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ORIGINAL PAPER

On site visual detection of *Porphyromonas gingivalis* related periodontitis by using a magnetic-nanobead based assay for gingipains protease biomarkers

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Abstract

Porphyromonas gingivalis (P. gingivalis) is a pathogen causing periodontitis. A rapid assay is described for the diagnosis of periodontal infections related to *P. gingivalis*. The method is making use of gingipains, a group of *P. gingivalis* specific proteases as a detection biomarker. Magnetic-nanobeads were labeled with gingipain-specific peptide substrates and immobilized on a gold biosensing platform via gold-thiol linkage. As a result of this, the color of the gold layer turns black. Upon cleavage of the immobilized substrates by gingipains, the magnetic-nanobeads-peptide fragments were attracted by a magnet so that the golden surface color becomes visible again. This assay is highly sensitive and specific. It is capable of detecting as little as 49 CFU·mL^{-1} of *P. gingivalis* within 30 s. Examination of periodontitis patients and healthy control saliva samples showed the potential of the assay. The simplicity and rapidity of the assay makes it an effective point-of-care device.

Keywords *Porphyromonas gingivalis* · Periodontal diseases · Colorimetric assay · Magnetic nanoparticles · Biosensor · Gingivitis · Diagnosis · Point of care

Introduction

Periodontal diseases are inflammatory diseases of microbial etiology affecting tooth supporting tissues [1]. Periodontal diseases comprise a wide range of inflammatory conditions that affect the supporting structures of the teeth such as the gingiva, bone and periodontal ligament, which may lead to tooth loss and contribute to systemic inflammation [2]. Gingivitis is characterized by the inflammation of the gums without loss of connective tissue attachment or bone. Gingivitis is a prerequisite for periodontitis, but does not necessarily lead to periodontitis [2] which affects approximately 7–15% of the adults in the Western world [3]. Periodontitis involves progressive loss of the alveolar bone around the

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teeth, and if left untreated, can lead to the loosening and subsequent loss of teeth. Epidemiological studies have linked periodontitis to other systemic illness such as atherosclerosis, cardiovascular diseases and rheumatoid arthritis [4, 5].

The microbiota of the human oral mucosa consists of a myriad bacterial species that normally exists in commensal harmony with the host [6]. In case of periodontitis a specific bacterial group, named as "red complex", including *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola,* show to be one of the factor that may be involved in the periodontaldisease [7]. Of these bacteria *P. gingivalis* participates intensively in the initiation and progression of this diseases.It invades teeth supporting tissues and evades the host defense mechanisms causing periodontitis [8]. It is armed with a plethora of virulence factors such as lipopolysaccharide (LPS), proteases such as gingipains, peptidyl arginine deiminase, hemagglutinins, fimbriae and other outer membrane proteins [3, 9, 10]. These virulence proteins facilitate *P. gingivalis* survival in the periodontal pocket, and contribute to the destruction of the teeth supportive tissues.

The current understanding of periodontitis pathogenesis suggests that gingipain proteases have an important role in the onset and progression of the disease [11]. This is because gingipains, which are trypsin-like cysteine proteases, account for at least 85% of the general proteolytic activity displayed by *P. gingivalis* [11, 12].

Up to date, many methods have been developed to diagnose periodontal diseases. Some of which include subjective observational indices, which are based on criteria such as bleeding on probing, measuring pocket depth, attachment loss and radiographic evidence of bone loss [7]. However, these methods are not capable to identify pathogens involved [11]. Alternative diagnostic methods available for identifying periodontal pathogens include culture-based, nucleic acid-based and antibody-based assays [13–16]. The advantages and disadvantages of these methods are summerized in Table 1.

More recently, research was being conducted on using the gingipains activity as biomarker for periodontitis. For example, Kaman et al. have developed highly specific fluorogenic protease substrates for the detection of *P. gingivalis* in situ. Applying the substates in a laboratory setting revealed that the substrates had a sensitivity up to 95% and a specificity up to 100% [11]. Alternatively, protease substrates were developed to detect human proteases which are involved in periodontitis, including cathepsin G and elastase. Althought the sensitivity and specificity of these protease substrates were low, the use of magnetic nanobeads to detect their proteolytic breakdown appeared promising [18].

