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Design of a dual aptamer-based recognition strategy for human matrix metalloproteinase 9 protein by piezoelectric biosensors

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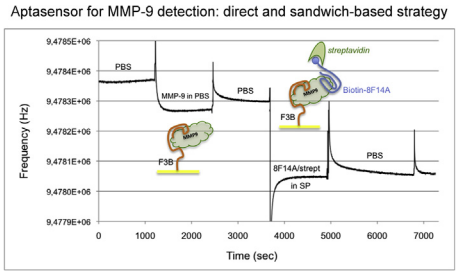
HIGHLIGHTS

- The first piezoelectric aptasensor for human matrix metalloproteinase 9 is presented.
- Specific and sensitive recognition is assured by a dual-aptamer strategy.
- Matrix effect of serum is evaluated and tackled by magnetic microbeads.
- Two comparable methods to quantify MMP-9 in serum, pre-treated or not, are shown.
- The detection limit in buffer is 1.2 pM, comparable to 6.8 pM in untreated serum.

GRAPHICAL ABSTRACT



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ABSTRACT

MMP-9, human matrix metalloproteinase 9, belongs to the family of zinc-dependent peptide-bond hydrolases and is involved in the degradation of the extracellular matrix (ECM). In clinics, it is well known that elevated MMP-9 serum levels are associated with cardiovascular dysfunctions, several aspects of the physiology and pathology of the central nervous system, neuropsychiatric disorders and degenerative diseases related to brain tumors, and excitotoxic/neuroinflammatory processes. Due to the large interest of diagnostics in this protein, efforts to set up sensitive methods to detect MMP-9 for early diagnosis of a number of metabolic alterations are rapidly increasing. In this panorama, biosensors could play a key role; therefore we explored for the first time the development of an aptamer-based piezoelectric biosensor for a sensitive, label free, and real time detection of MMP-9. The detecting strategy involved two different aptamers in a sandwich-like approach able to detect down to 100 pg mL^{-1} (1.2 pM) of MMP-9 as detection limit in standard solution. As proof of principle, commercial serum was investigated in terms of possible interferents, their identification and role in MMP-9 detection. The estimated detection limit for MMP-9 is about 560 pg mL^{-1} (6.8 pM) in untreated serum.

1. Introduction

MMP-9 is one of the 24 identified human matrix metalloproteinases (MMPs), zinc-dependent peptide-bond hydrolases that participate in extracellular matrix (ECM) degradation. Because

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of their implication in the changes and remodeling of ECM, MMPs are strictly regulated and generally are synthesized as proenzymes in the cytoplasm and activated by tissue or plasma proteinases [1,2]. MMPs participate in several physiological processes such as tissue remodeling, embryonic development, pregnancy, growth, and wound healing [3]. MMP-9 is synthesized and excreted as 92 kDa zymogen (proMMP-9) by mesenchymal, epithelial, and hematopoietic cells, then it is activated in situ by the converging plasmin/MMP-3 (stromelysin-1) cascade, which generates the active form of MMP-9 (83 kDa) [4]. In clinics, it is well known that elevated MMP-9 serum levels are associated with cardiovascular dysfunctions [3,5–7], several aspects of central nervous system physiology and pathology [8], and neuropsychiatric disorder [6]. It has also been implicated in degenerative CNS disorders [9], brain tumors, and excitotoxic/neuroinflammatory processes [10,11]. The MMP-9 involvement in the onset of several inflammatory diseases was also investigated: rheumatoid arthritis [12], periodontal disease [13], chronic wounds [14], skin inflammation [7], and inflammation of gastrointestinal [15] and renal tract [16]. Several lung diseases, such as acute and chronic asthma, emphysema, idiopathic pulmonary fibrosis, acute respiratory distress syndrome and some pulmonary infectious pathology, are characterized by elevated expression of MMP-9 and its high basal level in lung epithelium [17]. In particular, MMP-9 has been considered as promising target for cancer diagnosis on the basis of its massive up-regulation in malignant tissues and its ability to degrade all components of ECM [18], and interstitial connective tissues promoting angiogenesis, tumor invasion, and metastasis [19–21]. In healthy subjects, MMP-9 in plasma ranges from tens to hundreds ng/mL (from about 10 pM to 3 nM), whereas further increased levels of the protein are considered pathological (up to 1660 ng mL^{-1} , i.e. 20 nM) [22]. Considering the wide influence of MMP-9 expression on the onset of several diseases, the serum/plasma level of this analyte is becoming increasingly relevant in diagnostics [5]. In this framework, the efforts to set up innovative and sensitive methods to detect MMP-9 for early diagnosis of a number of metabolic alterations are increasing.

