that was followed by a rinsing step before the measurement was started. This system may be not suitable to construct a disposable sensor. Another approach was reported by Shin *et al.* (2013). They developed a sensor for MMP-9 detection using the peptide sequence Gly-Pro-Leu-Gly+Met-Trp-Ser-Arg-Cys with attached methylene blue as a redox probe. The proteolytic degradation of the peptide sequence was monitored by square wave voltammetry showing the detection range from 0.06 to 50 nM (Shin *et al.*, 2013). The sensitivity was high, however, the sensor may suffer from interference by MMP-2 as the peptide used was described by Netzel-Arnett *et al*. (1993) as a good substrate for both MMP-9 and MMP-2.

Generic sensor materials for the detection of proteases based on thin dextran hydrogel films cross-linked with peptides have recently been developed by our group. Degradation of the

hydrogel films was monitored using electrochemical impedance (Zheng *et al.*, 2011) and Quartz Crystal Microbalance (QCM) measurements (Stair et al., 2009). Good specificity for different proteases was achieved by choosing appropriate peptide cross-linkers. This method was used successfully to produce sensor materials suitable for the detection of three different proteases indicated in periodontal disease, human neutrophil elastase (HNE), cathepsin G and MMP-8 (Zheng *et al.*, 2011). In this work, the generic sensor materials were adapted for the detection of MMP-9. An important aspect of the sensor development was to limit non-specific proteolytic degradation of the hydrogel by MMP-2 as this enzyme is closely related to MMP-9. Both enzymes contain a fibronectin-like type II module (gelatin binding domain) and highly homologous catalytic domains (Olson *et al.*, 2000, Chen *et al.*, 2003). Three different peptides have been selected as cross-linkers, having specific cleavage sites for MMP-9 and being less susceptible for MMP-2 interference. The enzymatic rate constants for the hydrolysis of peptide Ser-Gly-Lys-Gly-Pro-Arg-Gln+Ile-Thr-Ala (SGKGPRQITA) by MMP-9 and MMP-2 were $k_{cat}/K_M=1.88x10^5$ $M^{-1}s^{-1}$ (Kridel *et al*., 2001), and $k_{cat}/K_M=8.4x10^4$ $M^{-1}s^{-1}$ (Chen *et al.*, 2003), respectively. The cleavage site was located between glutamine and isoleucine. Peptide sequence: Ser-Gly-Ala-Val-Arg-Trp+Leu-Leu-Thr-Ala (SGAVRWLLTA) had degradation rate constants $k_{cat}/K_M=2x10^5M^{-1}s^{-1}$ (MMP-9) and $k_{cat}/K_M=7.0x10^4$ $M^{-1}s^{-1}$ (MMP-2), with a cleavage site between tryptophan and leucine (Chen *et al.*, 2003). These two peptides were modified by adding lysine to obtain amine functionality at the C-terminal. A substrate available commercially for HPLC detection of MMP-9: Leu-Gly-Arg-Met+Gly-Leu-Pro-Gly-DNP (LGRMGLPG-DNP) with the cleavage site between methionine and glycine, ($k_{cat}/K_M=3.9x10^5$ $M^{-1}s^{-1}$, data provided by Merck Chemicals Ltd.) was adopted for our technology by replacing dinitrophenyl (DNP) with lysine to introduce an amino functional group at the C-terminal.

2 Materials and methods

2.1 Materials

Interdigitated gold electrodes were screen printed on 96% alumina and on silicon wafers with 20 nm SiO_2 and 30 nm CVD nitride by the Gwent Group Ltd. (Pontypool, UK); silicon wafers with a 40 nm thick thermal silicon dioxide were purchased from (University Wafer Inc., USA), proMMP-9 (monomer, human neutrophil, 92 kDa, purity >95%, specific activity >1400.0 mU/mg P determined by the producer), proMMP-2 (Human Rheumatoid Synovial Fibroblast, 72 kDa, purity >90%, specific activity >850.0 mU/mg P determined by the

producer), aminophenyl mercuric acetate were purchased from Merck Chemicals Ltd. (Nottingham, UK), peptides LGRMGLPGK, SGKGPRQITAK and SGAVRWLLTAK (95-98 % purity) were synthesized by Genscript Ltd. (Hong Kong). Each peptide was purified on a PL-HCO_3 solid phase extraction (SPE) column to remove TFA salts, freeze-dried and stored at -20 °C under an argon atmosphere. Columns PL-HCO_3 SPE 200 mg/6 ml were purchased from Agilent Technologies (Stockport, UK). Dextran from Leuconostocc ssp (2000 kDa), cysteamine hydrochloride (purity ≥98%), chromium etchant were purchased from Sigma-Aldrich (Dorset, UK). Spectra/Por Float-a-Lyser (MWCO 1000) were from Spectrum Laboratories Inc. (California, USA), photoresists SU8 2005 and S1818, EC solvent and Developer 351 were purchased from Chestech Ltd. (Rugby, UK), fluorogenic substrate Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Mca-KPLGL-Dpa-AR-NH_2) and OmniMMP™ fluorogenic control Mca-Pro-Leu-OH (Mca-PL-OH) were purchased from Enzo Life Sciences (Exeter, UK). Other chemicals were obtained from Sigma-Aldrich (Dorset, UK) and used without further purification. All solutions were prepared in Milli-Q water (18.2 MΩcm resistivity).

