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Colorimetric Assay for the Detection of Typical Biomarkers for Periodontitis Using a Magnetic Nanoparticle Biosensor

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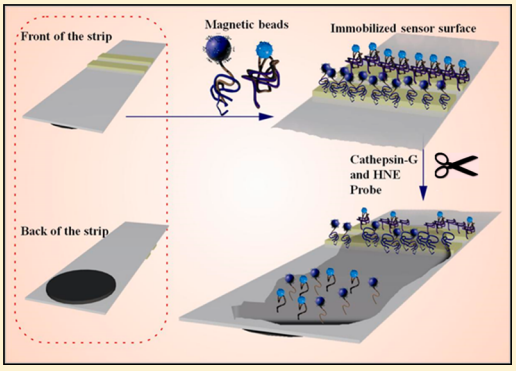
ABSTRACT: Periodontitis is a chronic disease which affects at least 10% of the population. If untreated, periodontitis can lead to teeth loss. Unfortunately, current diagnostic tests are limited in their sensitivity and specificity. In this study, a novel multiplex hand-held colorimetric diagnostic biosensor, using two typical inflammatory salivary biomarkers, Human Neutrophil Elastase (HNE) and Cathepsin-G, was constructed as proof of concept to potentially detect periodontitis. The biosensing method was based on the measurement of proteolytic activity using specific proteases probes. These probes consist of specific proteases substrates covalently bound to a magnetic bead from one end and to the gold sensor surface by the other end. When intact, this renders the golden sensor black. Upon proteolysis, the cleaved magnetic beads will be attracted by an external magnet revealing the golden color of the sensor surface observable by the naked eye. The biosensor was capable of specific and quantitative detection of HNE and Cathepsin-G in solution and in spiked saliva samples with a lower detection limit of 1 pg/mL and 100 fg/mL for HNE and Cathepsin-G, respectively. Examination of periodontitis patients' sample and a healthy control showed the potential of the multiplex biosensor to detect the presence of HNE and Cathepsin-G activity *in situ*. This approach is anticipated to be a useful biochip array amenable to low-cost point-of-care devices.



Periodontitis is a chronic disease affecting the supporting tissues of the teeth which affects at least 10% of the general population.[1−3] It is characterized by apical migration of epithelial attachment, loss of connective tissue, alveolar bone, and eventually tooth loss.[4−7] Periodontitis progression is usually site specific but is not consistent. Thus, it is difficult to clinically distinguish the progressed and nonprogressed inflamed sites. Moreover, the early stages of periodontal disease progression, particularly gingivitis, are often difficult to quantify because of the lack of a linear measurement tool.[8]

In clinical practice, periodontitis can be diagnosed by radiographic examinations.[9] However, still the best available diagnostic aid is the measurement of the depth of the tooth pocket. However, this only provides a retrospective analysis mostly when tooth attachment has already been lost.[10] So, the current diagnostic methodologies are limited to identify the cause of disease or patients at risk.[11,12] In addition, the clinical practice conducted by dentists, different microbiological and biochemical methods such as culturing, DNA probing, and polymerase chain reaction (PCR) are employed to detect bacteria in samples from periodontal pockets.[13,14] A general drawback of these methods is that they do not quantify the severity of the disease.

In this respect, a rapid point-of-care diagnostic biosensor would be of major benefit in the diagnosis, evaluation and treatment of periodontitis severity. So far, several studies aimed to develop new rapid point-of-care devices for the detection of periodontal diseases, using markers that pinpoint the severity of periodontitis.[15−17] For example, Ivnitski et al. have used a hand-held noninvasive electrochemical amperometric device to

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detect salivary peroxidase, which is related to periodontitis.[18] However, this method is nonspecific, expensive, and time-consuming. Another example is the benzoyl-DL-arginine-naphthylamide (BANA) test strips that were developed by Loesche et al.[16,17] and are currently in the market. This strip detects various bacteria (*Treponema denticola*, *Porphyromonas gingivalis*, and *Bacteroides forsythus*) found in adult periodontal plaque.[16,17] Even though this method has been in the market for a while, the strip only detects perio-pathogens, gives no information on disease severity, and lacks sensitivity and specificity.

Current understanding of the pathogenesis of periodontitis illustrated the role of *Porphyromonas gingivalis (P. gingivalis)* proteases in disease onset and progression.[19,20] Accordingly, these proteases were employed as a detection biomarker for periodontitis. To date, specific peptides substrate were identified to detect the proteolytic activity of *P. gingivalis* proteases *in vitro* and *in situ*.[19,21] The value of these substrates are beyond doubt, though, as protease activity in cases of periodontitis is a mixture of both host and microbiological origin, these substrates only cover one part of the pallet.