In line with the latter study, the goal of this study was to develop a *P. gingivalis* specific test using magnetic nanoparticle technology. Ideally, such a test can serve as a low-cost and specific colorimetric chair-side assay which can aid the dentist to diagnose periodontal diseases.

Table 1 Common *P. gingivalis* detection techniques

Detection method	Advantage	Disadvantage	Reference
Culture	Detecting and quantify oral cavities microbiota to create an antibiogram; has the ability to specifically distinguish between species	Time-consuming; laborious; has relatively low level of sensitivity	[14–16]
PCR	Reliable; able to detect low numbers of bacterial cells	Costly; laborious technique; bacterial DNA needs to be extracted and isolated from the sample; Crossreactivity with other (unknown) species may occur as the oral and subgingival microbiota is extensive and diverse	[14–16]
Antibody-based	High specificity; quantitative; applicable for multiplexing assays	Complex sample preparation; high instrument cost; requires trained personnel	[17]

Materials and methods

Magnetic-nanobeads with a mean diameter of 50 nm (according to manufacturer) and surface-coated with primary carboxylic groups was purchased from TurboBeads (Zurich, Switzerland). *N*-Hydroxysuccinimide (NHS), 1-(3-Dimethylaminopropyl)-3-Ethyl-Carbodiimide (EDC), greiner multiwell plate sealing film (A 5596) and pH test strips 0–14 pH, resolution: 1.0 pH unit (P4786) were purchased from Sigma-Aldrich (Dorset, UK). Magnetic sheets self adhesive A4 (Product Code: 15000SHEETA4) was purchased from Polarity Magnets Company (Wickford, UK). Peptide substrate (NH_2-Ahx-Arg-DLys-Ahx-Cys) was synthesised by Pepmic Co., Ltd. (Suzhou, China) with >98% purity. Brain Heart Infusion (BHI) medium and Trypticase Soy Agar (TSA) were purchased from Bio- Trading (Mijdrecht, The Netherlands).

Preparation of *P. gingivalis* culture supernatant

P. gingivalis culture supernatant containing secreted proteases was prepared as described previously [11]. In brief, *P. gingivalis* W50 was grown in BHI medium supplemented with 1 μg mL^{-1} hemin and 0.5 μg mL^{-1} menadione (Sigma, Zwijndrecht, The Netherlands) under anaerobic conditions at 37 °C for 72 h. The number of bacteria was determined by plating 10-fold serial dilutions on TSA plates. Following, plates were incubated at 37 °C under anaerobic conditions and enumerated after 3 days. The pure bacterial culture was serial diluted in culture broth (10^8, 10^7, 10^6, 10^5, 10^4, 10^3,10^2 and 10 CFU / mL), centrifuged for 10 min at 10,000 x *g* and filtered through 0.22 μm sterile filter (Millipore, Amsterdam, The Netherlands). Samples were used directly or stored at −20 °C for later use. The specificity testing was carried out by using culture supernatants from, 10^6 CFU / mL *Fusobacterium nucleatum* ATCC 33693 and 10^6 CFU / mL *T. forsythia* ATCC 43037. These bacterial strains were grown in 15 mL BHI medium under anaerobic conditions at 37 °C [11]. Next, the bacteria were pelleted by centrifugation for 10 min at 10,000 x *g* and the supernatant, containing secreted proteases, was sterilized by filtration through an 0.22 μm sterile filter (Millipore, Amsterdam, The Netherlands). Samples were stored at −20 °C for later use.

Saliva spiking

Commercially purchased human saliva (Lee Biosolutions Inc., St Louis, Missouri, USA) was spiked with *P. gingivalis, F. nucleatum* and *T. forsythia* pure culture supernatants (1:1 aliquots ratio). Saliva spiked with broth was used as a negative control to verify that the commercially purchased saliva does not react with the peptide labelled magnetic-nanobeads. The experiments were conducted in triplicate.