2.2 MMP-9 and MMP-2 activation

Matrix metalloproteinases (proMMP-9 and proMMP-2) were purchased as inactive forms where the active site of these enzymes is blocked by their propeptide domains. *P*-aminophenyl mercuric acetate (APMA) is a well-known compound to break the bond between propeptide and zinc located in the active domain. A number of autolytic cleavages occur after unblocking the zinc dependent active site. It was previously reported that activation of MMP-9 by APMA resulted in formation of truncated form of MMP-9 (67 kDa) (Shapiro *et al*., 1995). Activation of MMP-9 was optimized with APMA, and parameters which showed the highest activity and stability of MMP-9 were selected.

MMP-9 was activated by adding APMA, dissolved in 0.1 mM NaOH, to a final concentration of 2 mM. The volume ratio was 1:10 (APMA: proMMP-9). Samples were incubated for 30 min at 37 ºC, and stored at a temperature of 5 ºC. Activity of MMP-9 was tested in 50 mM Tris buffer, pH 7.5 containing 10 mM $CaCl_2$, 150 mM NaCl and 0.05% Brij-35 (w/v). Degradation of the fluorescence substrate: Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Mca-KPLGL-Dpa-AR) (10 µM) was monitored over 8 min at 25 ºC. A calibration curve for control samples (Mca-Pro-Leu-OH) was drawn and the degradation rate (pmoles/min) was calculated. The degradation rate for 200 ng/ml of MMP-9 was calculated to be 53

pmoles/min immediately after activation, and was stable after 24 h (56 pmoles/min) and 48 h (57 pmoles/min). Activated enzyme was stored up to three days and used for impedimetric measurements.

The same parameters were found to be optimal for MMP-2 activation, degradation rate for 200 ng/ml of MMP-2 was 20 pmoles/min. MMP-2 was activated immediately before impedimetric measurements and used the same day.

2.3 Fabrication of electrodes

Interdigitated gold electrodes (IDEs) with a diameter of 1.5 mm and paths and gaps of 0.1 mm were produced by microfabrication. Oxidised silicon wafers were cleaned with piranha solution (3:2 v/v H_2SO_4 and 30% H_2O_2) for 2 min, dehydrated, followed by chromium and gold thermal evaporation (30 nm and 150 nm, respectively) using an Edwards Coating System E306A. Photoresist S1818 was spin-coated on gold at 5000 rpm using a CHEMAT Technology spin-coater kw-4A. Wafers were prebaked for 10 min at 115 °C and exposed to UV light. The photoresist was developed in developer 351 diluted four times in Millipore water for 26 seconds. Uncoated gold was etched in potassium triiodide solution consisting of 5% potassium iodide and 2.5% iodine dissolved in water for 2 min followed by removal of chromium in chromium etchant (3 min). Afterwards, arrays were rinsed with acetone and isopropanol to remove the photoresist, and were cleaned in piranha solution for 1 min (3:2 v/v H_2SO_4 and 30% H_20_2).

2.4 Dextran oxidation

Oxidation of dextran (2000 kDa) was carried out in the presence of sodium periodate following a procedure described by Ruys *et al*., (1983). The oxidized dextran solution was then dialyzed in Spectra/Por Float-a-Lyser (MWCO 1000) against 2 l of ultrapure water for 4-5 days. Purified dextran was freeze-dried and stored at a temperature of -20 °C. The degree of dextran oxidation was verified by titration according to a procedure developed by Zhao and Heindel (1991). The degree of oxidation of aldehyde groups was 80% and was deemed sufficient for sensor fabrication. Our previous studies showed the highest degradation rate with 75% cross-linked hydrogel prepared for other proteases (Stair *et al.,* 2009).