Human Neutrophil Elastase (HNE) is serine protease. It is the metabolic product of the neutrophils which plays a destructive role in the process of periodontal tissue breakdown.[22] Thus, a high level of HNE in the gingival crevicular fluid (GCF) was noted.[20] Therefore, this protease can be used as a potential indicator for inflammation severity at individual sites.[21] Notably, it is not only the amounts of this protease that differ but also the activity of the protease. For example, the proteolytic activity of HNE in GCF is higher during periodontal disease.[23]

Another host-cell derived serine protease with potential for periodontitis diagnosis is Cathepsin-G. In response to periodontitis this protease is secreted in the extracellular spaces where they degrade gingival tissue components such as collagen.[24] Furthermore, there is an indication that Cathepsin-G is linked with the progression of gingivitis and chronic adult periodontitis.[24] Immunohistochemical studies of Cathepsin-G have shown that this protease is expressed and localized in the inflamed gingiva with an increased activity related to the severity of periodontal inflammation. These results suggested the possible involvement of Cathepsin-G in the degradation of inflamed gingival connective tissue[24] since it was reported that Cathepsin-G level was elevated in the GCF.[25]

A number of colorimetric protease assays based on the cleavage of gold nanoparticles (AuNPs) aggregates cross-linked with peptides sequences has been developed.[26−29] To date, novel colorimetric protease biosensors were based on the use of magnetic nanoparticles (MNPs) as they possess high capture efficiency due to the high surface/volume and thus offering numerous binding sites.[30] Moreover, MNPs can readily conjugate with a biorecognition element to provide a sensing probe and so can be utilized in the development of point-of-care (POC) diagnostic biosensors. Our previous studies assigned successful application of MNPs in the development of sensitive and specific colorimetric biosensors for Prostate Specific Antigen.[31]

In this work we present a prototype multiplex colorimetric rapid point-of-care diagnostic system using salivary fluid as a noninvasive matrix for the diagnosis of periodontal disease.[32] The designed colorimetric biosensor consists of black color MNPs−HNE (or Cathepsin G) substrate complex covalently attached to a gold sensing platform. This construct results in a layer of magnetic beads adsorbed on the sensing platform surface masking its golden color. Under HNE or Cathepsin G proteolytic activity, the peptide linkage between MNPs and the gold sensor surface will be cleaved. An external magnet will then collect the cleaved MNPs from the gold sensor surface making the golden color visible to the naked eye. This sensing platform offers a highly specific and sensitive colorimetric biosensor.

EXPERIMENTAL SECTION

Materials and Reagents. Carboxyl-terminated beads of 50 nm diameter were provided by Turbo beads (Switzerland) via Sigma-Aldrich (Dorset, U.K.). Self-adhesive Magnet sheets were purchased from Polarity Magnets Company. The recombinant Human Neutrophil Elastase (HNE) was purchased from Merck Chemical (Nottingham, United Kingdom). The recombinant Cathepsin-G protease was purchased from Elastin Products Company Inc. The peptide sequences (HNE specific peptide, GSGSGGGAAPVAAKGGGSGSC and Cathepsin-G specific peptide, Ahx- GPQGIWGQR-Ahx)[33] were synthesized by Pepmic Co., Ltd. (Suzhou, China). The coupling agents *N*-hydroxysuccinimide (NHS) and 1-(3-(dimethylamino)propyl)-3-ethyl-carbodiimide (EDC) and the plastic pH indicator strip were purchased from Sigma-Aldrich (Dorset, U.K.). The self-adhesive tape was purchased from Whatman (London, U.K). The saliva was purchased from Lee Biosolutions Inc. (Missouri, U.K.). The wash/storage buffer (10 mM Tris base, 0.15 M sodium chloride, 0.1% (w/v) bovine serum albumin, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium azide, pH 7.5), the coupling buffer (10 mM potassium phosphate, 0.15 M sodium chloride, pH 5.5), the HNE reaction buffer (100 mM Tris-HCl, 500 mM sodium chloride, pH 7.5), and Cathepsin-G assay buffer (0.05 M sodium acetate, 0.1 M sodium chloride, pH 5) were prepared from chemicals of analytical grade.

Conjugation of the Human Neutrophil Elastase (HNE) and Cathepsin-G Substrate Peptides to Magnetic Beads. The magnetic bead suspension (150 mg/mL) was placed in an ultrasonic bath (Grant, XUBA3 model, Spain) for 5 min at full frequency of 44 kHz and three times washed with coupling buffer. After washing, the beads were mixed with the peptide (1.0 mg/mL) and the coupling agents EDC (0.57 mg/mL) and NHS (12 μg/mL). The mixture was shaken gently at room temperature for 24 h. Any uncoupled peptides were removed by washing the beads 3 times with washing buffer. Finally, the beads were stored at 4 °C in storage/wash buffer until use.[31]

Gold Sensor Platform Preparation. Clear adhesive tape was plated with a thin layer (30 nm) of gold. Subsequently, a narrow piece (1.5−2.0 mm in width) was cut and stacked over the plastic pH strip to provide a physical support for the biofunctionalization process of the detective sensor.