Different bioanalytical approaches have been developed for detecting MMPs levels and activities [23–28]. Among these, fluorometric immunocapture [27,28] assays take advantage of combining fluorescent substrates with immunochemical recognition. Nevertheless, fluorescent tests with synthetic substrates can take place almost only with purified enzymes since the presence of interfering molecules in biological fluids, mainly inhibitors, can affect the assay due to the fact that MMP specificities are similar. On the other hand, immunochemical approaches sometimes cannot discriminate between zymogens and active enzymes [29]. Separative approaches, such as chromatography coupled to mass spectrometry (LC/MS/MS) were also developed [30–32], but involving sample pre-treatment, i.e. enzymatic digestion with trypsin. Zymography, a method based on the electrophoresis of SDS-PAGE gels copolymerized with a protein substrate, was also explored for evaluating MMP-2 and MMP-9 activities. In particular, on the basis of the characteristic molecular weight, the identification of the zymogen and the active forms of gelatinases was possible [33–35]. However, this approach results complex and is subjected to possible underestimation of the active MMP-9 level. In addition, it is not sensitive enough to detect small amounts of MMPs and it is often not possible to discriminate between different MMPs because the substrates are usually degraded by more than one MMP. So the zymographical techniques are often flanked by a complementary technique, such as immunohistochemistry, for valid conclusions [35]. All the above-mentioned assays for MMP-9 detection are sensitive (60 pg mL^{-1} for assay with chromogenic peptide substrate [25]; 2.9 ng mL^{-1} for LC/MS/MS [31]; 10 pg mL^{-1} for zymography [35]), but the procedures are expensive and time-consuming, as well as requiring sample pretreatments and qualified personnel.

In the current panorama of methods devoted to MMPs detection for clinical purposes, biosensors could represent a successful alternative to traditional bioanalytical methods for MMP-9 detection, thanks to their sensitivity and selectivity, their ease of use, portability, and ability to be applied to complex and untreated matrices. Moreover, biosensors are often reusable for tens of measurements, allowing a significant decrease of the costs. Up to now only few papers reported the development of biosensing-based approaches to this issue, by electrochemical [36–38] and optical [39–41] transduction and in most of cases MMPs were assayed only in buffer solutions. The collagenolytic activity of MMP-9 in PBS solution was evidenced in real-time and kinetically characterized by a conventional SPR technique [42]. An immuno-based SPR imaging via quantum dots amplification was designed for the sequential analysis of MMP-9, HER2, Fas and Ang-2 proteins and applied as proof of concept only in buffer [39]. The only example in testing such a biosensor for metalloproteases in human plasma and whole blood is reported for MMP-2 and is based on fluorescence resonance energy transfer (FRET) [43]. To our knowledge, only a piezoelectric biosensor is reported in the literature [44] for MMP-1 immuno-based detection, again only in standard solutions.

In this framework, and in the perspective of developing fast analytical tools for early diagnostics, here we engineered for the first time the development of a piezoelectric-based biosensor for MMP-9 detection by exploiting as recognition bioelement an aptamer duo working in a sandwich-type assay, a very common strategy for the development of sensitive and selective aptamer-based biosensors [45–48]. In fact the dual-aptamer strategy is useful to get highly sensitive assays because the signal generation requires simultaneous binding of two aptamers to the same target molecule, so the incidence of non-specific binding events is much lower than that of assays based on direct detection (one receptor) [49]. In addition, the dual-site binding assay can achieve better detection limit because the background is reduced [50] and the second aptamer could be used at the same time as amplification agent, for example increasing the mass of the complex on a QCM surface to enhance the sensitivity of a piezoelectric-based aptasensor [51]. Aptamers are single strand oligonucleotides selected by an in vitro process, SELEX, against a variety of targets and promise to rival antibodies in molecular diagnostics. In last years their use in clinical diagnostics have appeared coupled to biosensor-based methods [52–55]. Aptamer selection allows obtaining highly pure molecules with high yield and reproducibility. They are stable to a variety of physical–chemical conditions (pH and temperature) and to long-term storage. Moreover, they can be handled at room temperature without any risk of irreversible denaturation. Their small size makes aptamers easy to be chemically modified, without loss of affinity for the target, and enables them to access protein epitopes that might otherwise be blocked or hidden [56–59]. The selection of aptamers for MMP-9 detection is an emerging field, and only one pioneering work was recently presented by Toulmé and coworkers [60]. The targeting ability of the aptamer, its selectivity in recogniting a specific MMP species, and also in discriminating between the zymogen and the active form of MMP-9 were demonstrated both by SPR and through *ex vivo* imaging studies on slices of human brain tumors. Next step would be then the application of these promising results for diagnostic purposes. Thus here this anti-MMP-9 aptamer was used as primary recognition element in a piezoelectric–based sensing in combination with a second one, binding to a different MMP-9 site, in a sandwich-like assay, with the aim to develop a label free aptasensor for MMP-9 detection. Moreover, after a preliminary assessment in buffer solution, the

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aptasensor behavior was exploited in human serum, to evaluate its applicability to complex mixtures, eventually avoiding any sample pre-treatment except a simple dilution (in buffer).

The piezoelectric transduction was achieved by a Quartz Crystal Microbalance (QCM), chosen for its sensitive, label free, and real time ability in detecting affinity interactions [61,62]. The final detecting strategy was optimized in a dual aptamer-based assay in which a secondary aptamer was used as signal enhancer targeting MMP-9 on a secondary epitope, i.e. a sandwich-like assay with two different aptamers. The analytical performances of the aptamer-based sandwich assay calculated as detection limit reach 1.2 pM (99.6 pg mL^{-1}) in buffer solution and 6.8 pM (0.56 ng mL^{-1}) in untreated serum. Commercial serum was tested on the aptasensor as proof of concept, showing that IgG interference recorded by the direct assay can be overcome by the sandwich format, thanks to the high selectivity of the secondary aptamer.

