Analytica Chimica Acta 1153 (2021) 338280

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

One-step, wash-free, bead-based immunoassay employing bound-free phase detection

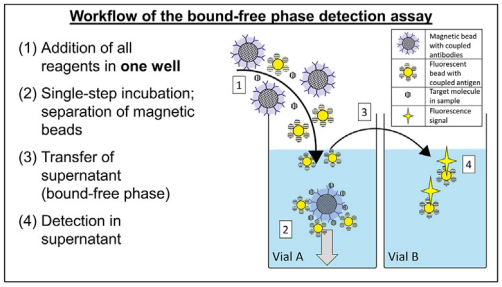
Benita Johannsen [a, **], Michal Karpíšek [b, c], Desirée Baumgartner [d], Vanessa Klein [a, 1], Nagihan Bostanci [e], Nils Paust [a, d], Susanna M. Früh [a, d], Roland Zengerle [a, d], Konstantinos Mitsakakis [a, d, *]

[a] Hahn-Schickard, Georges-Koehler-Allee 103, 79110 Freiburg, Germany
[b] BioVendor—Laboratorní Medicína a.s., Research & Diagnostic Products Division, Karasek 1767/1, Reckovice, 62100, Brno, Czech Republic
[c] Masaryk University, Faculty of Pharmacy, Palackeho Trida 1946/1, 61242, Brno, Czech Republic
[d] Laboratory for MEMS Applications, IMTEK—Department of Microsystems Engineering, University of Freiburg, Georges-Koehler-Allee 103, 79110, Freiburg, Germany
[e] Section of Periodontology and Dental Prevention, Division of Oral Diseases, Department of Dental Medicine, Karolinska Institutet, Alfred Nobels Allé 8, 14104, Huddinge, Stockholm, Sweden

HIGHLIGHTS

- Bound-free phase detection (BFPD) assay is a wash-free heterogeneous immunoassay.
- One-step versatile assay compatible with diverse complex fluids (saliva, serum).
- Demonstration of BFPD multiplexing capacity using 3 oral markers of periodontitis.
- CRP BFPD assay verified with European Certified Reference Material.
- Adaptation of assay detection range through the magnetic/fluorescent beads ratio.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 December 2020
Received in revised form
14 January 2021
Accepted 31 January 2021
Available online 5 February 2021

Keywords:
Immunoassay
Bound-free phase

ABSTRACT

We present a simple and fast one-step heterogeneous immunoassay, with performance characteristics that can enable easy and versatile adaptation to miniaturized, automated point-of-care systems. This novel analytical method uses magnetic and fluorescent beads as capture and detection agents respectively. Its main feature is the measurement of the fluorescent signal in the bound-free phase for (semi-) quantitative detection of analytes. Thus, no washing is required and the workflow consists only of sample and reagent supply, incubation, separation and detection. The immunoassay concept is demonstrated with C-reactive protein (CRP), a systemic inflammation marker. CRP in only 5 μL of undiluted serum was measured in the range 20–140 mg L^{-1} (includes clinically relevant cut-off values). The limit of detection (LOD) was 22.1 ± 6.3 mg L^{-1} (incubation 15 min). A CRP certified reference material was measured on five different days. Intra- and inter-assay coefficients of variation were 4.6 ± 1.9% and 5.6% respectively.

* Corresponding author. Hahn-Schickard, Georges-Koehler-Allee 103, 79110, Freiburg, Germany.
** Corresponding author.
E-mail addresses: benita.johannsen@hahn-schickard.de (B. Johannsen), konstantinos.mitsakakis@hahn-schickard.de (K. Mitsakakis).
[1] Current address: Eurofins Genomics Germany GmbH, Anzinger Str. 7a, 85560 Ebersberg, Germany.

https://doi.org/10.1016/j.aca.2021.338280

Micro/nanoparticles
Oral biomarkers
Inflammation
Saliva

To demonstrate the compatibility of the assay concept with additional matrices and concentration ranges, three oral inflammation markers, namely matrix metalloproteinases 8 and 9 (MMP-8, MMP-9) and tissue inhibitor of metalloproteinases 1 (TIMP-1), were measured in saliva in the ranges 0.47 −30 ng mL^{-1} for MMP-8 and MMP-9, and 0.69−44 ng mL^{-1} for TIMP-1. LODs were 0.24 ng mL^{-1}, 0.38 ng mL^{-1} and 0.39 ng mL^{-1} respectively (incubation 20 min). Multiplexing capacity of the assay concept was also shown with these markers. The demonstrated excellent reproducibility of the results, combined with the versatility and low complexity of the introduced immunoassay concept, make it an attractive candidate for applied analytical chemistry and automated point-of-care testing.

1. Introduction

Immunoassays are an important tool in diagnostics [1–3]. They provide very specific results due to the antibody-antigen reaction, which follows a lock and key principle [2], and are used in a diverse range of fields, from analytical chemistry to clinical diagnostics, and in application areas like cardiovascular, autoimmune or infectious diseases, oncology, oral health, food analysis and environmental safety [1–3]. Application-dependent requirements have guided the development of a large variety of immunoassay types and detection technologies available at research and commercial level [1,3,4]. Many of the aforementioned applications strongly favor a move towards de-centralized testing [3,5]. Especially in healthcare applications, diagnosis at such settings poses some specific requirements. Analysis must be rapid due to the nature of some diseases, e.g. sepsis, tropical fever and acute respiratory tract infections, and antibiotic prescriptions (turnaround times > 30 min are barely acceptable). The analysis procedure must be as easy as possible for the user, meaning that all necessary reagents must be pre-stored ideally at room temperature. Another important design issue with immunoassays is their versatility. In particular, as different application fields use different sample matrices (e.g. blood, saliva, urine), and require more than one biomarker for a diagnostic decision [4–8], an immunoassay should be compatible with different sample matrices and its format should allow multiplexing.