Sensing Monolayer Immobilization. The HNE and Cathepsin-G magnetic-peptide solution was mounted over the gold sensing surface and left at room temperature until dryness. Consequently, an external magnetic field was applied using a permanent magnet with field strength of 3360 gauss and 573 gauss at 1 mm and 10 mm distances, respectively. The magnet was passed over the functionalized strip from a distance of 3−5 mm to remove any nonimmobilized magnetic beads.

Biosensing of HNE. The current detection method is based on the measurement of HNE proteolytic activity using a specific substrate peptide. The probe was designed to be specific for HNE. Accordingly, the substrate sequence

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AAPVAAK,[33] with an 8-residue linker on either terminal of the peptide, was used. The N-terminal of the peptide was attached to the magnetic bead. A cysteine residue was inserted at the C-terminal, permitting a gold−sulfur irreversible interaction[35] for the establishment of a self-assembled monolayer (SAM) of peptide and magnetic bead on the surface of the sensor platform (Figure 1). Conversely, a round permanent paper magnet was fitted on the back and beneath the platform. Next, the HNE protease was dropped over the functionalized sensor platform. During the proteolytic cleavage, the permanent magnet would attract the cleaved magnetic beads and cause a visual observation of the bright gold surface, affording qualitative evaluation of the examined samples. In addition, a quantitative evaluation was manageable by using different concentrations of HNE solution (100 ng/mL, 10 ng/mL, 1 ng/mL, 100 pg/mL, 10 pg/mL, and 1 pg/mL). The experiments for each concentration were conducted three times.

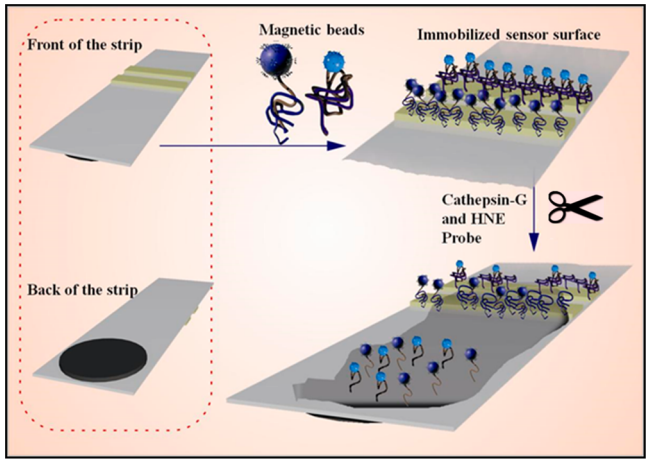

Figure 1. Schematic image of the multiplex colorimetric assay. Colorimetric assay built specifically for HNE and Cathepsin-G simultaneous detection. HNE and Cathepsin-G-induced dissociation of the magnetic bead complex exposes the gold color of the bare sensor surface. The assay chip is functionalized with Cathepsin-G and HNE substrate peptide-magnetic beads separately, and the protease solution is blotted onto the functionalized beads.

Biosensing of Cathepsin-G. The current detection method is based on the measurement of Cathepsin-G proteolytic activity using a specific substrate peptide. The probe was designed to be specific to Cathepsin-G. Accordingly, the substrate sequence AAPPFFK[33] with Ahx (aminohexanoic acid) linkers on either terminal of the peptide was used. The N-terminal of the peptide was attached to the magnetic bead. A cysteine residue was inserted at the C-terminal, permitting a gold−sulfur irreversible interaction[34] for the establishment of a SAM layer of the peptide and magnetic bead on the surface of the sensor platform (Figure 1). Similarly, a round permanent paper magnet was fitted on the back and beneath the platform (Figure 1). Next, the Cathepsin-G protease was dropped over the functionalized sensor platform. As described previously, during proteolytic cleavage, the permanent magnet would attract the cleaved magnetic beads and cause a visual observation of the bright gold surface, offering qualitative evaluation of the examined samples. In addition, a quantitative evaluation was manageable by using different concentrations of Cathepsin-G solution, (100 ng/mL, 10 ng/mL, 1 ng/mL, 100 pg/mL, 10 pg/mL, 1 pg/mL, and 100 fg/mL). All experiments were conducted three times.

Saliva Collection. Commercially purchased saliva was spiked with the HNE and Cathepsin-G protease (using 50 μL of saliva and 20 μL of various HNE protease concentrations). A negative control using nonspiked saliva was tested to check whether the commercially provided saliva reacted with the already functionalized magnetic beads. The experiments were conducted three times.