2. Materials and methods

2.1. Materials, reagents, and procedures

The active human MMP-9 (83 KDa) was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). The protein was stored as received (5 μg in 50 mM HEPES, 10 mM $CaCl_2$, 20% glycerol, 0.005% BRIJ®-35 Detergent, pH 7.5) at −80 °C and dilutions were performed in PBS (8 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl and 2 mM KH_2PO_4, pH 7.4) just before use. Both the 3′-biotinylated 2′fluoro-pyrimidine-RNA aptamers F3B and F3R [60] (36 nt) were identified and synthesized at the ARNA Laboratory INSERM U869 (Bordeaux, France). The F3B RNA aptamer, containing 2′-fluoro, pyrimidine ribonucleosides and 2′-O-methyl, purine ribonucleosides to confer it RNAse stability, was used as primary aptamer in all the experiments, whereas its scrambled sequence, F3R, was used as negative control to assess the specificity of F3B toward MMP-9. Before use, the oligonucleotides were purified by electrophoresis on 20% polyacrylamide/7 M urea denaturing gels. The 3′-biotinylated 8F14A DNA aptamer was used as secondary aptamer for the dual detection and was obtained by standard selection procedure against a MMP-9 fragment containing the catalytic domain. The selection was carried out by Novaptech (Pessac, France) from a DNA library containing 10^{14} candidates with a 30 nucleotide random window. From the selected pool we identified a family of strong binders that contained G residues driving the folding into quadruplexes as characterized by circular dichroism and UV-monitored thermal denaturation at 295 nm (not shown). We focused our study on the strongest binder, the 8F14 aptamer that does not compete with F3B for binding to MMP-9, a key condition for developing a sandwich assay. The selected candidate was further truncated down to a species, 8F14A, 5′ TCG TAT GGC ACG GGG TTG GTG TTG GGT TGG 3′, which retained the binding properties of the parent aptamer 8F14. The 5′-biotinylated DNA aptamer TBA (Thrombin binding aptamer from Tombelli et al. [63]) that also fold as a quadruplex was from Eurogentec (Seraing, Belgium) and was used as negative control to check the specificity of 8F14A toward MMP-9 in the sandwich assay. Immobilization of F3B/F3R aptamers and binding experiments of primary aptamer F3B with MMP-9 were performed in PBS buffer (8 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl and 2 mM KH_2PO_4 pH 7.4). All the experiments with the secondary aptamer 8F14A were performed by using the SELEX buffer, i.e. SP buffer (SP 1X: 50 mM Tris−HCl, 50 mM NaCl, 100 mM KCl, 5 mM $CaCl_2$ and 1 mM $Mg(CH_3COO)_2$ pH 7.5). Regeneration solution was composed by 25 mM EDTA, 3.6 M urea, and 40% formamide [64]. NH_3, H_2O_2, ethanol, and sodium acetate were purchased from Merck (Milan, Italy); NaOH, 11-mercaptoundecanol, bis-2-metoxyethyl ether, bromoacetic acid, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC), ethanolamine, streptavidin (from *Streptomyces avidinii*), biotin and dextran (from *Leuconostoc mesenteroides*) used for the modification of the gold surface of the sensor and for the immobilization of the receptors were purchased from Sigma (Milan, Italy); epichlorohydrin and N-hydroxysuccinimide (NHS) were from Fluka (Milan, Italy). Human serum albumin (HSA), human whole serum, and human purified IgGs were from Sigma−Aldrich, Milan, Italy. HSA and IgGs were used to test the specificity of the F3B aptamer in binding MMP-9 in serum. Dynabeads® Protein G Immunoprecipitation Kit and Dynabeads® Protein A Immunoprecipitation Kit were from Invitrogen and were here used for the selective removal of IgG, and IgA/M/E respectively from commercial serum, as suggested by the manufacturer for immunoglobulins solutions up to 2.5 mg ml^{-1}. In particular, 1:1 volume of serum (1:100 diluted in PBS) and Dynabeads® Protein A/G were mixed in tube, and incubated for 40 min under continuous gentle shaking. After that, the beads were collected on the tube wall by placing the tube near a magnet, allowing the recovery of the surnatant (Ig's free).

2.2. Piezoelectric apparatus and aptamer immobilization

9.5 MHz AT cut quartz crystals (14 mm, 165 μm) with gold evaporated (42.6 mm^2 area, Ø 7.4 mm) on both sides were obtained by Elbatech (Marciana (LI), Italy). Prior to use, the quartz crystal was cleaned for 10 min in a boiling solution of H_2O_2 (33%), NH_3 (33%) and milliQ water in a 1:1:5 ratio; then thoroughly washed with distilled water, dried, and used immediately afterward. Finally, the crystal was housed inside a PVC cell so that only one side of the crystal was in contact with the solution in the cell well. All the measurements were carried out at room temperature (T ~ 20 °C) and in static conditions. F3B aptamer was immobilized by using the well-known thiols/carboxylated dextran chemistry for the immobilization of biotinylated sequences via streptavidin binding [65]. After preparation of the streptavidin surface, 200 μL of a 1 μM PBS solution of the F3B biotinylated aptamer was first submitted to thermal pre-treatment to guarantee the proper folding of the sequence. It consisted in heating up to 99 °C for 1 min, and cooling down at 0 °C for 1 min. Afterward, the aptamer solution was rapidly added to the cell and the immobilization was allowed to proceed for 30 min. To avoid any further unspecific binding on the streptavidin layer, the crystal surface was finally saturated with a 1 g L^{-1} solution of biotin. The DNA aptamer 8F14A was also denatured before the sandwich assay, heating the SP solution of aptamer (1 μM) up to 75 °C for 5 min and cooling down at 0 °C for 5 min [66]. After baseline equilibration, the crystal was ready for measurements. Frequency variations were continuously recorded using a quartz-crystal analyzer (Model QCA922, Seiko EG&G, Chiba, Japan), and the resonance frequency was displayed in real-time using a computer connected to the instrument interface.