2.5 Hydrogel formation

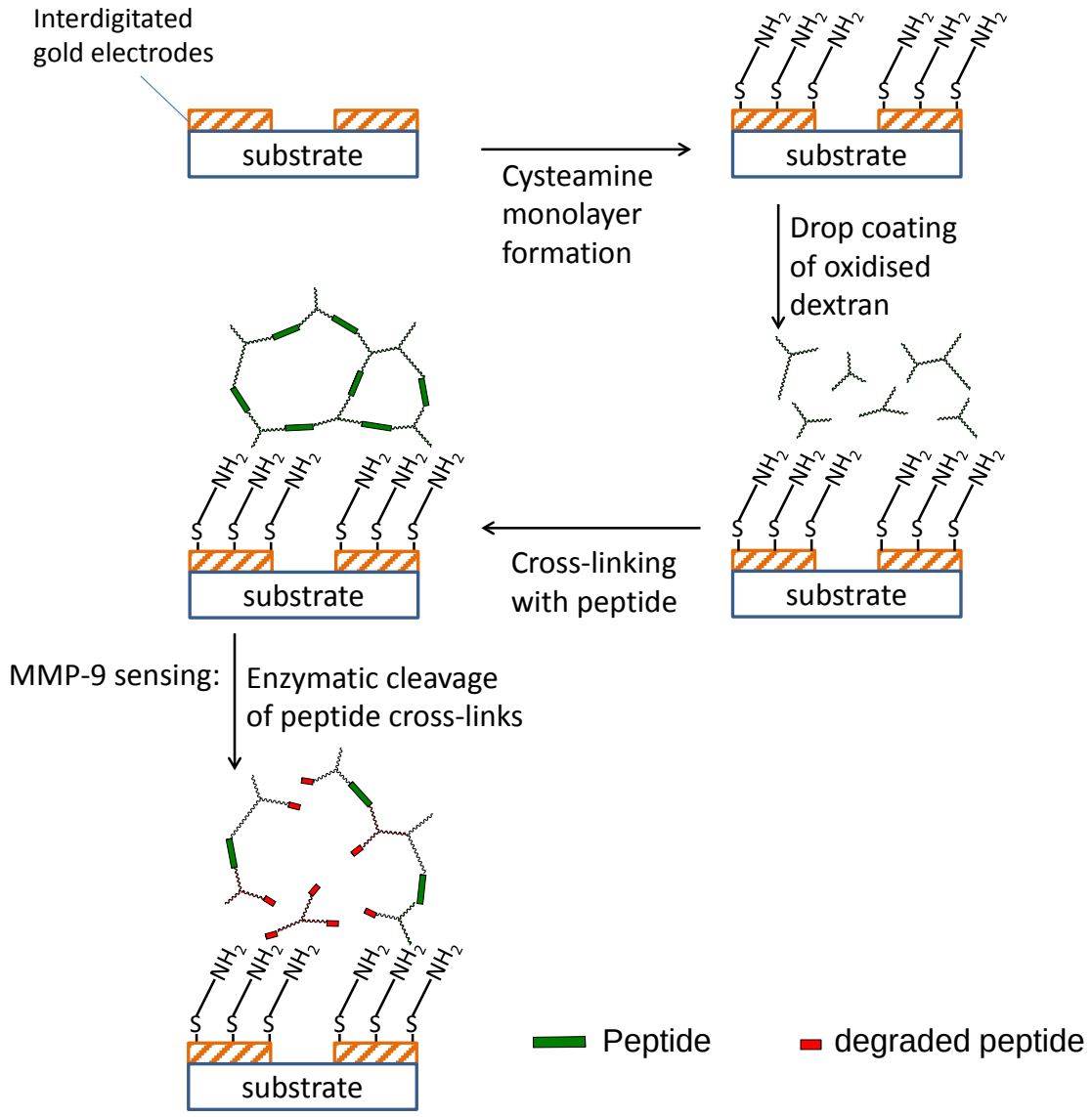
A two step-coating method, previously developed by our group (Zheng *et al.*, 2011) was adopted to deposit MMP-9 sensitive films. A schematic of the sensor coating process is shown in Scheme 1.

2.5.1 Hydrogel formation on interdigitated gold electrodes (alumina substrate)

Screen printed IDEs (96 % alumina substrate) were cleaned with piranha solution (3:2 v/v solution of sulfuric acid and 30% hydrogen peroxide) for 2 min. A photoresist SU8 2005 was deposited to insulate the tracks and define the area of hydrogel deposition. Afterwards, IDEs were immersed in 10 mM of cysteamine in 4:1 ethanol and water solution for 1-2 hours 21 °C to form a self-assembled monolayer. Electrodes were thoroughly rinsed with water after monolayer modification and blow-dried with nitrogen. Dextran (3.3 mg) was dissolved in 250 µL of pH 8.0 sodium phosphate buffer (10 mM), containing isopropanol (20 %), diluted 5000 times with the same buffer/isopropanol mixture, and 0.2 µL of the resulting solution was drop-coated on the electrodes. Samples were dried for 24 hours over calcium chloride at 30 °C followed by peptide coating. Peptides LGRMGLPGK (2.4 mg), SGKGPRQITAK (3.0 mg) and SGAVRWLLTAK (3.1 mg) were dissolved in 62.5 µL of phosphate buffer (10 mM), pH 8.0, then 62.5 µL of methanol was added. The resulting solution (0.2 µL) was drop-coated on the dextran layer. Samples were cured for 48-60 hours over sodium bromide, at 30 °C. Afterwards, sensors were rinsed by dipping them quickly three times in ultrapure water. Sensors were stored at 5 °C and used within two weeks.

2.5.2 Hydrogel formation on interdigitated gold electrodes (silicon substrate)

Interdigitated gold electrodes obtained by microfabrication or screen printing on silicon substrates were cleaned and coated with a cysteamine monolayer using the same experimental conditions as described above for screen printed IDEs on alumina substrates. Dextran (3.3 mg) was dissolved in 250 µL of phosphate buffer (10 mM), pH 8.0, diluted 1250 times and 0.4 µL of the resulting solution was drop-coated on the cysteamine monolayer. Samples were dried for 24 h over calcium chloride at 30 °C followed by peptide coating. Peptide LGRMGLPGK (2.4 mg) was dissolved in 125 µL of sodium phosphate buffer (10 mM), pH 8.0, diluted 1000 times in the same buffer. The resulting solution (0.4 µL) was drop-coated on the dextran layer. Samples were cured for 48 - 60 h over sodium bromide, at 30 °C. The mole ratio of functional groups (cysteamine: dextran: peptide) was calculated to be approximately 1:4:1 for microfabricated electrodes. Sensors were stored at 5 °C and used within two weeks.