Periodontal Patient Samples Testing. To study the clinical applicability of the developed biosensors, GCF from patients suffering from periodontitis was utilized. The study was approved by the Institutional Ethical Board of the Academic Medical Center of Amsterdam, The Netherlands, and informed consent was obtained from all donors. The patient sample was examined by the standard qPCR method at the Department of Periodontology laboratories and showed a positive result. Consequently, this sample was tested on HNE specific, Cathepsin-G specific, and multiplexing platforms. The patient samples were blotted on the platform containing the SAM layer of HNE and Cathepsin-G beads. The experiments were conducted in triple.

Multiplexing the Colorimetric Sensor Probe for Periodontal Inflammation Detection. Two narrow pieces (1.5−2.0 mm in width) were cut and stacked over the plastic pH to provide a physical support for the biofunctionalization process of the detective sensor. The magnetic-peptide solutions of each HNE and Cathepsin-G were mounted over each gold sensing surface and left at room temperature until dryness. Consequently, an external magnetic field was applied using a permanent magnet with strengths of 3360 gauss and 573 gauss at 1 mm and 10 mm distances, respectively. The magnet was passed over the functionalized strip from a distance of 3 mm to 5 mm to remove any nonimmobilized magnetic beads. A round permanent paper magnet was fitted on the back and beneath the platform. Next, several tests were conducted, where the HNE and Cathepsin-G proteases were blotted over the functionalized sensor platform. The experiments were repeated three times.

RESULTS AND DISCUSSSION

Progress in nanotechnology in the field of biosensors has improved and enhanced the application of biosensors.[35] Generic colorimetric sensors which monitor biomarkers via degradation of thin films in the presence of an analyte is a promising approach for sensing biological processes in a disposable sensor format.[33] The choice of the thin film material is based on the selection of a generic material that could be cleaved specifically by the biomarker of interest. A high degree of specificity could be achieved using films composed of a known natural substrate. However, colorimetric biosensors are capable of detecting protease activity providing accurate diagnostic and monitoring techniques for many diseases, such as prostate cancer[31,36−38] and inflammations such as periodontal diseases.[38]

As reported in the literature, the HNE protease level and activity increases during periodontal disease resulting in the loss of the attachment between teeth and supporting tissues.[38] Similarly, Cathepsin-G protease levels and activity are also elevated during periodontal inflammation resulting in the degradation of inflamed gingival connective tissues.[24] Furthermore, Cathepsin-G is correlated with periodontal disease severity.[8] Thus, both proteases could both serve as potential

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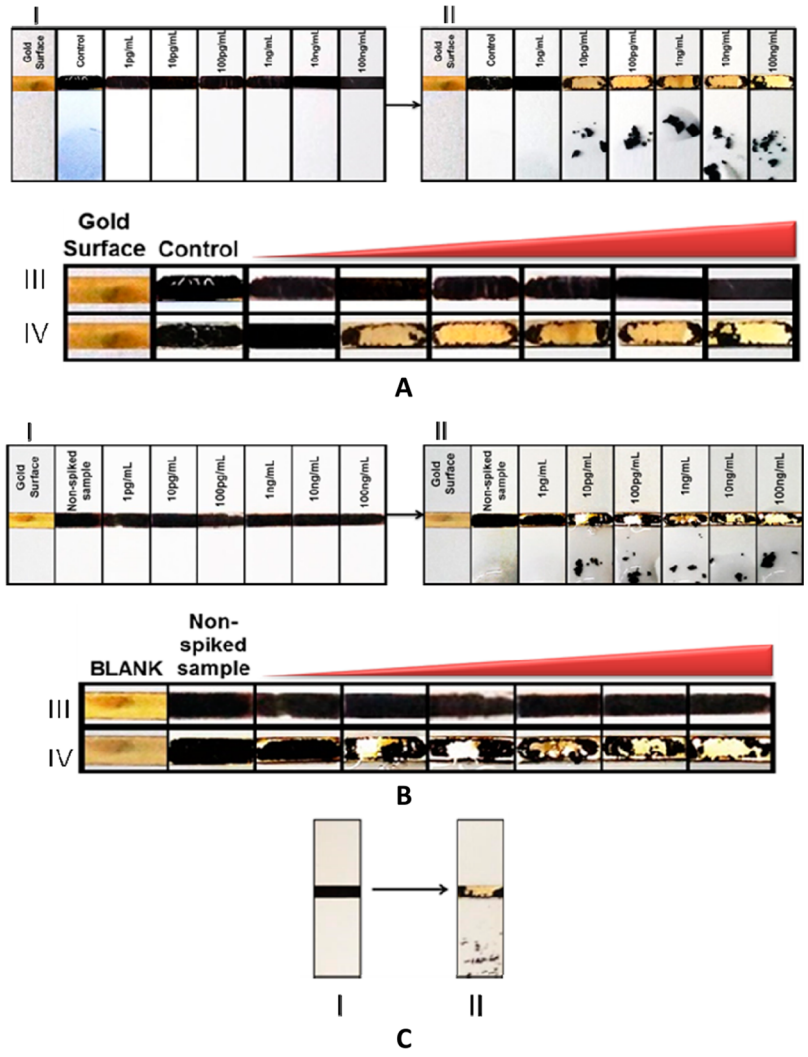