2.3. Protein measurements on QCM

Analytical data, frequency shifts (in Hertz) due to the mass change at the sensing surface, were measured by subtracting to the baseline frequency recorded in the proper blank solution (PBS for the F3B/MMP-9 binding and SP for the MMP-9/8F14A one) the frequency recorded after the binding of the biomolecule. Direct measurements of MMP-9 on the primary aptamer F3B were performed as follows: 100 μL of MMP-9 protein at different concentrations were added to the cell and left in contact with the immobilized aptamer F3B for 30 min (Fig. 1).

The surface was finally equilibrated with PBS buffer and regenerated with 25 mM EDTA, 3.6 M urea, and 40% formamide solution, for 30 s [64]. The surface was then washed with PBS to

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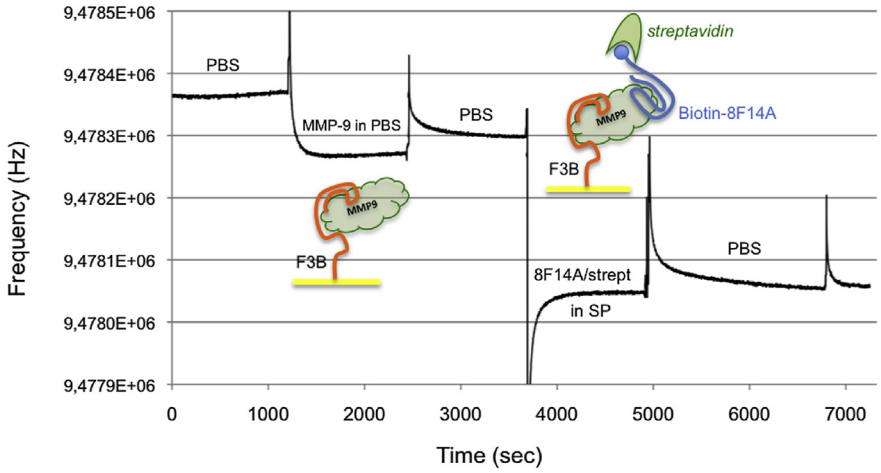

Fig. 1. Sensorgram obtained by the sandwich approach. From a stable baseline in PBS buffer, MMP-9 (50 nM) is added as analyte and left in contact with F3B aptamer. After buffer washing, the biotinylated 8F14A/streptavidin complex is added in SP buffer (the SELEX buffer for 8F14A/MMP-9 binding), and allowed to react on MMP-9. Finally, PBS was added for washing.

remove the unbound protein and the analytical datum of the direct recognition, expressed as difference before the addition of MMP-9 and after the washing step, was related only to the mass stably bound to the F3B aptamer. The sandwich assay was carried out as follows: 100 μL of 8F14A (500 nM) and streptavidin (1 μM) were incubated in SP buffer in vial for 30 min at room temperature to obtain their binding in solution. After that, the obtained complex was added on the F3B/MMP-9 complex on the biosensor to obtain the secondary recognition.

3. Results and discussion

3.1. MMP-9 direct detection via F3B aptamer

In order to develop a QCM-based aptasensor for the detection of MMP-9 by its direct recognition through the F3B aptamer, as preliminary step, the effect of a thermal unfolding/refolding step before its immobilization on the sensor surface was investigated. This is in fact a key issue in aptasensor development [67]. To this aim, 200 μL of 1 μM biotinylated F3B in PBS buffer was thermally treated as reported in the experimental section and then promptly added to the streptavidin-modified surface of the crystal and let react for 30 min. On a different crystal, the immobilization step was performed without the thermal pre-treatment to evaluate the binding ability of F3B obtained by either protocol.

Different concentrations of MMP-9 were tested in triplicate on both sensors, *i.e.* 10, 25, and 50 nM. After each binding, the surface was regenerated by dissociating the analyte−receptor complex with the regeneration solution for 30 s. Both aptasensors provided good responses but the one carrying the thermally treated F3B showed a significantly higher response both in terms of sensitivity and reproducibility, confirming that the pre-treatment promotes the correct folding of the aptamer and a higher binding ability to the analyte (Fig. S1 of Supplementary material). This, for its application in biosensing, is a key information for the subsequent optimization of the method. As a negative control, the aptamer F3R (scrambled sequence of F3B) was immobilized on a new sensor surface and tested on the same MMP-9 solutions applied for F3B, leading to no binding (Fig. S1 of Supplementary material). Afterward, the calibration curve of MMP-9 in buffer was performed in the concentration range of interest 0.1–25 nM (8.3–2075 ng mL^{-1}). The dose−response curve obtained is reported in Fig. 2, displaying a high linear correlation ($R^2 = 0.999$) up to 25 nM, with a %RSD = 7.3. The experimental detection limit resulted 5 nM ($\Delta f = 5.53 \pm 1.28$ Hz, $\sigma = 2$ Hz), slightly higher than the concentration range referred to a healthy subject (from ~10 pM to ~3 nM) and therefore susceptible to possible false results (positive or negative) for samples at the non-pathological/pathological edge.