Enzyme-linked immunosorbent-assay (ELISA), which uses an enzymatic reaction to detect proteins, is a state-of-the-art technology for many diagnostic applications that require protein quantification [1,2,4,5]. Using particles as a solid phase (heterogeneous bead-based ELISA) shortens the procedure in terms of incubation duration, allows the miniaturization and integration of immunoassays into microfluidic systems, simplifies the washing procedure [5,9–11], and contributes to the aforementioned requirement of full integration. Most significantly, the use of magnetic particles adds the advantage of easy and efficient separation using an external magnet.

Nevertheless, magnetic bead-based heterogeneous ELISAs still require one or more washing steps [5,9,12–15], with the disadvantages that more steps are required and more liquid reagents are involved and must be pre-stored to provide integration in a point-of-care (PoC) system. Furthermore, washing steps may not always remove all unbound detection labels. Compared to heterogeneous assays, the homogeneous assays are by definition wash-free [4,11,16–18], but most of them require special equipment or special detection methods [16], and a common challenge is the high background noise, which reduces the sensitivity of the assays [17]. The introduction of fluorescent labels to magnetic particle-based assays can replace the indirect labeling of an enzymatic reaction with a direct label bound to the detection protein. This can lead to a reduced number of reagents and assay/washing steps, enable multiplexing [5,8,19], and offer high sensitivity [4,10,11,20]. Nevertheless, the assay formats, even of commercial systems, are difficult to transfer to PoC settings due to long turnaround times (incubation and detection) or because they require special benchtop processing and readout equipment not suitable for standard microtiter plate readout [8,19,21–28].

In this context, and taking into account the key aforementioned requirements for easy integration into a rapid PoC system, we developed a competitive, one-step, two-bead-based, wash-free, heterogeneous immunoassay that detects the analyte in the bound-free phase (bound-free phase detection assay - BFPD). It utilizes only one liquid reagent, the assay buffer, as well as magnetic particles as solid phase for capture and fluorescent particles for detection. The bound-free phase contains the reagents and surplus fluorescent beads that did not react with the solid phase, after separation from the latter. The assay requires only one incubation step with a duration of 15–20 min (protein dependent). The importance of the newly-developed method is the combination of structural (magnetic and fluorescence particles) and functional (heterogeneous, wash-free nature, detection in bound-free phase) features that enable a rapid, versatile, easily adaptable, platform-independent assay realization, with drastically reduced number of assay steps that can be easily integrated into automated liquid handling platforms, as well as into PoC systems where automation is essential.

In this work we describe the technical features of this immunoassay method and we provide its analytical characterization. We also demonstrate its versatility through a proof-of-concept operation with two different sample matrices (serum and saliva); two different concentration ranges (mg L^{-1} and ng mL^{-1}); single, duplex and triplex configurations; and four different biomarkers. In particular, we tested our assay with C-reactive protein (CRP), a major marker of inflammation which circulates in the blood [29,30], as well as matrix metalloproteinases 8 and 9 (MMP-8, MMP-9) and tissue inhibitor of metalloproteinases 1 (TIMP-1) [31], which are biomarkers (among others) that are reported to be related to periodontitis [32,33], a major oral disease affecting the tissue that surrounds the teeth and causes gum inflammation. These specific markers and matrices were selected in order to demonstrate the analytical performance and versatility of our BFPD assay (rather than their clinical significance and diagnostic relevance to systemic and periodontal inflammation), in application areas where recently published clinical work indicates a correlation between oral, systemic diseases and inflammation [34–36]. The field of oral health and early diagnosis of periodontal disease is gaining increased attention as it is a public health issue [37–39]. Therefore, the combination of detecting systemic and periodontal inflammation could be a promising approach for early risk detection, prevention of additional systemic diseases like Alzheimer's, cardiovascular diseases and diabetes [40–43], and for personalized monitoring [34,36,44,45].

2. Materials and methods

2.1. Preparation of proteins for saliva assays

Antigens for the MMP-9 and TIMP-1 assays were produced by BioVendor (Czech Republic) with a mammalian transient expression system using HEK-293 cells and an in-house vector pBV1. After molecular cloning, a purification using affinity chromatography was conducted. Quality control of the MMP-9 and TIMP-1 antigens was done with a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12% SDS PAGE). The antigen for the MMP-8 assays was purchased from Sino Biological (China). Antibodies for the MMP-8 (monoclonal), MMP-9 (mono- and polyclonal were both tested; polyclonal was selected) and TIMP-1 (polyclonal) assays were produced using the hybridoma technology following immunization of BALB/c mice with recombinant proteins. Production of polyclonal antibodies was performed in cooperation with a contractual partner. Production of the antibodies for all the saliva markers received a favorable evaluation by a competent authority in accordance with the Act No. 246/1992 Col. of the laws of the Czech Republic. Monoclonal antibodies were purified on a column with immobilized protein G (BioRad, USA). The specific polyclonal antibodies were purified by immunoaffinity chromatography. Antibodies were stored at 4 °C and their functionality was tested in immobilized antigen and sandwich ELISA. The specificity of each antibody was tested in indirect ELISA across MMP-8, MMP-9 and TIMP-1 recombinant proteins. No response to cross-reactivity was observed even at the high concentrations (10 μg mL^{-1}). Furthermore, the three proteins of our study were tested for specificity also against other proteins of the MMP family (i.e., MMP-2, MMP-3), and again without observing any cross-reactivity.