Figure 2. Colorimetric HNE sensor probe (specific HNE substrate peptide covalently bound to a magnetic bead) under the effect of different HNE concentrations in buffer **A**. Spiked saliva samples **B**, patient sample **C. I and II** are Image of the sensor before and after pipetting different concentrations of HNE. **III** and **IV** are Zoomed in, visual comparison of the golden color of the exposed probe before and after HNE activity.

markers for the detection of periodontal diseases and disease progression.

In this study, the generic sensor concept was initially demonstrated using the periodontal markers HNE and Cathepsin-G and their peptide sequences AAPVAAK and AAPFFK, respectively.[38,39] However, to ensure protease accessibility, the chosen specific HNE peptide sequence was linked to an additional 7-residue linker on either terminal; while the Cathepsin-G sequence was attached with Ahx-linkers on either terminal of the peptide. These linkers were placed to enhance the access of the protease to the substrate sequence near the sensor surface and magnetic bead layer by providing additional degree of freedom to the target molecule. The N-terminal of the peptide was attached to the magnetic bead and a cysteine residue was added at the C-terminal, allowing a gold−sulfur interaction,[34] and resulting in the formation of a SAM layer of peptides (HNE and Cathepsin-G) and magnetic bead on the gold sensor surface. Subsequently, the sensor chips were tested separately with purified proteases, saliva spiked with HNE and Cathepsin-G, and periodontal patient samples.

Determine the Limit of Detection of HNE and Cathepsin-G. The constructed sensor was tested to detect the proteolytic activity of HNE and Cathepsin-G, by blotting 100 ng/mL of both protease solutions independently, over the functionalized gold sensor. The catalytic effect of both proteases induced the release of the peptide-magnetic bead moiety from the sensor surface, and the cleavage process was accelerated by the presence of the round paper magnet attached at the back of the sensor. The dissociation of the peptide-magnetic bead moiety from the gold sensor surface exposed the golden color of the sensor surface to be visible to the naked eye as seen in Figure 2A (for HNE) and Figure 3A (for Cathepsin-G).

In general, this biosensing method is pliable to a quantitative detection of HNE as well as Cathepsin-G. After blotting different concentrations (1 pg/mL, 10 pg/mL, 100 pg/mL, 1 ng/mL, 10 ng/mL, and 100 ng/mL) of the HNE protease and different concentrations (100 fg/mL, 1 pg/mL, 10 pg/mL, 100 pg/mL, 1 ng/mL, 10 ng/mL, and 100 ng/mL) of the Cathepsin-G protease independently onto the functionalized gold sensor. In the presence of the permanent magnet attached to the strip, we observed a steady increase in the visible bare gold surface in comparison with the protease solution concentration (Figure 2A and Figure 3A). This method proved to be sensitive and specific allowing the detection limit to be as low as 1 pg/mL for HNE and 100 fg/mL for Cathepsin-G as

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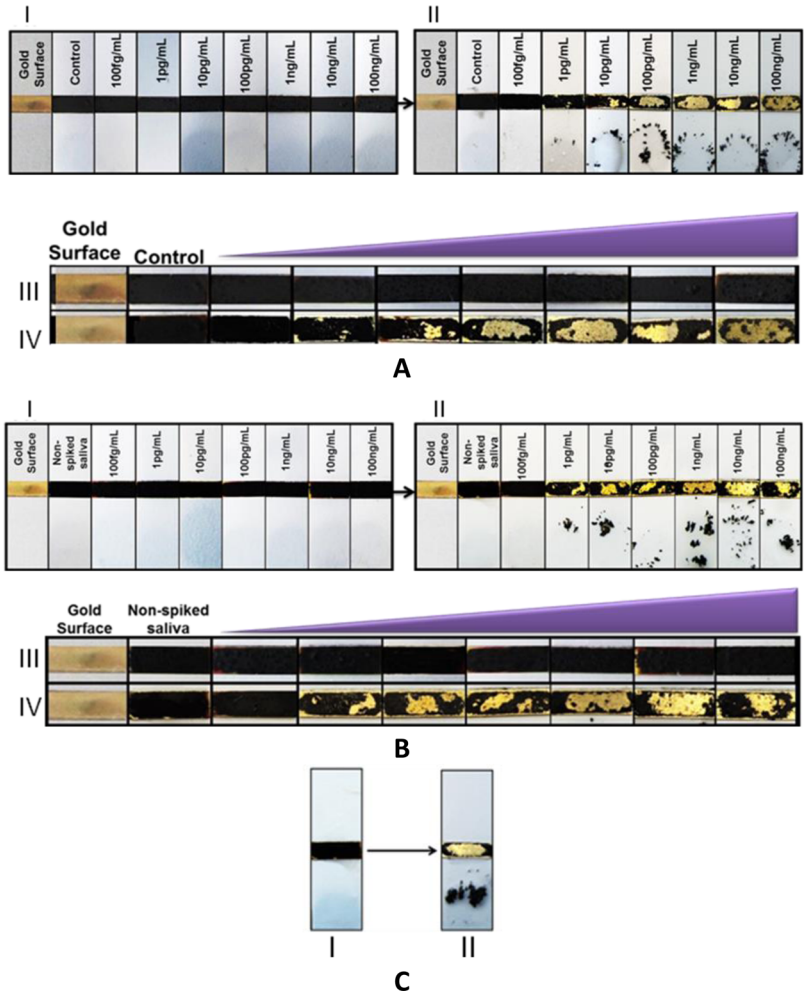