3.2. Aptasensor behavior in untreated serum

As a proof of concept, the system was evaluated on commercial human serum. The whole serum (MMP-9 concentration unknown) was tested on the F3B receptor as it was, and diluted down to 1:1000 in PBS buffer. Dilutions were tested on different sensors, modified with the receptor or only with streptavidin (control surface), since interferences could interact in a non-specific manner either with F3B or with the sensor surface. Crystals were all saturated with biotin solution before sample testing. Results evidenced that at 1:10 dilution factor, the ratio specific (*i.e.* interactions ascribable to receptor binding) vs. unspecific signal (*i.e.* interactions with the sensor surface) is 28.5, becoming 91.0 for 1:50 dilution of serum (Fig. S2). Moreover, signals displayed a linear trend (signal vs. dilution factor) on the aptasensor, while no relation was found on the control surface, meaning that the F3B receptor binds not only to the specific target MMP-9 but also to some serum component. To further elucidate the origin of the unspecific binding, the most abundant proteins present in serum, albumin (HSA) and immunoglobulins G (hIg G), were independently tested on the aptasensor to evidence their possible contribution.

PBS solutions of HSA (526 μM, 35000 mg L^{-1}) and hIgGs (7 μM, 1120 mg L^{-1}) were tested both on specific (with F3B immobilized) and control surfaces (without F3B), showing that the main contribution is due to hIgGs (here tested for a 1:10 concentration with respect to the minimum serum level, ≈10000 mg L^{-1}), and confirming that it prevalently occurs on the F3B aptamer and only minimally on the sensor surface (Fig. S3). Thus an important matrix effect was found which imposed further assay optimization to allow MMP-9 detection in human speciments *i.e.* serum.

3.3. Dual recognition for selectivity enhancement

To circumvent this problem, we took advantage of a second aptamer, 8F14A, obtained by standard selection procedure against a MMP-9 fragment containing the catalytic domain. This aptamer was part of a family of strong MMP-9 binders characterized by a G-

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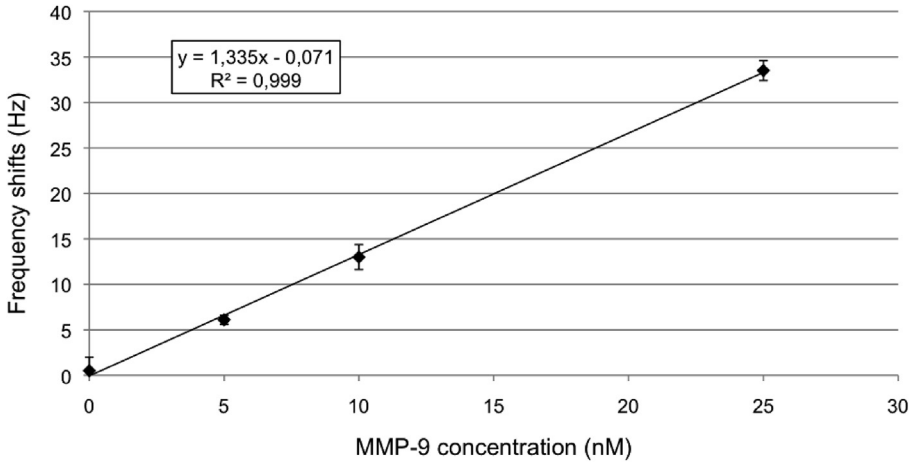

Fig. 2. Dose-response curve obtained on F3B aptamer with the direct method for MMP-9 detection in the range 0.1–25 nM in PBS buffer (n = 3 for each concentration point).

quadruplex structure, and does not compete with F3B for binding to MMP-9 when tested in solution (see paragraph 'Assessment of the sandwich strategies and negative controls' of Supplementary material, Figs. S4 and S5), a key condition for developing a sandwich assay.

Each step of the sandwich assay was optimized to find the best conditions in terms of 8F14A concentration (500 nM, Fig. S6), its contact time on MMP-9 (30 min, Fig. S7), and contact time of 1 μM streptavidin on 8F14A (10 min, Fig. S8 of Supplementary material), as well as possible unspecific interaction occurring on the biosensor surface (see 'Assessment of the sandwich strategy and negative controls' paragraph of Supplementary materials). The final F3B/MMP-9/8F14A/streptavidin adduct proved to be fully regenerable among measurements by using the regeneration solution (30 s), followed by equilibration in PBS buffer. The aptasensor performances obtained by the sandwich assay were finally compared to those obtained by the direct assay of MMP-9 on F3B (Fig. 3).