Scheme 1. Schematic of sensor fabrication and the sensing process

2.6 Monitoring of hydrogel degradation

Film degradation was monitored by electrochemical impedance spectroscopy. Measurements were conducted in a customized cell (1 ml volume) connected to an Autolab PGSTAT30 with FRA2 (Windsor Scientific, UK). FRA version 4.9 software was used for all analyses. A small perturbation potential (10 mV) was applied to the system and impedance was monitored in a frequency range of 10 - 10 000 Hz. Measurements were conducted in 50 mM Tris buffer (pH 7.5) containing calcium chloride (10 mM) and sodium chloride (150 mM). Studies were carried out in the absence or presence of 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide at a temperature of 21°C. The cell was filled with buffer (950 µL), and the measurement started followed by the addition of enzyme (50 µL) or buffer (50 µL) when a

stable background signal was observed. The percentage change of impedance (ΔZ) was calculated from the magnitude of the impedance recorded before (Z0) and after (Z) exposure of the sensor to different concentrations of enzyme:

$$\Delta Z = \frac{Z - Z0}{Z0} \times 100\%$$

The detection limit was calculated as the concentration of MMP-9 that gave a change of impedance equal to the mean value of the blank plus three times the standard deviation (SD) of the blank after 5 min of the degradation process.

3 Results and discussion

3.1 Cross-linker selection

Electrochemical impedance spectroscopy was employed to monitor the activity of MMP-9 and MMP-2 in the following studies. One of the most important tasks was to select a suitable substrate for MMP-9. Peptide cross-linkers with good MMP-9 sensitivity were chosen for the cross-linking of oxidised dextran films (LGRMGLPGK, SGKGPRQITAK, SGAVRWLLTAK), and hydrogels were formed on screen printed, interdigitated gold electrodes on alumina substrates as described above (see Materials and Methods and Scheme 1). As the peptides contained cleavage sites for MMP-9, an exposure of the film to a solution containing active MMP-9 was expected to break the cross-links causing a change in the impedance of the hydrogel coated electrodes (Scheme 1). Screen printed electrodes were selected for sensor development as a large number of electrodes are required for clinical trials, and the screen printing technology enables inexpensive disposable sensor systems. The impedance spectrum did not show a resistive region in the presence of charge transfer buffer, thus the impedance was monitored without the addition of charge transfer probe. Impedance was recorded at 10 Hz, 100 Hz, 1 kHz, 10 kHz. The highest sensor responses were recorded at 1 kHz. Impedance changes were observed after the addition of MMP-9 with all three peptides (Figure 1). Sensors coated with dextran cross-linked with the peptide sequence LGRMGLPGK showed a rapid response and the highest sensitivity. An impedance response was usually detectable 50 s after the addition of active MMP-9 (200 ng/ml). The signal increased by 6.3 % after 20 min of the degradation process. Sensors coated with peptides SGKGPRQITAK and SGAVRWLLTAK responded about 3 min later after their exposure to MMP-9, and the impedance increased by approximately 2% after 20 min of hydrogel degradation (Figure 1).

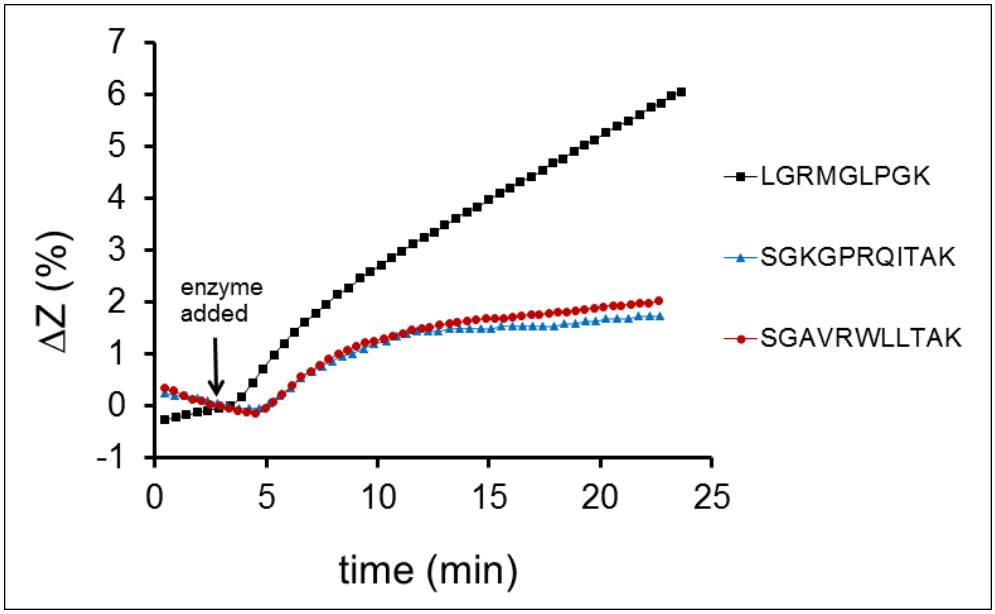


Figure 1. Sensor response to 200 ng/ml MMP-9 at 1 kHz in Tris buffer, pH 7.5. Peptide cross-linked hydrogel films were deposited on screen printed gold electrodes on alumina

3.2 Biosensor selectivity

The peptide cross-linker with the highest sensitivity for MMP-9 (LGRMGLPGK) was tested with MMP-2, a gelatinase which may interfere with MMP-9 detection in blood specimens. An insignificant response of less than 1% was obtained after the exposure of the film to MMP-2 for 20 min demonstrating good selectivity of the sensor (Figure 2). The underlying reason for this high selectivity may be the presence of arginine in this sequence. Studies performed by Chen *et al.* (2003) have shown that selectivity of MMP-2 and MMP-9 is controlled by the S2 sub-site of the catalytic domains. Sequences of catalytic domains of MMP-9 and MMP-2 are His-Ala-Leu-Gly-Leu-Asp-His (HALGLDH) and His-Ala-Met-Gly-Leu-Glu-His (HAMGLEH), respectively. Therefore, MMP-9 has a preference for Arg at P_2 which is able to interact with Asp at S_2. (Chen *et al.*, 2003; Kriedel *et al.*, 2001). These studies also clearly indicate that the increase of the impedance resulted from the proteolytic activity of the enzymes.