Figure 3. Colorimetric Cathepsin-G sensor probe (specific HNE substrate peptide covalently bound to a magnetic bead) under the effect of different Cathepsin-G concentrations in buffer **A**. Spiked saliva samples **B**, patient sample **C**. Parts I and II are images of the sensor before and after pipetting different concentrations of HNE. Parts III and IV are zoomed in, visual comparison of the golden color of the exposed probe before and after Cathepsin-G activity.

shown in Figure 2A and Figure 3A, respectively. This detection limit was determined by identifying the lowest protease concentration incapable of cleaving the peptide-magnetic beads composite covalently attached to the golden sensor surface i.e., sensor golden surface area is invisible to the naked eye due to the intact SAM layer. The nonspecific protease (blank) showed to have no reaction with HNE and the Cathepsin-G peptide-magnetic complex, since the sensor demonstrated no disruption of the SAM layer (Figure 2A and Figure 3A). This assay is evidently better than the other previously reported[39] method by Kamarun et al.[39] using the hydrogel degradation films approach, which was suitable for the detection of HNE in a range of 0.72 to 30 U/mL, and the Stair et al.[33] method which detected 10 U/mL^{-1} HNE and 100 mU/mL^{-1} Cathepsin-G. Moreover, upon comparison with other techniques applied for periodontitis detection, such as PCR and culture,[19] this colorimetric assay is proved to be more sensitive and rapid as the results are obtained within 20−30 s. Whereas, PCR and culture techniques require long processing time and so the results are not obtained rapidly.[13,14] Furthermore, it is worth mentioning that this colorimetric assay is cheap, instrument-free, and can be used by nonskilled personnel as routine screening at the dentist practice, unlike PCR and culture techniques.

Determine the Limit of Detection of HNE and Cathepsin G in Spiked Saliva. The constructed sensor was validated by spiking saliva with different concentrations of HNE (1 pg/mL, 10 pg/mL, 100 pg/mL, 1 ng/mL, 10 ng/mL, and 100 ng/mL) and different concentrations of Cathepsin-G (100 fg/mL, 1 pg/mL, 10 pg/mL, 100 pg/mL, 1 ng/mL, 10 ng/mL, and 100 ng/mL), independently. Each concentration of the spiked saliva was blotted onto the functionalized gold sensor for 5 s or so, under the effect a permanent magnet attached to the strip. Clearly, a steady increase of the visible bare gold area in comparison with the HNE and Cathepsin-G enzyme solution concentration was perceived (Figure 2B and Figure 3B). The limits of detection for both spiked proteases were comparable to those obtained with the purified proteases. The nonspiked saliva was used as the negative control; the result proved no cleavage of the magnetic beads-peptide and no significant change in the sensor surface black color (Figure 2B and Figure 3B). The detection limit observed was as low as 1 pg/mL for HNE and 100 fg/mL for Cathepsin-G as shown in parts A and B of Figure 3, respectively.

Detection of HNE and Cathepsin-G Proteolytic Activity in Clinical Material. Our sensor was also validated by testing several samples obtained from patients with periodontal disease. A full positive result was shown by the

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clear cleavage of the magnetic beads-peptide layer of HNE and Cathepsin- G, and the consequent appearance of the sensor golden surface color (Figure 2C and Figure 3C). The nonspiked saliva was used as the negative control for both proteases; the result proved no cleavage of the magnetic beads-peptide without any disruption of the SAM layer.

Multiplex Colorimetric Periodontal Inflammation Sensor Probe under the Effect of Different Periodontal Protease Markers. A further development of the sensor probe was carried out where both markers were tested at once. Two pieces of the gold sensor surface were placed onto the pH strip and the magnetic-peptide complex of Cathepsin-G (top band) and HNE (bottom band) were mounted over the gold sensor surface and allowed to dry at room temperature ensuring proper gold sensor surface functionalization (Figures 4, 5, and 6). At this stage, the golden color of the sensor chip was completely concealed. Afterward, an external magnetic field was passed over the functionalized gold sensor surfaces to remove any nonimmobilized magnetic bead. The cleavage process was hastened by the existence of the round paper magnet which was placed at the back of the sensor. The sensor was then ready for Cathepsin-G and HNE detection.