The mass enhancement due to the supra-molecular architecture built on the sensing surface led to a significant improvement of the recorded response, obtaining to scale MMP-9 concentration down to 5 pM (415 pg mL^{-1}), with a calculated detection limit of 1.2 pM (99.6 pg mL^{-1}), as displayed in Fig. 4. The high correlation of the low-concentration segment of the calibration curve (1–2500 pM), $R^2 = 0.997$, and the averaged percentage coefficient of variation (% CV), of 7%, demonstrated the high performance of the sandwich-based assay in standard solutions.

3.4. Microbeads treatment for IgG removal from serum

To verify that serum immunoglobulins represent the main source of unspecific binding on F3B (direct detection), commercial human serum was pre-treated with magnetic microparticles (2.8 μm) to eliminate immunoglobulins from the mixture, previously demonstrated to be effective [68]. Microbeads, coated with protein G or A, were thus used to remove IgG, and IgA/IgM/IgE/IgG, respectively. The comparative responses obtained on F3B aptasensor before and after 1:100 diluted serum pretreated with magnetic particles were thus evaluated. In particular, in the case of Dynabeads® G for IgG removal, a signal reduction of 65% was found,

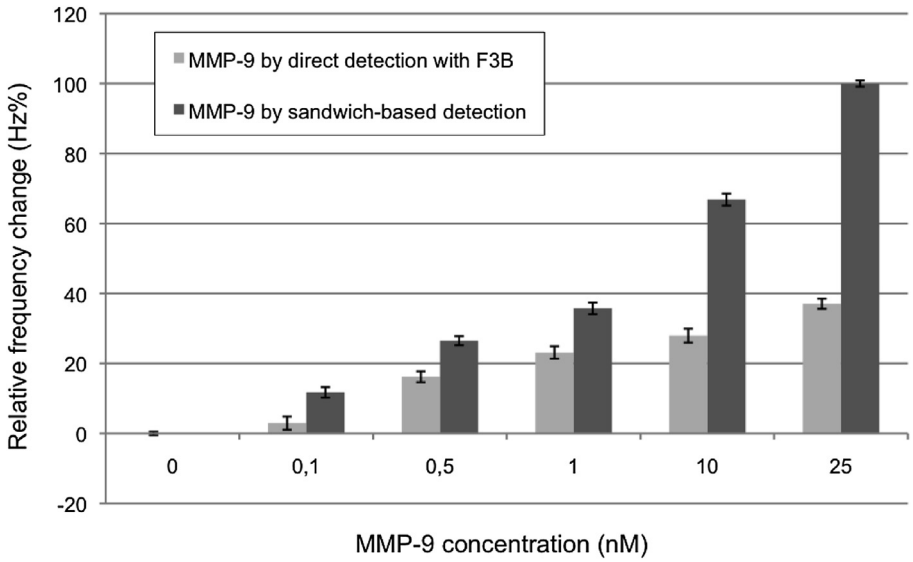

Fig. 3. Comparison between results obtained in HBS buffer by the direct detection of MMP-9 and the sandwich-like approach with 8F14A/streptavidin adduct. Delta shifts in frequency (Hz) are here expressed as relative change compared to the maximum shift observed at 25 nM by the sandwich.

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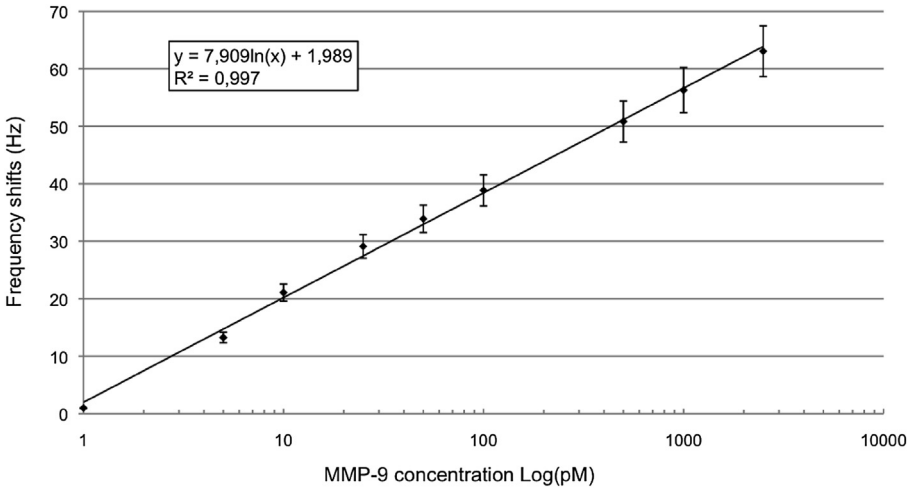

Fig. 4. Semi-logarithmic plot of MMP-9 calibrated in buffer at low concentrations (<2.5 nM) via sandwich assay, displaying the improved experimental detection limit, estimated to be 1.2 pM.

whilst in case of Dynabeads® A for IgA, IgM, IgE, IgG removal, the reduction of 71% of the unspecific binding was obtained (Fig. 5).

These results confirmed that the unspecific binding of serum on F3B can be minimized by immunoglobulins removal by simple and fast pretreatment with affinity chromatography with protein G or A. Moreover, the unspecific signal recorded on undiluted serum due to HSA became negligible at 1:100 sample dilution ($\Delta f < 1$ Hz after 1:100 dilution).