2.2. Preparation of saliva assay standards

The saliva samples used for the preparation of multi-analyte standard were collected under approval from the local ethical committee. Concentrations of MMP-8, MMP-9 and TIMP-1 were determined using an ELISA (BioVendor) and were mixed with the saliva to reach an appropriate analyte value. Mixed saliva samples were filtered with a pore size of 0.4 μm (Millipore, USA) and centrifuged at 10,000×*g* for 10 min. The supernatant was subsequently lyophilized and stored at −80 °C. Before use, the lyophilized standards were rehydrated to the highest concentration and then diluted to reach the concentration range of the calibration curves.

2.3. Preparation of tosylactivated magnetic beads for the CRP assay

Magnetic beads for the CRP assay with a tosylactivated surface and a diameter of 2.8 μm (Dynabeads, M − 280) were purchased from Thermo Fisher Scientific (USA). Anti-human polyclonal CRP antibodies (A80-125A; Bethyl, USA) were coupled on the surface of the magnetic beads. The manufacturer provided information that these antibodies react specifically with human CRP, while they may cross-react with CRP from other species. The coupling of these antibodies on the surface of the magnetic beads was achieved by slightly adapting the manufacturer's protocol. After incubating the antibodies onto the bead surface and incubating the beads in phosphate-buffered saline (PBS) at pH 7.4 with 0.5% Bovine Serum Albumin (BSA) (Carl Roth, Germany) under rotation, an additional step for deactivating the surface reactive groups was introduced. Therefore, the tube was placed on a magnet for 2 min, the supernatant was discarded and 1000 μL of 50 mM ethanolamine (Carl Roth, Germany) in sterile 50 mM PBS (pH 8.0) was added. Incubation was performed at 37 °C for 1 h under rotation. Thereafter, the supplier's protocol was used. The beads were stored at 2.0% solid in storage buffer (PBS, 0.1% BSA, 0.03% Synperonic P84 (Sigma-Aldrich, USA), 0.05% Sodium azide (Carl Roth, Germany)) at 4 °C.

2.4. Preparation of carboxylated magnetic beads for the saliva assays

Magnetic beads with a carboxyl-activated surface (carboxylic acid groups on the surface) and a diameter of 2.8 μm (Dynabeads, M − 270) were purchased from Thermo Fisher Scientific. Capture antibodies (specific for MMP-8, MMP-9, TIMP-1) were coupled to the magnetic beads using the following protocol: 8.3 mg of beads were washed four times with 25 mM MES (2-(N-morpholino) ethanesulfonic acid) buffer (pH 6). For activation of the surface, a solution containing 25 mM EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride) (Thermo Fisher, USA) and 25 mM NHS (N-Hydroxysuccinimide) (Thermo Fisher, USA) was prepared in 25 mM MES buffer (pH 6). 1500 μL of the activation solution was added, followed by incubation at room temperature (RT) for 30 min under rotation. After three washing steps, an antibody solution (135 μL of the antibody, 237 μL of MES at pH 6.2) was added and incubated at RT for another 23 h. The reaction was terminated by adding 2 M glycine to a final concentration of 200 mM and the suspension was incubated at RT for 2 h. Afterwards the beads were washed three times with PBS and stored in PBS with 0.05% Thimerosal (2% solid) at 4 °C.

2.5. Preparation of fluorescent beads for the CRP assay

For the CRP assay, native CRP protein (C7907-26, 95−98%, highly purified, United State Biological, USA) was coupled to fluorescent beads (F8810, red (excitation (ex) 580 nm/emission (em) 605 nm), 0.2 μm, Thermo Fisher Scientific). An optimized protocol was developed, which did not require centrifugation after bead surface activation. 5 mg of beads was transferred to a tube and washed in 1000 μL of 25 mM MES buffer (pH 6.1). The supernatant was discarded after centrifugation (Eppendorf, 5415D, 16,100×*g*, max. 10 min). This procedure was performed twice. Surface activation was achieved by adding 125 μL of 8 mM NHS (Sigma-Aldrich, USA) and 125 μL of 8 mM EDC (Sigma-Aldrich, USA) after the washing buffer was discarded. After incubation at RT for 1 h under rotation, 70 μg of CRP protein was added. 25 mM MES buffer (pH 6.1) was added to give a final volume of 500 μL. The CRP proteins were incubated at RT for 2 h under constant rotation. Post-saturation of free reactive groups was conducted by adding 500 μL of PBS with 1.0% BSA. After incubation at RT for 30 min under rotation, 50 μL of 1 M ethanolamine in PBS (pH 8.0) was added to hydrolyze any still active groups. After two final washing steps with storage buffer, the beads were stored at 2.0% solid at 4 °C.