Preperation of peptide labeled magnetic-nanobeads

Magnetic-nanobeads suspension (15 mg/mL, Turbobeads, Zurich, Switzerland) was dispersed in an ultrasonic bath (Grant, XUBA3 model, Spain) for 5 min, then collected using a magnet separator and washed three times with the coupling saline buffer (10 mM potassium phosphate, 0.15 M sodium chloride, pH 5.5). Next, 1 mL of the peptide substrate (1.0 mg mL^{-1}, 'NH_2-Ahx-Arg-DLys-Ahx-Cys [11]', >98% %, Pepmic Co. Ltd., Suzhou, China), EDC (0.57 mg $mL^{-}1$, Sigma-Aldrich, UK) and NHS (12 μg/mL, Sigma-Aldrich, UK) were added to 30 mg /mL magnetic-nanobeads suspension in coupling buffer. The mixture was then shaken gently at room temperature for 24 h. Next, magnetic-nanobeads were collected using the magnet separator and the supernatant was discarded. The collected peptide labeled magnetic-nanobeads were then washed three times with washing buffer (10 mM Tris base, 0.15 M sodium chloride, 0.1% (*w/v*) bovine serum albumin, 1 mM ethylenediaminetetraacetic acid, 0.1% sodium azide, pH 7.5) and stored in washing buffer at 4 °C for later use [19, 20] (Fig. 1a).

Preparation of *P. gingivalis* assay platform

Greiner multiwell plate sealing film (A 5596, Sigma-Aldrich, UK) was coated with gold at the school of Engineering at Cranfield University, United Kingdom. The coated sheet was then chopped into small rectangular pieces (1 mm × 2 mm). Each piece was lodged over a solid support to bestow assay platform base (Fig. 1b). Afterwards, 20 μL of the peptide labeled magnetic-nanobeads suspension was dropped over the gold platform and set aside at room temperature till dryness (Fig. 1c). A gingipain-specific peptide substrate was covalently bound to the carboxylated magnetic-nanobeads by an amide linkage on one hand and to the gold platform via disulfide linkage handled by the cystine residue thiol on the other hand. During substrate synthesis, the lysine amino terminal was blocked to avoid incorrect coupling. The substrate sequence did not contain any carboxy terminal which may lead to polymerization. However, noncovalent adsorption of the labeled magnetic-nanobeads over the gold platform unavoidably occured, thus, a magnet (12.5 mm × 12.5 mm × 5 mm, Polarity Magnets Company, Wickford, UK) with a field strength of 3360 gauss and 573 gauss at 1 mm and 10 mm distance, respectively, was passed over the platform from a distance of 3–5 mm to remove the non-covalently adsorbed labeled magnetic-nanobeads. Later, a 2 mm diameter adhesive circle magnet (prepared by punching an adhesive magnet sheet purchased from Polarity Magnets Company, Wickford, UK) was fixed on the platform backside beneath (2–3 mm) the sensing platform as shown in Fig. 1d.

Measurement of gingipain proteolytic activity

In this colorimetric detection method, the probe was aimed to detect the proteolytic activity of gingipains as a diagnostic biomarker for periodontitis using a specific peptide substrate. *P. gingivalis* culture media was dropped over the functionalized (self-assembled monolayer (SAM)) platform triggering magnetic-nanobeads cleavage (Fig. 1e). The permanent magnet which was stacked at the back of the strip attracted the released magnetic-nanobeads prompting visual observation for a qualitative evaluation of the tested samples. Quantitative evaluation was tested by using different *P. gingivalis* concentrations with the following CFU values; 4.5×10^8 CFU/ mL, 4.5×10^7 CFU/ mL, 4.5×10^6 CFU/ mL, 4.5×10^5 CFU/ mL, 4.5×10^4 CFU/ mL, 4.5×10^3 CFU/ mL, 4.5×10^2 CFU/ mL, 45 CFU/ mL and 4.5 CFU/ mL.