Therefore, the residual signal on F3B after IgA/E/M/G removal ($\Delta f = 6.43 \pm 1.66$ Hz), should be attributable to MMP-9. To prove this, the secondary recognition by the F14A/streptavidin adduct was carried out on the all three samples, revealing that the relative signals were independent from immunoglobulins concentration (Fig. 5). In fact, signals elicited by the secondary binding (light grey) in the tested samples are very similar.

In particular, the signal inferred from serum treated for IgA/E/M/G removal was 26.43 ± 2.06 Hz, corresponding to 21.98 ± 1.01 pM MMP-9 in diluted serum (2.20 ± 0.01 nM in undiluted serum), as calculated by the sandwich-based calibration of Fig. 4. Therefore, by this approach the interfering effect is bypassed and does not affect MMP-9 measurement.

In support of this conclusion, two additional controls were successfully performed: a) 1 nM (83 ng mL^{-1}) MMP-9 in PBS was subjected to the same procedure and tested before (56.26 ± 1.66 Hz) and after microbeads treatment (54.32 ± 2.73 Hz) to check that microbeads do not affect MMP-9 concentration in the sample (*i.e.* analyte depletion) and possibly its detection; b) serum samples 100-folds diluted and then pre-treated or not with Dynabeads® A (each in triplicate) were separately added on the F3B aptamer and then revealed by the sole secondary recognition to verify that the unspecific binding of serum immunoglobulins on F3B does not affect the final reading of MMP-9 by 8F14A/streptavidin. In fact, binding sites of F3B involved in the unspecific binding with serum immunoglobulins, could at the same time interfere with the specific binding with MMP-9 molecules, leading to a lower binding capability of the receptor toward its specific target. Controls confirmed that pre-treatment with Dynabeads® A does not affect MMP-9 content and that the secondary detection of the protein on F3B aptamer is not influenced by the presence of immunoglobulins, presumably because when the specific target is present in solution together with IgGs, the F3B aptamer assumes its preferred and energetically favored folding apted to selectively bind MMP-9, thus excluding other unspecific binding.

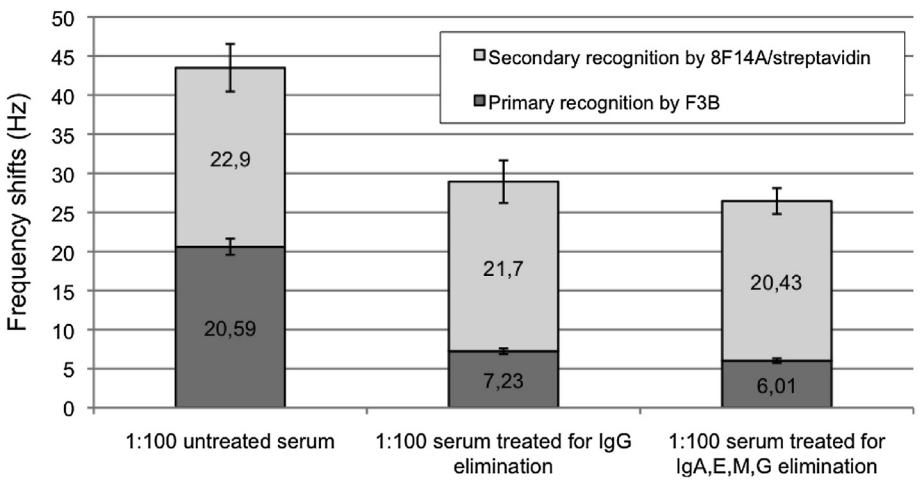

Fig. 5. Responses obtained before and after pretreatment of serum (1:100 diluted) with microbeads for IgG removal and for IgA/M,/E/G removal. The overall signal is here split in two, the first refers to F3B binding (dark grey) and the second one to the secondary recognition by 8F14A/streptavidin adduct (light grey).

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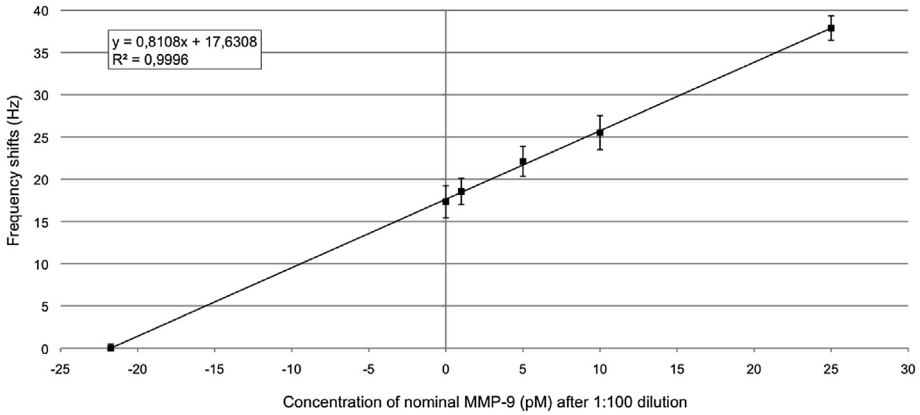

Fig. 6. Multiple standard addition method for MMP-9 quantification by secondary recognition in untreated 1:100 serum, in which MMP-9 spikes span from 1 to 25 pM.