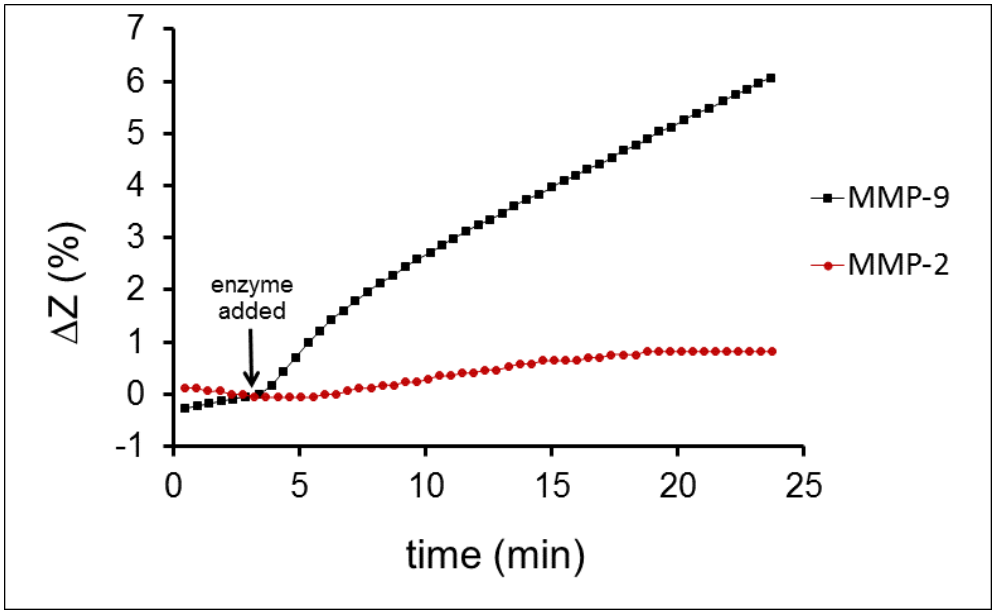


Figure 2. Sensor response to 200 ng/ml MMP-2 or MMP-9 at 1 kHz in Tris buffer, pH 7.5. Peptide cross-linked hydrogel films were deposited on screen printed gold electrodes on alumina

3.3 Monitoring of MMP-9 activity on interdigitated screen printed gold electrodes

Further studies were conducted in the presence of potassium ferricyanide (5 mM) and potassium ferrocyanide (5 mM) to allow measurement of changes in the charge transfer resistance at lower frequencies and thereby improve sensor performance. The modification of screen printed IDEs was characterized by monitoring the impedance in the frequency range from 10 Hz to 10 kHz. Figures 3a and 3b show Bode plots for each modification step. The impedance increased after each modification step in the frequency region of the spectra that was controlled by interfacial electron transfer and diffusion of the redox probe confirming the modification of the electrode surfaces. Impedance spectra were fitted to the Randles equivalent circuit and the charge transfer resistance (R_{CT}) was estimated. The R_{CT} of a bare electrode was 221.5 Ω and increased after monolayer, and dextran/peptide modification to 3770 Ω and 6090 Ω, respectively. Activity of MMP-9 was monitored using the film cross-linked with LGRMGLPGK. Before the addition of the enzyme, a stable impedance was observed which suggests that the hydrogel was strongly attached to the surface. Similarly to the previous experiments, sensors responded quickly, usually 50 seconds after their exposure to enzyme showing an increase in the impedance of approximately 18% after 20 min of proteolytic degradation (200 ng/ml MMP-9) (Figure 3c). The increase in impedance was also