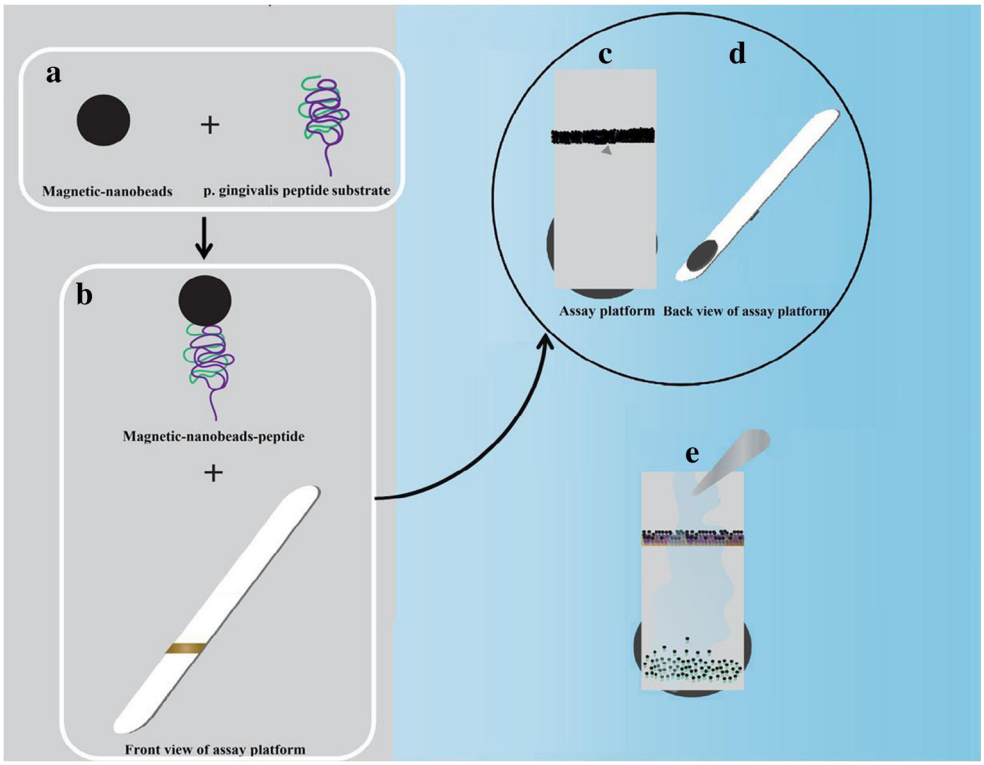

Fig. 1 Schematic overview of *P. gingivalis* protease detection assay. **a** Preperation of peptide labeled magnetic-nanobeads; **b** Immobilization of peptide labeled magnetic nano-beads over assay gold platform; **c** Ready to use assay platform; **d** Back view of ready to use assay platform; **e** Detection assay

Moreover, a quanttative measurment of the disappearance of magnetic-nanobeads peptide fragment and appearance of golden color was achieved by analyzing the images using ImageJ (National Institute of Health) [21].

Testing of periodontitis patient's samples

To test the clinical application of the constructed method for *P. gingivalis* related infections detection in situ [22], gingival crevicular fluid attained from patients suffering from periodontitis were examined. This 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. All patients' samples showed positive periodontitis results by standard qPCR method performed at the Department of Periodontology, VU University Amsterdam. Patients' samples were examined using the designed assay. The experiments were conducted in triplicate.

Results and discussion

P. gingivalis is an invasive and evasive opportunistic oral pathogen [3, 23]. It invades periodontal tissues and evades the host defense mechanisms. It utilizes a panel of virulence factors such as gingipain proteases which play a critical role in periodontal inflammation [6]. Therefore, the *P. gingivalis* specific gingipains are considered biomarkers for periodontal diseases [24]. At present, nanotechnology bioapplication in diagnostic analytical techqniques, in particular those capable of identifying proteases activity, as a disease biomarker were very attractive due to their high specificity and sensitivity [22, 25–32].The ultimate goal of this study was to develop a low-cost diagnostic tool for *P. gingivalis* related periodontitis measuring gingipain protease activity.