3.5. Detection of MMP-9 by 8F14A directly in diluted serum bypassing microbeads pretreatment

Last, untreated serum spiked with different MMP-9 concentrations (0.1, 0.5, 1.0, and 2.5 nM) was serially tested in triplicate (after 1:100 dilution) on a fresh-prepared crystal carrying the F3B aptamer.

In the meantime, 8F14A aptamer and streptavidin were co-incubated in SP buffer and then added on the pre-formed F3B/MMP-9 complex obtained after spiked sample addition. In this experiment, frequency shifts considered are referred to the sole secondary binding of 8F14A/streptavidin, since this contribution is ascribable to the sole MMP-9 content and allows its detection by avoiding immunoglobulins removal. The dose—response obtained was linear within the tested range, allowing the fitting and the estimation of the unknown concentration of MMP-9 in undiluted serum through the multiple standard additions (Fig. 6). The target quantification in non-spiked serum ($\Delta f = 17.63 \pm 1.21$ Hz) led to an MMP-9 content of 2.17 ± 0.05 nM (SDint = 0.20, SDslo = 0.16), i.e. 180.11 ± 4.15 ng mL^{-1} in undiluted serum (calculated on the fitting of Fig. 6), independently from the medium effect possibly present on the F3B aptamer. This value is in line with mean MMP-9 values in healthy subjects and is in very good agreement with the content inferred from serum treated with Dynabeads® A and tested by the sandwich strategy (2.20 ± 0.01 nM, by linear equation of Fig. 4). The averaged reproducibility (%CV) obtained was about 8%.

To estimate the sensitivity of this approach, we finally tested serum dilutions from 1/50 to 1/400 (in PBS buffer) obtaining the linear trend reported in Fig. 7, from which we inferred 6.8 ± 0.25 pM (564 ± 21 pg mL^{-1}) as calculated detection limit of MMP-9 in untreated serum.

4. Conclusion

MMP-9 is fast attracting the interest of clinical analysts and analytical biochemists due to its role as highly informative biomarker in early cancer diagnosis and other diseases. Up to now, very few attempts in developing biosensor-based methods for MMPs detection in real matrices are reported in literature. In this paper, a piezoelectric-based aptasensor for the detection of MMP-9 was developed for the fist time. Detection limit at picomolar level (100 pg mL^{-1}) is obtained in buffer solution by applying a dual aptamer-based strategy. This result is of interest in comparison with more expensive, time consuming and complex methods

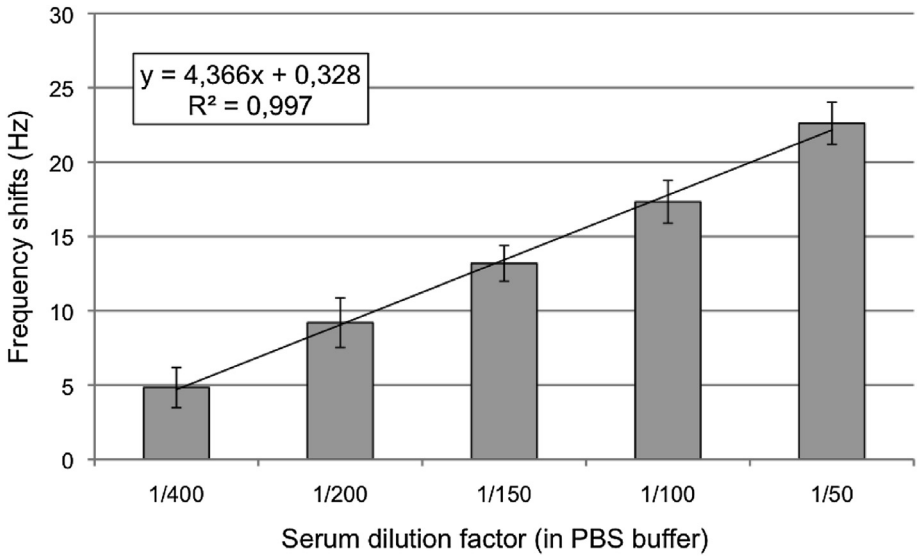

Fig. 7. Frequency shifts relative to MMP-9 detection in untreated serum except dilution, in the range 1/50-1/400 in PBS buffer. Signals reported are referred to the sole secondary recognition by 8F14A/streptavidin adduct on MMP-9 already bound on the F3B primary aptamer.

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reaching similar detection limits in buffer solutions. The mass enhancement due to the sandwich assay was a key step both from the sensitivity and the selectivity point of view. On human undiluted serum, a matrix effect was found and attributed to the direct unspecific interaction of the primary aptamer with immunoglobulins, which can be removed by a single serum pretreatment with magnetic microbeads coated with Protein A or G. Quantification of MMP-9 was carried out on human serum, both pretreated and untreated. In the latter case, MMP-9 quantification was conducted by multiple standard additions method assisted by the selectivity of the 8F14A aptamer. Good agreement for MMP-9 quantification was found with good reproducibility (8%). These findings open new perspectives in the application of real-time and low cost aptasensing to molecular analysis of MMP-9 for the anticipated diagnostics. The progress can be foreseen in the parallel, real-time analysis of different clinical markers directly in biological media, as very recently reviewed by our group [69].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2015.07.009.

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