2.6. Preparation of fluorescent beads for saliva assays

Fluorescent beads for MMP-8 (F8807, dark red (ex 660 nm/em 680 nm)), MMP-9 (F8810, red (ex 580 nm/em 605 nm)) and TIMP-1 (F8811, yellow-green (ex 505 nm/em 515 nm)) were purchased from Thermo Fisher Scientific (USA). The fluorescence excitation and emission wavelengths of the fluorescent beads were chosen carefully in order to allow multiplexing without non-specific cross-talk. For all three, the same coupling protocol was used. 10 mg of the beads was transferred to a tube. For activation of the bead surface, 35 mM EDC and NHS were mixed 1:1 and 160 μL was added to the beads. After incubation at RT for 1 h on a shaker (600 RPM), MMP-8, MMP-9 or TIMP-1 antigen (250 μg) was added to the beads. MES buffer was added to give a total volume of 400 μL. The mixture was incubated at RT for 2 h on a shaker (600 RPM). After a desalting

step at RT for 2 h to remove MES, the bead surfaces were quenched with a 200 mM glycine solution and incubated overnight at 4 °C. The beads were stored in PBS at 4 °C.

2.7. BFPD CRP assay (singleplex)

Undiluted, CRP-free human serum (HyTest, Finland) spiked with CRP antigen (C7907-26, United State Biological, USA) was used to generate the calibration curves for the proof-of-concept of the BFPD assay. Sample dilution buffer from BioVendor (Czech Republic) was used as the assay buffer. A certified reference material (CRM) was purchased (European Commission—Joint Research Centre, Belgium; ERM-DA474/IFCC) for the method verification. The magnetic beads, fluorescent beads, serum and assay buffer (more details in Table 1) were incubated in a single step at 37 °C for 15 min on a shaker (1000 RPM, BioShake iQ, QInstruments, Germany). Afterwards, the bound phase was separated from the bound-free phase with a magnet (~2 min), and 50 μL of the bound-free phase was pipetted into a detection well. The fluorescence intensity of the bound-free phase was detected using a microtiter plate reader for the CRP assay (Spark M – 10, Tecan, Switzerland; for the specific filter properties, see Supplementary S1).

2.8. BFPD saliva assays (single- and multiplex)

The saliva assays (MMP-8, MMP-9 and TIMP-1) were incubated at 37 °C for 20 min and PBS was used as the assay buffer. For the multiplex saliva assays, all beads, assay buffer and samples were pipetted into the same well (more details in Table 1). There were no further changes in the amounts of magnetic or fluorescent beads compared to the amounts used in the singleplex experiments, and the total assay volume (76 μL during incubation) was also kept the same. After an incubation at 37 °C for 20 min, the bound phase was separated with a magnet and 50 μL of the bound-free phase was pipetted into a second well for readout on an Array Reader F-series (BioVendor Instruments, Czech Republic; for the specific filters and measurement parameters, see Supplementary S1).

3. Results and discussion

In this work we present the BFPD immunoassay for applicability in serum and saliva. For method verification we used the inflammation marker CRP, which is not only used to specify the severity of an infection, but also to support decisions to prescribe antibiotics and to monitor treatment responses [29,46–48]. The BFPD assay format that was developed and validated with CRP in human serum was then transferred to BioVendor (different assay handling and detection setup), that developed the saliva assays for MMP-8, MMP-9 and TIMP-1, which can be used for the evaluation of oral health [33,49–51]. These three biomarkers were used in order to demonstrate the versatility of the assay concept in terms of: (i) easy adaptation to other protein assays (even at 1000× lower concentration ranges) and measurement setups, thereby allowing third parties to develop and/or adapt BFPD assays; (ii) multiplexing; and (iii) expanding the application of the BFPD assay to other complex matrices such as saliva.

3.1. BFPD assay method description

The BFPD assay method in competitive format is illustrated in Fig. 1A. The assay components comprise magnetic and fluorescent beads as capture and detection agents respectively, as well as one assay buffer. These, together with the sample, are incubated in a single reaction well (one-step assay). Afterwards, the bound phase is separated with a magnet from the bound-free phase and a specific amount of the latter is transferred into a detection well, where the fluorescence intensity is quantitatively measured. Assay multiplexing is enabled by the introduction of multiple capture and fluorescent particles.

3.2. CRP assay and method verification

The CRP assay was optimized for a dynamic range that included in its linear range the cut-off values that various clinical studies [46,47] have described as crucial for decisions on whether to prescribe antibiotics. A certified reference material (CRM) was used for method verification. Its concentration was 41.2 mg L^{-1} (very near the CRP clinical cut-off value of some studies [46,47]) with an uncertainty of 2.5 mg L^{-1} (Supplementary S2). The CRM was measured on five different days with five repetitions per day [52] and the concentration was calculated based on the calibration curve shown in Fig. 1B (equation in Supplementary S3). Fig. 1C shows the CRP concentration of the CRM measured on five different days, with five repetitions each day, using the same standard curve obtained at the beginning of the five days in order to simulate a situation comparable to a PoC device, where the calibration curve is obtained batch-wise and stored in the device [53]. The results of the method verification show that the mean value of each measuring day was in the range of the CRM concentration (Fig. 1C). The mean value of CRM over all days was 40.5 ± 2.3 mg L^{-1}, deviating by only 1.7% from the expected reference value. The intra-assay coefficient of variation (CV) for the specific CRM concentration was calculated for each day, with an average over the five days of 4.6 ± 1.9%. The inter-assay CV was calculated from all data over the five days and was 5.6%. A single factorial analysis of variance (ANOVA) was conducted using the method described by Pum et al. [52] and Andreasson et al. [54] to obtain information on the measurement uncertainty. With the assumption of a normal distribution and a Gaussian factor of 2, the analysis resulted in a measurement uncertainty of 8.4% (for more detailed information on the ANOVA results see Supplementary S3). This shows that the assay was able to detect the CRM with a high precision and a variation below 6% using the same calibration curve, a short incubation duration, no washing steps and only 5 μL of undiluted serum.