Detection assay using *P. gingivalis* protease culture supernatant

The proteolytic activity of *P. gingivalis* culture supernatant, and thus gingipain presence, was detected by dripping culture supernatant over the functionalized assay platform (Fig. 1). Cleavage of the specific peptide bond by the proteases leads to dissociation of the magnetic-nanobeads-peptide fragments, away from the detection platform surface due to the subsequent attraction by an external magnet. As a result, the golden color of the detecting platform is restored and can be observed by the nakedeye (Fig. 1d). Kaman et al., reported thatpeptide substrates containing single D-amino acids have a high specificity (up to 100%). In addition, the effect of spacer Ahx moiety length on the detection sensitivity was evaluated in our previous work [33]. Significant improvement in sensitivity was observed when the spacer length on each termini of the substrate sequence was from 4 to 8 carbons. Thus, the current study substrate was designed to have a spacer length on each terminus within the optimum length required, *i.e.* 6

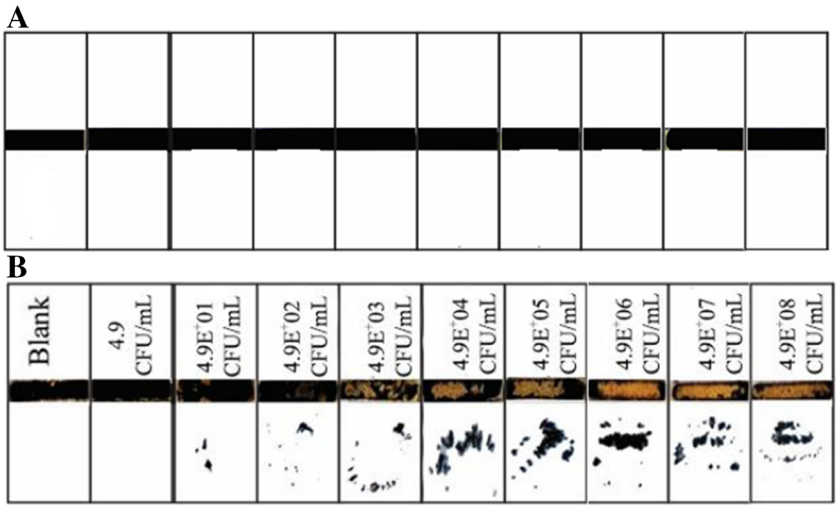

Fig. 2 Colorimetric *P. gingivalis* protease sensor probe under the effect of varying concentrations of *P. gingivalis* culture supernatant. **a** Before; and **b** after application of the samples

carbon via hexyl moiety. The amount of the peptide substrate required for peptide-magnetic-nanoparticles conjugation step was optimized in our previous work to achieve optimal monolayer performance and stability [26].

To test the sensitivity of the assay, serial diluted *P. gingivalis* culture was prepared and spotted onto the assay platform. Figures 1b and 2a showed the results before and after sample application, respectively. It was observed that there is a gradual increase in the visible golden color area which is proportional to the *P. gingivalis* concentration. This is related to the activity of the secreted proteases which results in the cleavage of the magnetic-nanobeads-peptide fragments. The designed technique showed a limit of detection (LOD) of 49 CFU/ mL within 30 s.This technique exhibited higher sensitivity and shorter response time in comparison to the work with the FRET-based assay [11]. This is mainly due to the amplification of the signal; using the magnetic-nanobeads in the assay rather than using the organic fluorescent dyes. Currently, other researchers had pointed up an immunochromatographic device with monoclonal antibodies against *P. gingivalis* outer membrane protein (OMP) 40 (HBP35) for semi-quantification of *P. gingivalis* in subgingival plaque [34]. Another research group demonstrated a portable electrochemical DNA sensor linked to PCR. A comparison of assays lower limit of

Table 2 New trends for the detection of *P. gingivalis* related periodontitis

Method	Sample	Detection limit (CFU mL^{-1})	Advantages	Disadvantages	Year Ref
Magnetic-nanobeads protease based assay	Saliva	49	Feasible; highly sensitive; high specificity; applicable for "on-site" detection; visual read out of the results; benefit clinical diagnosis of periodontal disease	Requires minimal detection platform manipulation	Current Work 2018
Immunochromatography based device DK13-PG-001	GCF Saliva	10^4	Requires minimal sample manipulation; easy-to-read results; feasible for chair-side detection; benefit clinical diagnosis of periodontal disease	Needs trained personnel and instrument to read out the result	[9, 14, 34]
Electrochemical DNA sensor linked with PCR	GCF Saliva	10^4	Measurement principle is simple; easy and can be applied to other targets; Suitable for primary screening of periodontal bacteria.	Need trained personnel and instrument to read out the result	[35]
FRET	GCF Saliva	10^5	Easy to perform; does not require enzyme pre-enrichment and purification steps; specific; offers the potential for development into a chairside test; bacteria as a source of the enzymes need not necessarily be viable as long as there is bacteria-derived enzyme activity	Requires fluorimeter to read out the results	[11]