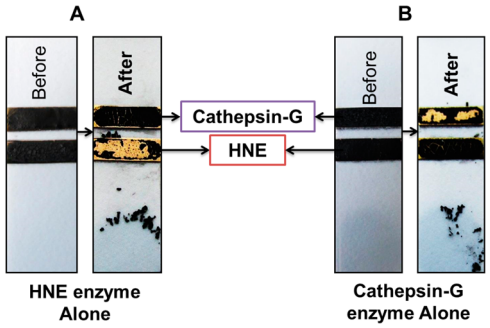

Figure 4. Multiplex colorimetric periodontal inflammation sensor probe exposed to different periodontal protease markers. (A) Examination of HNE protease alone (at 100 ng/mL). (B) Examination of Cathepsin-G alone (at 100 ng/mL) (top band, Cathepsin-G; bottom band, HNE).

The first set of experiments conducted on the multiplex colorimetric sensor probe was the examination of the activity of each individual protease on the functionalized sensor. The concentration of each protease was selected based on the highest activity seen in previous results (Figure 2A and Figure 3A). In both HNE and Cathepsin-G results (Figure 2A and Figure 3A), the highest concentration used was 100 ng/mL. The results obtained for both HNE (Figure 2A) and Cathepsin-G (Figure 3A) showed that the concentration selected had maximum cleavage.

In theory, there is no claim that the HNE peptide sequence can be cleaved by the Cathepsin-G protease or vice versa. The reason for this is that these sequences are specific and sensitive to the specific protease, which has been proven in a previous study.[39] Therefore, when the HNE protease alone was blotted onto the sensor strip, there was no cleavage seen on the top band containing Cathepsin-G and maximum reaction was seen in the bottom band containing HNE (Figure 4A). Similarly, when the Cathepsin-G protease alone was blotted, there was no cleavage seen in the bottom band and there was maximum reaction seen in the top band (Figure 4B). These results indicate that the peptide sequences for both proteases are specific and sensitive. Furthermore, no cross-reaction was observed.

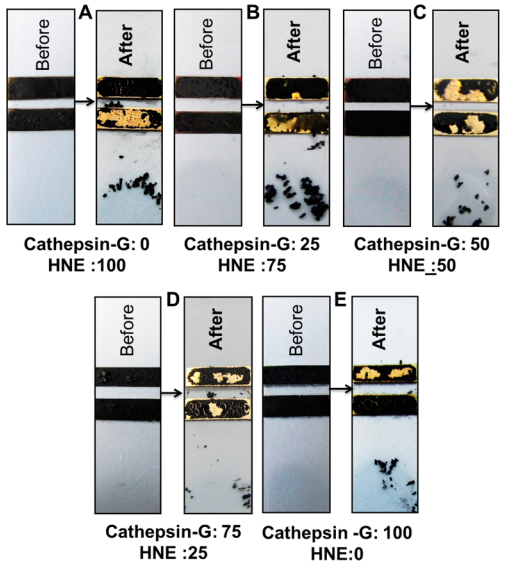

Figure 5. Multiplex colorimetric periodontal inflammation sensor probe exposed to 100 ng/mL mixed proteases (HNE and Cathepsin-G) at different ratios (Cathepsin-G/HNE). (A) Image of the assay before and after exposure to mixed proteases with the ratio of Cathepsin-G = 0 and HNE = 100. (B) Image of the assay before and after blotting mixed proteases with the ratio of Cathepsin-G = 25 and HNE = 75. (C) Image of the assay before and after blotting mixed proteases with the ratio of Cathepsin-G = 50 and HNE = 50. (D) Image of the assay before and after blotting mixed proteases with the ratio of Cathepsin-G = 75 and HNE = 25. (E) Image of the assay before and after exposure to mixed proteases with the ratio of Cathepsin-G = 100 and HNE = 0 (top band, Cathepsin-G; bottom band, HNE).

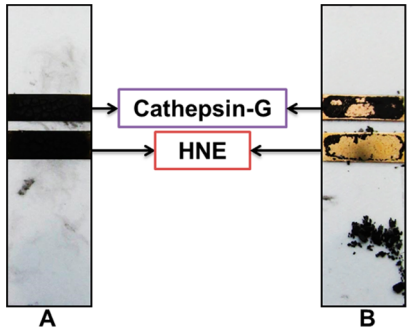

Figure 6. Colorimetric multiplex sensor probe application on patient sample. (A) Examination of healthy control saliva (negative result). (B) Examination of periodontitis patients' saliva (positive result).