Table 1
Reaction setup for BFPD assays. *amounts for the final setup used for both single- and multiplex experiments.

	CRP	MMP-8	MMP-9	TIMP-1
Amount of magnetic beads [μg]	230	160*	186*	160*
Amount of fluorescent beads [μg]	20	1.13*	0.23*	1.29*
Assay buffer	Sample dilution buffer	PBS	PBS	PBS
Volume of assay buffer [μL]	70	55	55	55
Sample matrix	Serum	Saliva	Saliva	Saliva
Volume of sample [μL]	5	21	21	21
Incubation duration [min]	15	20	20	20
Measured range	20–140 mg L^{-1}	0.47–30 ng mL^{-1}	0.47–30 ng mL^{-1}	0.69–44 ng mL^{-1}

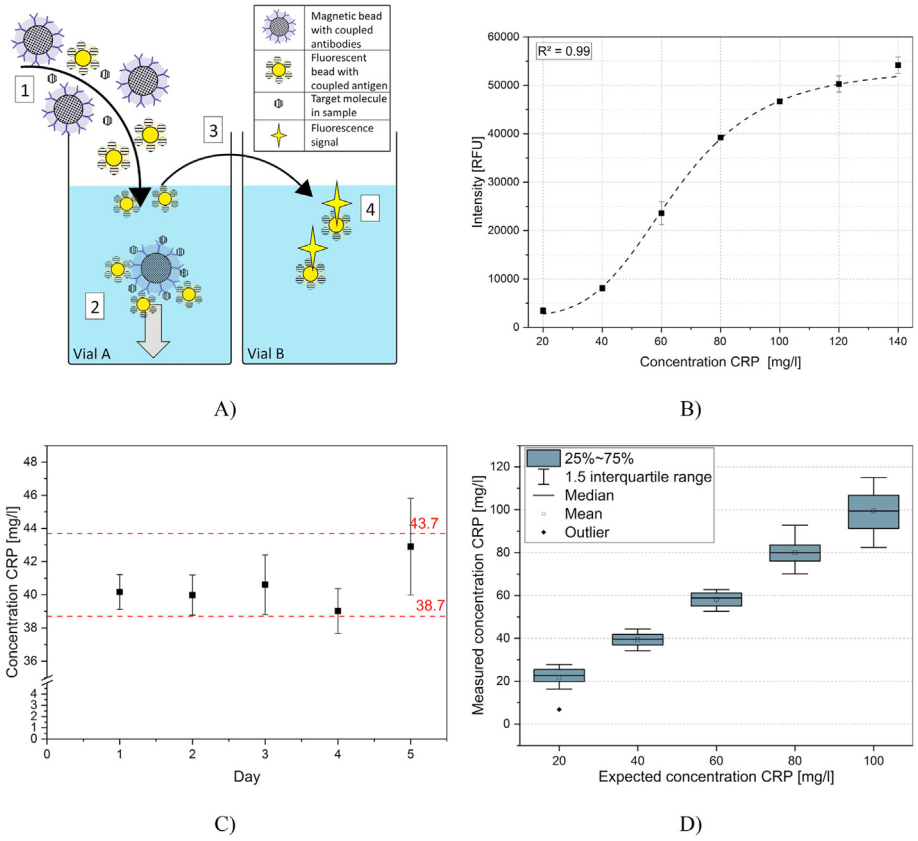


Fig. 1. A) Illustration of the workflow for the BFPD assay. 1) Addition of all reagents to the reaction vial at the same time. 2) Incubation at 37 °C for 15 min (CRP) or 20 min (MMP-8, MMP-9 and TIMP-1). Immune complex formation between the antibodies on the magnetic beads and the target molecule in the sample or the equivalent antigen on the fluorescent bead (competitive assay format). Separation of the complex using a magnet (keeping the magnetic beads in the first vial). 3) Transfer of only the supernatant (bound-free phase) into a second vial for 4) detection. B) Calibration curve (N = 3) for method verification with a certified reference material (CRM) (blank of 2070.7 ± 526.0 RFU) using a logistic sigmoidal fit (Supplementary S3). C) The CRM concentration measured on five different days and five times each day. The supplier's value is shown with a dashed line. D) Reproducibility assessment: The boxplots show the data over four days (N = 16, except the 20 mg L^{-1} concentration with N = 14; two samples were detected as out of range). The corresponding calibration curves are available in the Supplementary S4.

In case of a sample-to-answer CRP detection (starting from whole blood) washing would not be required either, as the serum (or plasma) separation can be done using centrifugation steps, which can be easily integrated in miniaturized systems, when it comes to PoC integration [55].