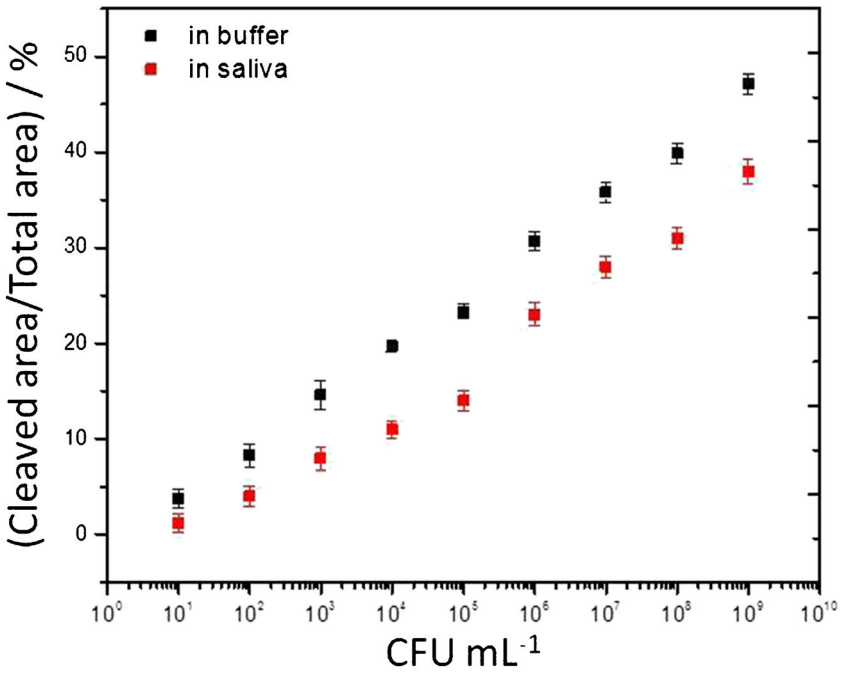

Fig. 3 Graph shows the quantitative data using ImageJ software in buffer and in saliva for various concentrations of bacteria; Cleaved area is defined as the area of the restored golden platform color after sample application; Total area is defined as the total area of the black color platform before application

detection (LOD), advantages and disadvantages are summarized in Table 2.

Spiked saliva testing

The feasibility of the current assay in *P. gingivalis*-related periodontitis as a diagnostic device was examined by using spiked saliva as an oral non-invasive diagnostic fluid. Varying *P. gingivalis* concentrations were spiked into the purchased saliva in a 1:1 aliquots ratio and applied to the functionalized biosensor. Figure 3 shows the quantitative data (detection responses) for various bacterial concentrations in buffer and saliva. Clearly, a gradual increase in the visible golden area with the increase of bacterial concentration was observed (Fig. 3). Unspiked saliva samples were used as a negative control and showed no cleavage of the magnetic-nanobeads-peptide fragments, no disruption of the SAM layer and no significant change in the platform color. In principle, the designed assay appears suitable for the analysis of patients' specimens at the site of collection. This enables the dental practitioner to perform an "on-site" test, to identify the periodontal pathogen *P. gingivalis* and to execute a proper treatment protocol.

Patient sample testing

To evaluate the performance characteristics of the designed assay in a clinical setting, the technique was tested with *P. gingivalis* patient CGF samples. Accordingly, 3 specimens from patients diagnosed with *P. gingivalis*-related periodontitis were tested. The presence of *P. gingivalis* was confirmed by positive cultures and qPCR as described previously [11]. Notably, the platform golden color was restored and can be observed by bare eye (Fig. 4). Furthermore, the assay results were positive and comparable with culturing technique.