confirmed by the impedance spectrum measured after degradation (Figure 3a). The charge transfer resistance estimated from an equivalent circuit fit showed an increase from 6090 Ω to 10530 Ω during degradation. The production of hydrogel on screen printed IDEs might have resulted in the deposition of very thin, but highly cross-linked films. This could be explained by the partial penetration of dextran inside the pores. Therefore, a thinner layer of dextran was formed with large number of aldehyde groups accessible for subsequent cross-linking with peptide. In the previous studies carried out by Stair *et al*. (2009) and Zheng *et al*. (2011) similar sensor behaviour was detected using quartz crystals and microfabricated IDEs coated with peptide cross-linked hydrogels. They observed an impedance increase and quartz crystal frequency decrease initially after the addition of proteases. It was explained that initial degradation of the film resulted only in breaking the peptides bonds while the hydrogel was still attached to the surface. As a result, thickening of the hydrogel was observed and thus the increase of the impedance. This could explain the changes of the impedance of the highly cross-linked films deposited on the rough electrodes. This is also consistent with studies carried out by Anne *et al.* (2012) and Shin *et al*. (2013) who showed that on a rougher surface, significantly higher amounts of peptide were not degraded compared to a very even surface. The percentage of non-cleavable peptides was usually 40% of total coverage on the polished gold electrodes possibly due to the presence of molecular-scale grooves. On an extremely flat gold surface, less than 10 % of peptides were not degraded (Anne *et al*., 2012). Therefore, it is possible that swelling of the film was observed instead of its dissolution as a result of partial degradation of peptides on screen printed IDEs. Additionally, films may be protected in pores. However swelling due to a reduced cross-link density caused by the cleavage of peptide cross-links does not explain the increase in impedance as a greater influx of water should also result in an increase in the ferri/ferrocyanide concentration in the film. There are three possible explanations for this behaviour (Zheng *et al.*, 2011): (i) Only the surface of the film is highly cross-linked. Degradation of the top layer of the film exposes unreacted aldehyde groups, which can react with the protein. Protein binding could reduce the concentration of ferri/ferrocyanide in the film. (ii) If the electrode surface is initially incompletely covered with hydrogel, expansion of the film due to swelling could result in a greater surface coverage thereby impeding the access of charge transfer reagent to the surface. (iii) Peptide cleavage results in the formation of carboxylic acid groups and amino groups. The negative charge of the carboxylate group at pH 7.4 could cause the ferri/ferrocyanide to be expelled. However, this effect would be counteracted by the formation of positively charged amino groups.

The increase in impedance was also seen previously when using oxidised dextran films cross-linked with a different peptide (AAPVAAK) on screen printed electrodes for the detection of human neutrophil elastase (HNE) (see general discussion of (Zheng et al., 2011)). In this case, the impedance was also stable in buffer and increased rapidly when exposed to solutions containing HNE. Although unexpected, this result was highly reproducible in both this and previous studies on such electrodes.

Linear calibration curves were obtained by plotting impedance changes versus MMP-9 concentration after 5, 10 and 20 min of the film degradation. The enzyme was detected in a clinically relevant range from 50 to 400 ng/ml (Figures 3c and 3d). The detection limit (15 ng/ml) was lower than the level of MMP-9 reported in patients with RR MS which is beneficial as levels of the enzyme may vary between patients (Avolio *et al*., 2003), and also differences may be observed in different specimens such as serum and plasma (Gerlach *et al*., 2007).