3.3. Reproducibility assessment

Since the CRM, with its commercially available concentration, could not be used over the entire CRP measurement range, we evaluated the reproducibility of the assay in the following way: spiked CRP-free serum standards were measured (N = 4 for each, all four calibration curves can be seen in Supplementary S4) in the same plate and on four different days, resulting in one calibration curve on each day. Spiked standards were then measured (N = 4 for each) in a second plate on each day ('expected concentration' in Fig. 1D) and their concentration was calculated based on the corresponding calibration curve of that day ('measured concentration' in Fig. 1D). The analysis showed a linear behavior between the measured and expected concentrations between 20 mg L^{-1} and 100 mg L^{-1} (median and mean values for the measured concentrations are summarized in Table 2). A scatter plot with a linear fit curve gave a slope of 0.98 with an R^2 of 0.95 (Supplementary S4). Only one outlier was measured, for 20 mg L^{-1}, which can be expected as the concentration 20 mg L^{-1} is close to the limit of detection (LOD) of 22.1 ± 6.3 mg L^{-1} and the limit of quantification (LOQ) of 25.3 ± 4.8 mg L^{-1} (equations [3,56] in Supplementary S4). The assay showed a reproducible and robust behavior, with the mean concentration CVs below 10%, except for 20 mg L^{-1} with 15.3% (concentration close to the LOD, equation in Supplementary S4). A concentration CV of 6.5 ± 3.1% in the linear range (40 mg L^{-1} to 100 mg L^{-1}) was calculated by using the mean concentration CVs of each day over four days. These data show that the assay had concentration CVs below 7% in the linear range over four days, and thus allows quantitative measurement of crucial, clinically relevant concentrations in this range [46,47]. The recovery rate of the spiked CRP concentration in the linear range, using the data obtained over four days, was 98.3 ± 2.6% (Supplementary S4). This is very close to 100% and in the acceptable range of 80–120%, according to Andreasson et al. [54]. This thorough analytical characterization shows that the BFPD assay, which implements a simple and fast

Table 2
Overview of the median and mean values of the different CRP expected concentrations for reproducibility assessment.

Expected concentration [mg L^{-1}]	Median [mg L^{-1}]	Mean [mg L^{-1}]
20	23.1	22.9
40	40.7	39.9
60	58.3	57.9
80	80.0	80.3
100	99.5	98.9