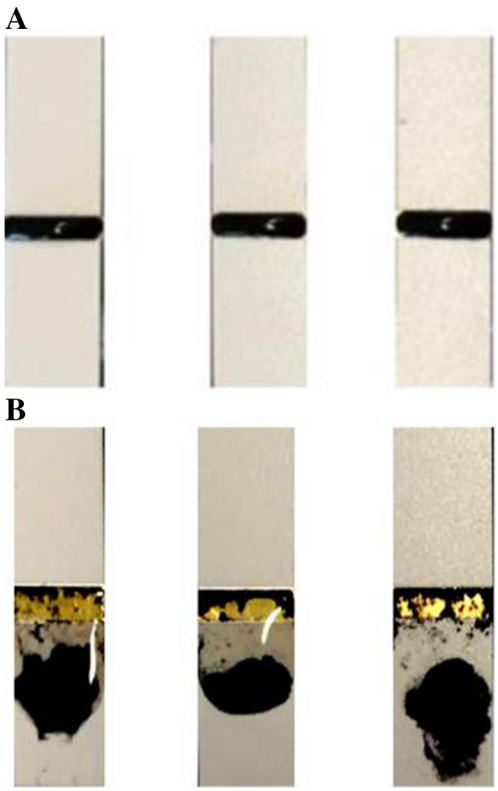

Fig. 4 Example of the colorimetric *P. gingivalis* protease biosensor with patient material; **a** Before adding the periodontitis patient sample; and **b** after addition of saliva from *P. gingivalis* related periodontis patients.

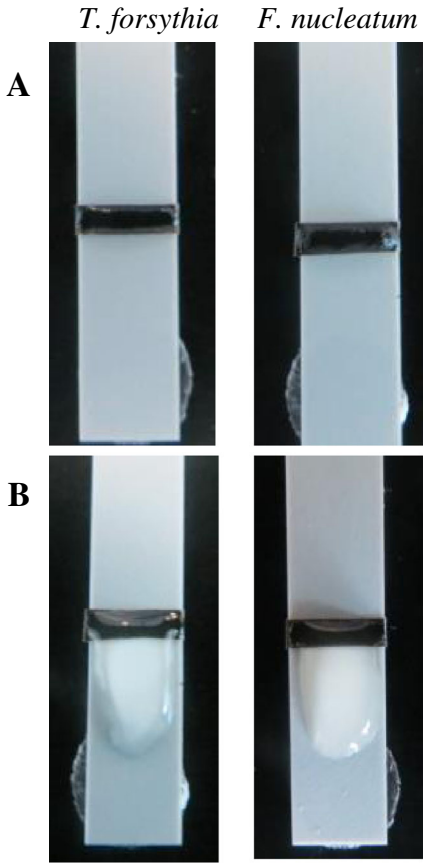

Fig. 5 Cross-reactivity study of the *P. gingivalis* protease biosensor with *T. forsythia* and *F. nucleatum* culture supernatants **a** Before application of the culture; and **b** after application of the culture

Cross-reactivity studies

Likewise, the specificity of the detection assay in the presence of other saliva pathogens proteases such as *F. nucleatum* (10^6 CFU / mL) and *T. forsythia* (10^6 CFU / mL) was assessed. The assay showed no disruption of the detecting platform and no appearance of the gold color (Fig. 5). These results confirm that the current assay is specific towards the detection of *P. gingivalis* and has no cross-reactivity with the other oral pathogens tested in vitro.

Conclusions

P. gingivalis proteases showed significant interest being one of the promising biomarkers associated with periodontal diseases. In this article, a novel and low-cost analytical assay capable of detecting *P. gingivalis* proteases were pointed up, using magnetic nanobeads covalently linked with a gingipain specific peptide substrate. The sensor was tested with serial diluted *P. gingivalis* culture, saliva spiked samples and patient material. The detection limit of the assay was 49 CFU/mL and the presence of *P. gingivalis* was detected within 30 s. Noteworthy, *P. gingivalis* protease detection was based on the appearance of the gold platform color which can be observed visually. This assay can be performed "on site" by non-skilled personnel without the requirement of expensive instrumentation. However, future work must be conducted to proof method selectivity and precision by using independent methods such as HPLC to measure the substrate concentration before and after gingipain application. In addition certified reference material should be used as assay control.

Compliance with ethical standards

The author(s) declare that they have no competing interests.

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