The second set of experiments conducted was the examination of mixed proteases (HNE and Cathepsin-G) at different ratios. The selected concentration of both proteases was again 100 ng/mL. The various ratios of the mixed proteases used were 0 μL of Cathepsin-G to 100 μL of HNE (Figure 5A), 25 μL of Cathepsin-G to 75 μL of HNE (Figure 5B), 50 μL of Cathepsin-G to 50 μL of HNE (Figure 5C), 75 μL of Cathepsin-G to 25 μL of HNE (Figure 5D), and 100 μL of Cathepsin-G to 0 μL of HNE (Figure 5E).

The examination of mixed proteases at different ratios was conducted to determine the variance in the cleavage reaction for both markers. Therefore, the mixtures of proteases with

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different ratios were blotted onto the sensor. The first result obtained showed there was no reaction seen on Cathepsin-G (top band) and maximum cleavage was seen on the HNE sensor (bottom band) where a golden color change was visible as seen in Figure 5A. However, the results of the ratio of mixed protease (25 μL of Cathepsin-G to 75 μL of HNE) showed a minimum reaction on the Cathepsin-G (bottom band) and a decline in the cleavage of the HNE sensor as the visible color change to gold is reduced compared to the 0:100 ratio of HNE (Figure 5B).

On the other hand, the results of the mixture of proteases under the 50:50 μL ratio (Figure 5C) demonstrated a visible equal reaction of both Cathepsin-G and HNE (top and bottom bands). There was an equal color change from black to gold after blotting the mixture of protease as shown in Figure 5C. Moreover, the results of the mixture of proteases under the 75 μL Cathepsin-G/25 μL HNE ratio, showed an increase in the color change from black to gold of the Cathepsin-G sensor, while a decline in the cleavage of the HNE sensor was observed (Figure 5D), indicating the reaction worked in a similar fashion to the ratio provided. However, the cleavage reaction of Cathepsin-G 75 μL was not equivalent to the HNE result 75 μL.

The final results (Figure 5E) obtained showed there was no reaction seen on the HNE sensor (bottom band) and maximum cleavage was seen on the Cathepsin-G sensor (top band) where a golden color change was visible to the naked eye. However, the results obtained of Cathepsin-G (100) was not equivalent to the HNE cleavage (100), since there was more reaction seen in the HNE results (Figure 5A) compared to the Cathepsin-G results (Figure 5E). In general, these results observed showed similar activity and cleavage of the protease to the ratios provided.

The final set of experiments conducted were the authentication of the multiplex sensor by testing several samples provided by patients with periodontal disease. A full positive result was observed and determined by the clear cleavage of the magnetic beads-peptide layer of Cathepsin-G (top band) and HNE (bottom band), since a consequent appearance of the sensor golden surface color was observed. However, the cleavage of the magnetic beads peptide layer of Cathepsin-G was seen to be lower than HNE but there was still a visible gold surface seen and the result was positive (Figure 6). The results obtained showed a variation in the activity of the Cathepsin-G and HNE; this indicated that the sensor is specific and sensitive since the results were not the same for every patient sample. Normal individuals' saliva samples were also tested and no reaction was seen and the black color of the beads was maintained on the surface sensor (Figure 6A).

CONCLUSION

The generic sensor material developed was capable of monitoring HNE and Cathepsin-G protease activity separately and in combination, qualitatively, and quantitatively via the degradation of the specific peptide substrates. The developed low-cost colorimetric sensor can be utilized with no prior technical knowledge and without the aid of any sophisticated instrumentation. Certainly, the approach is grounded on a one-step sensitive monolayer preparation using the SAM method. In addition, this assay is a washless process based on the analyte protease-induced detachment of the monolayer. The recognition device does not require any labeling or amplification schemes. Moreover, the protease recognition signal is an increase of the golden surface that is observable by the naked eye. To carry out periodontal diseases diagnostics, the small sensor strip is proficient for the quantitative HNE detection in solution and saliva, attaining a lower detection limit of 1 pg/mL with good specificity and reproducibility. Similarly, the small sensor strip is efficient for quantitative Cathepsin-G detection in solution and saliva, attaining a lower detection limit of 100 fg/mL with good sensitivity and reproducibility, Furthermore, this small sensor strip has been shown to be proficient as a multiplex detecting both HNE and Cathepsin-G simultaneously. Also the peptide sequences selected proved to be specific and sensitive since the proteases reacted with the correct peptide. In addition, in a small proof of concept study, this small sensor strip has been shown to be proficient when testing periodontal patient's samples proving that there is a potential future in using this application for the detection of periodontal diseases. In overall, this colorimetric assay can be used not only for the diagnosis of periodontal disease but can be adapted for other infectious diseases.

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Notes

The authors declare no competing financial interest.

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