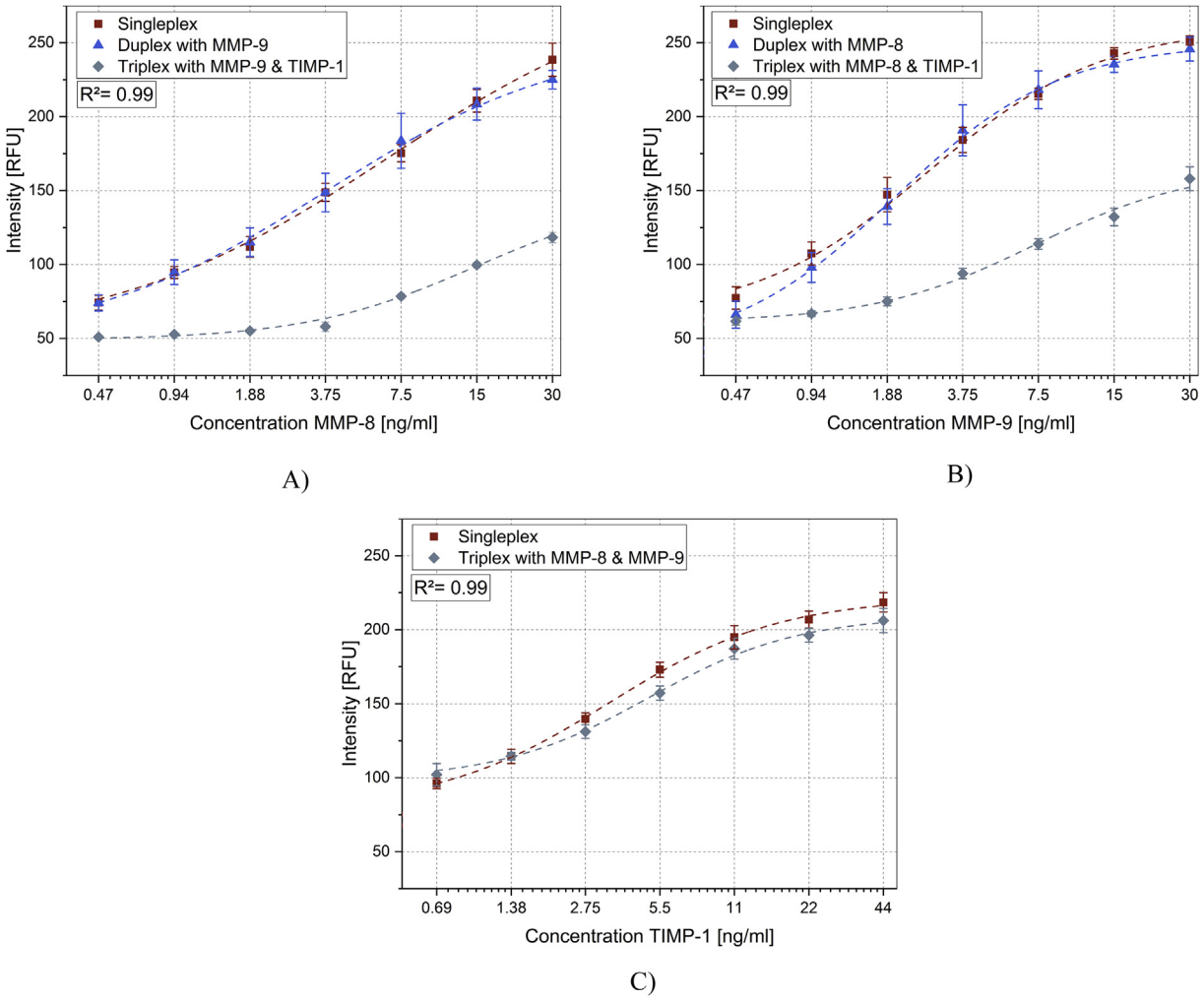


Fig. 2. A) Calibration curve for MMP-8 measured in singleplex (N = 6), duplex (N = 8) together with MMP-9 (where all MMP-8 and MMP-9 magnetic and fluorescent beads are incubated in the same well) and triplex (N = 5) together with MMP-9 and TIMP-1 (where all MMP-8, MMP-9 and TIMP-1 magnetic and fluorescent beads are incubated in the same well). B) Calibration curve of MMP-9 measured in singleplex (N = 6), duplex (N = 8) together with MMP-8 and triplex (N = 5) together with MMP-8 and TIMP-1. C) Calibration curve for TIMP-1 measured in singleplex (N = 6) and triplex (N = 5) together with MMP-8 and MMP-9.

workflow, exhibits excellent reproducibility and overall performance in the absence of any washing steps and in a complex sample like serum.

3.4. Salivary marker assays

There are several biomarkers that are related to oral health, for example, the active form of MMP-8 that is already used in PoC applications for detection of periodontitis [57,58]. In addition, literature reports the relation of MMP-8, MMP-9 and TIMP-1 (either alone, or in combination) with periodontitis or gingivitis [6,7,49–51,59,60]. We chose to address these three markers, from the analytical perspective in order to use them as 'models' and demonstrate (i) the multiplexing capacity of our newly-developed assay concept as well as (ii) the compatibility with a second complex matrix like saliva, in addition to serum. Given that even after centrifugation and filtering, saliva still contains ~5500 proteins [61], demonstrating that the BFPD assay is compatible with this sample matrix and in a wash-free manner, renders it a strong candidate for saliva-based diagnostics in general, which is a rapidly increasing field, even for infectious diseases [62,63], due to the non-invasive nature of saliva. In case of integrating the current assay configuration into saliva-based sample-to-answer analysis, there are also methods developed to avoid washing even in the sample treatment step, therefore the whole analysis could be performed in a wash-free way [64].

Fig. 2 shows the comparisons between the singleplex, duplex and triplex measurements. In all three configurations, all three target molecules were present in the reaction well. In singleplex, duplex and triplex assays, the magnetic/fluorescent beads of one, two or three markers were simultaneously incubated in the reaction well. The singleplex and duplex calibration curves for MMP-8 and MMP-9 were comparable (Fig. 2A and B). The triplex calibration curves showed an increase in the LOD (in comparison to the singleplex ones) of: 3.7× for MMP-8; 3.5× for MMP-9; and 2.6× for TIMP-1 (Table 3). Furthermore, the triplex calibration curves for MMP-8 and MMP-9 exhibited a reduced signal of the measured concentration when the TIMP-1 magnetic/fluorescent beads were present (Fig. 2A and B) than the single/duplex calibration curves (when the TIMP-1 magnetic/fluorescent beads were absent). On the contrary, the triplex calibration curve for TIMP-1 was in good agreement with its singleplex calibration curve (Fig. 2C). This can presumably be attributed to known biological regulation mechanisms, in which TIMP-1 acts as an inhibitor for MMPs by forming

Table 3
Comparison of the singleplex (s), duplex (d) and triplex (t) assay configuration performance for the three oral markers (MMP-8, MMP-9, TIMP-1) by calculating the LOD and LOQ values (Sigmoidal fit curve equations in Supplementary S5).

	MMP-8			MMP-9			TIMP-1		
	s	d	t	s	d	t	s	d	t
LOD [ng mL^{-1}]	0.24	0.37	0.89	0.38	0.35	1.32	0.39	–	1.03
LOQ [ng mL^{-1}]	0.34	0.48	1.20	0.53	0.48	1.59	0.49	–	1.44
Blank [RFU]	49.8 ±5.2	46.5 ±10.2	47.5 ±2.6	63.8 ±1.9	40.5 ±2.5	62.6 ±2.2	71.6 ±4.9	–	89.0 ±4.1

complexes with them [65,66]. Therefore, we presume that in the triplex assay: (i) the epitopes of MMP-8 and MMP-9 are not captured by their corresponding magnetic beads when the former are within MMP-TIMP complexes, leading to a reduced detection of MMP-8 and MMP-9 in the triplex case (Fig. 2A and B); whereas (ii) the epitope of TIMP-1 is captured by the TIMP-1 magnetic beads not only when it is free but also when it is in the MMP-TIMP complexes, leading to negligibly reduced detection in the triplex case (Fig. 2C). This different behavior may also be explained by the different competition and binding kinetics of TIMP-1 antibody/antigens compared to those of the MMPs. For example, with the TIMP-1 polyclonal antibodies, different epitopes might be detected, and therefore the reaction capacity and kinetics of the assay in triplex format might not be as affected by the formation of MMP-TIMP complexes as in the case of MMP antibodies. Further and more detailed analysis of the biomolecular interactions will be necessary for clarification, but this is not examined in this work, which focuses on demonstrating the capacity of the assay *per se*, rather than exploring specific biomolecular interaction mechanisms. In this context, it is important that in the triplex configuration, the assay showed high reproducibility, as shown by the low mean signal CVs of the triplex measurements: 2.8 ± 1.2% for MMP-8; 3.8 ± 0.8% for MMP-9; and 3.9 ± 1.6% for TIMP-1 (Fig. 2A-C triplex measurements). Thus, it is possible to use the triplex assay for a fast detection of all three markers after adapting the assay to the relevant detection range for the respective application. It should also be noted that our measurement setup does not contain simply spiked markers in a buffer, but our measured standards are reconstituted lyophilizates of actual, pooled saliva. Therefore, our results represent realistic conditions, like the interaction between MMPs and TIMP-1, which is reflected in the reduced signal in the case of triplex assays.