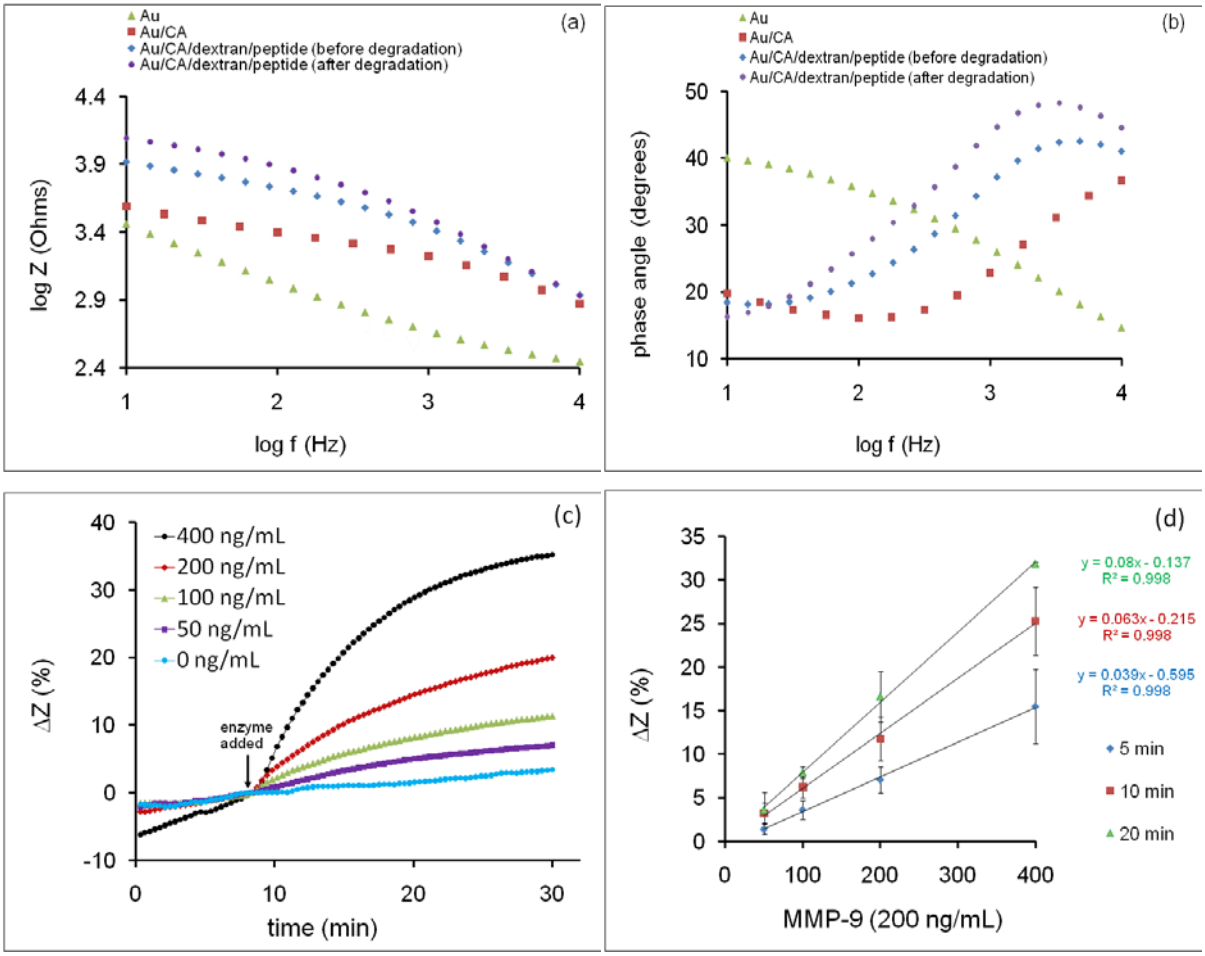


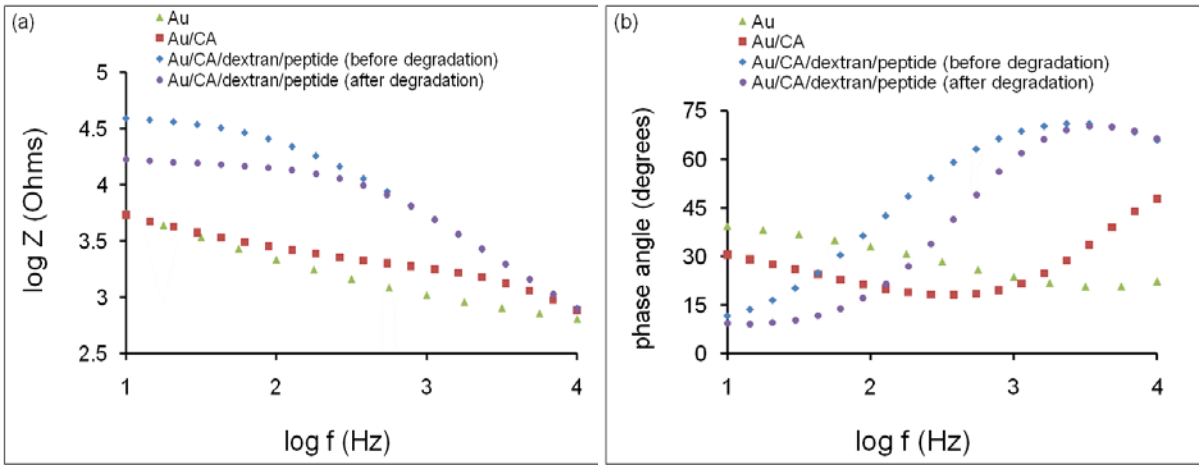
Figure 3. (a and b) Impedance spectra of screen printed IDEs before and after coating with a cysteamine monolayer, after coating with peptide cross-linked dextran hydrogel and after hydrogel degradation by 400 ng/ml MMP-9 in charge transfer buffer, pH 7.5. (c) Sensor

responses to different concentrations of MMP-9 (ng/ml) in charge transfer buffer, pH 7.5 at 10 Hz (MMP-9 was added at 8.3 min as indicated by the arrow.) and (d) the calibration curves showing ΔZ after exposure of the sensor to MMP-9 for different periods of time. The error bars represent ±SD (n=3). Sensors were produced on screen printed IDEs (silicon substrate). RSD calculated after 20 min of the film degradation was 33.3; 29.4; 5.82; 14.4; 0.66 % for 0; 50; 100; 200; 400 ng/ml MMP-9, respectively.

3.4 Monitoring of MMP-9 activity on microfabricated interdigitated gold electrodes

Interdigitated gold electrodes produced by microfabrication were used to verify the influence of the roughness of electrodes on sensor responses. The modification of microfabricated IDEs was also characterized by monitoring the impedance in the frequency range from 10 Hz to 10 kHz. Figures 4a and 4b show Bode plots for each modification step and after degradation in the presence of MMP-9. Data were fitted to the Randles equivalent circuit and the charge transfer resistance (R_{CT}) was estimated. The R_{CT} of a bare electrode was 413 Ω and increased after monolayer, dextran/peptide modification to 1500 Ω and 32100 Ω, respectively. This confirmed the successful modification of electrode surfaces. The charge transfer resistance of the hydrogel modified electrodes was significantly larger for the microfabricated electrodes compared to the screen printed electrodes (see Figure 3a). This indicates that much better coating quality was achieved on the smoother surface of the microfabricated electrodes. The maximum phase angle observed for screen printed electrodes coated with peptide cross-linked hydrogel was 43 degrees (Figure 3b) due to high surface roughness of the electrodes and therefore the hydrogel film, while the maximum phase angle for microfabricated electrodes was about 73 degrees in the capacitive region (Figure 4b) indicating lower roughness of the hydrogel coating as compared to screen printed IDEs (Figure 3b). Figure 4 shows impedance spectra recorded in the absence and presence of MMP-9 in charge transfer buffer. The impedance spectrum after degradation was recorded 20 min after the addition of enzyme to the cell and showed a significant decrease in impedance. The highest sensor response, around 40 %, was detected in the resistive part of the spectrum at low frequencies between 10 - 100 Hz. This indicates that the hydrogel was dissolved and electron transfer between the electrode and the redox couple was facilitated. The impedance changes were not observed in the capacitive region, at higher frequencies between 1 kHz - 10 kHz. This may be attributed to the hydrophilicity of the films and uptake of electrolyte before hydrogel degradation.

Impedance changes at different concentrations of enzyme (50-200 ng/ml) were measured at 100 Hz (Figure 4c). The background impedance was stable before the addition of MMP-9. Rapid degradation was recorded during the first 10 min after the exposure of the sensor to MMP-9 (200 ng/ml), where impedance decreased by about 38 %. The degradation rate can be improved further by employing the optimum temperature for MMP-9 activity, which is 37°C (Increasing the temperature from 25°C to 37°C increases k_{cat} of MMP-9 approximately by a factor of 4 (Fasciglione *et al.*, 2000)). The impedance further decreased by 5% in another 10 min (Figure 4c). Dextran was probably cross-linked only on the surface as a result of using a two step-coating method for sensor preparation and thus rapid degradation of the film was monitored over the first min. The low degradation rate at the end of the measurement (Figure 4c) may suggest that most of the substrate was degraded by the enzyme. However, significantly higher impedance of this sensor after 20 min of hydrogel degradation compared to electrodes coated with a monolayer (Figure 4a) indicates that only part of hydrogel was dissolved after proteolytic degradation. These results suggest that dextran was covalently bound to the amino terminated monolayer. Incomplete degradation of peptides could be also possible. As mentioned above, this was observed by Anne *et al.* (2012) even on extremely flat gold surfaces. The stability of the dextran layer and incomplete dissolution may also result from the high molecular weight of dextran that was used in our studies (2000 kDa).



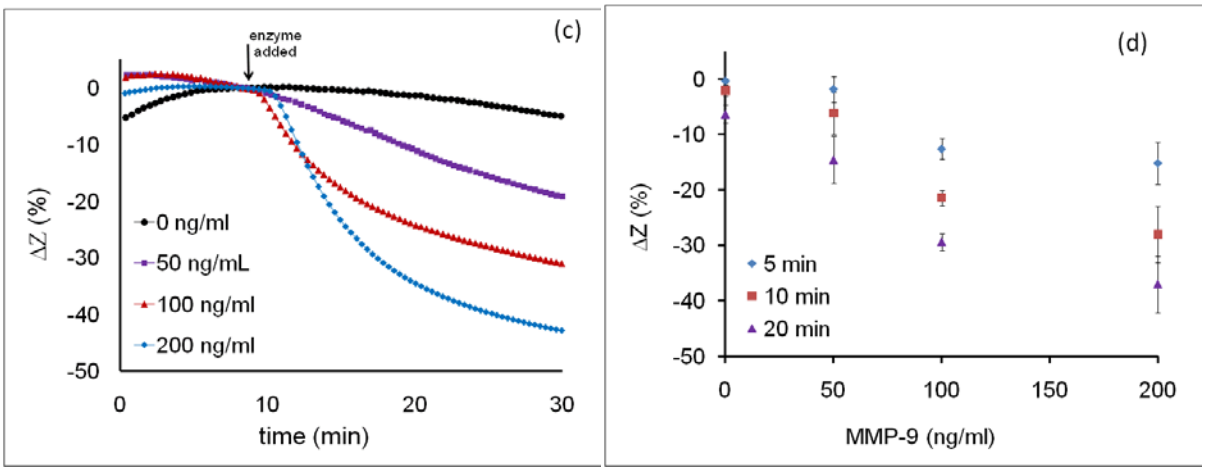


Figure 4. (a and b) Impedance spectra of microfabricated IDEs before and after coating with a cysteamine monolayer, after coating with peptide cross-linked dextran hydrogel and after hydrogel degradation by 200 ng/ml MMP-9 in charge transfer buffer, pH 7.5. (c) Sensor responses for different concentration of MMP-9 (ng/ml) in charge transfer buffer, pH 7.5 at 10 Hz (MMP-9 was added at 8.3 min as indicated by the arrow.) and (d) the calibration curves showing ΔZ after exposure of the sensor to MMP-9 for different periods of time. The error bars represent ±SD (n=3). RSD calculated after 20 min of the film degradation was -25.2; -29; -5.3; -13.6 % for 0; 50; 100; 200 ng/ml MMP-9, respectively.

4 Conclusions

MMP-9 was successfully detected in a clinically relevant range for relapsing remitting multiple sclerosis. Importantly, the sensor materials showed a negligible response to identical concentrations of MMP-2 which may also be present in blood specimen, demonstrating a possibility for using the MMP-9 sensor for analysis of plasma samples. Two different processes of hydrogel degradation were observed on electrode surfaces with different roughness resulting in an increase of impedance on rough electrodes and a decrease of impedance on smoother, microfabricated electrodes. It is assumed that the films were more likely to remain on the rough electrodes after degradation as some of the hydrogel was trapped in the pores and a smaller percentage of peptide cross-links was expected to be cleaved. For smooth electrodes, a larger percentage of peptide-crosslinks was expected to be cleaved by the enzyme resulting in the dissolution of a larger proportion of the films. Electrodes prepared by both, microfabrication and screen printing, are suitable to monitor MMP-9 activity. Not only are the measurements straightforward and MMP-9 is detected in real-time, but also the fabrication process is simple, which will allow mass production of

these sensors. The sensor performance and its repeatability will be further improved by deposition of the hydrogel by an automated dispensing system such as BioDot. Moreover, materials used for the development of disposable MMP-9 sensor are generic and can be used for detection of other proteases by changing the peptide sequence.

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