This adaptation of the detection range (e.g. here as shift from high to low concentrations) can be accomplished through the ratio of the absolute amounts of the magnetic and fluorescent beads. Fig. 3A and B show the calibration curves (measured as singleplex) for MMP-8 and MMP-9. They could be tailored (shifted) and the dynamic range could be controlled by changing the ratio of the magnetic and fluorescent bead amounts. Total volume, incubation duration and temperature, as well as the sample/standard volumes, were kept the same. Before the shift, for both MMP-8 and MMP-9, the absolute amounts of magnetic and fluorescent beads were 105 μg and 0.17 μg respectively. The absolute amounts for the calibration curves after the shift (oral calibration curves with a detection range between 0.47 ng mL^{-1} and 30 ng mL^{-1}) are given in Table 1. The optimization of the calibration curve (after the shift) led to a ~20× lower LOD for MMP-8, from 4.62 ng mL^{-1} to 0.24 ng mL^{-1} (Fig. 3A). For MMP-9 the LOD was ~ 5× lower, from 1.92 ng mL^{-1} to 0.38 ng mL^{-1} (Fig. 3B). Such parametrization simplifies the process during development and highlights the analytical importance of using two beads in our immunoassay concept. In principle, our assay concept has such flexible configuration that the fluorescent agent could also be quantum dots or fluorophores with high quantum yield, as long as it can be dispersed in the assay buffer and remain in the bound-free phase (supernatant) after magnetic bead separation. However, the selection of beads as fluorescence agents is additionally highly advantageous because it enables the aforementioned parametric tailoring of each assay's analytical performance and detection range by means of the ratio of these two bead amounts. A further advantage of selecting beads as fluorescent agents and therefore using two types of beads is the fast incubation due to the high surface to volume ratio of the micro/nanoparticles and the faster kinetics of the antibody-antigen reaction [1,4,11], which appears to be independent of the measured order of magnitude of the concentration (15 min and 20 min incubation times for mg L^{-1} and ng mL^{-1} concentration ranges, respectively).

4. Conclusions and outlook

In this work we developed the BFPD assay, a bead-based, competitive heterogeneous immunoassay method whose main features are the rapid incubation time, the use of two beads for enhanced mixing, capture and detection, the latter taking place in the bound-free phase and without the need for any washing steps. We developed assays for four different protein markers, namely CRP and three saliva markers of periodontitis, MMP-8, MMP-9 and

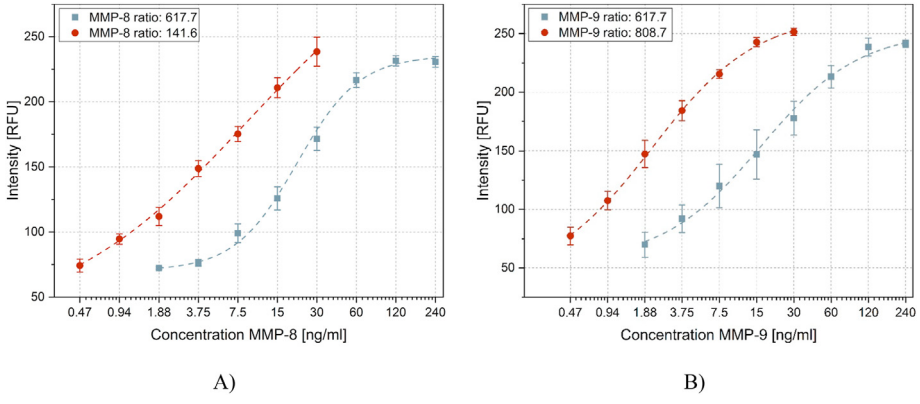


Fig. 3. The shift of the calibration curve from high to low concentrations was achieved by changing the ratio between the magnetic and fluorescent bead amounts. A) The calibration curve shift for MMP-8. The measurements before and after the shift were conducted on three different plates, with a total of N = 6 in both cases. The blank before and after the curve shift was 73.21 ± 2.9 RFU and 49.8 ± 5.2 RFU, respectively. B) The calibration curve shift for MMP-9, with a similar experimental setup (measurements on three different plates before and after the shift, with a total of N = 6 in both cases). The blank before and after the curve shift was 48.9 ± 2.9 RFU and 63.8 ± 1.9 RFU, respectively.

TIMP-1. For the three latter, we also successfully demonstrated the assays in duplex and triplex format. We evaluated the analytical features of the assay and optimized its performance. The versatility and easy adaptability of the BFPD assay was shown by means of: compatibility with two different complex matrices (serum, saliva); suitability for measurement in concentration ranges across three orders of magnitude (mg L^{-1} range for CRP; ng mL^{-1} range for saliva markers); and easy transfer and adaptation to diverse assay handling and detection instruments. The importance of the short incubation duration (15 min for the CRP and 20 min for the saliva markers) and the very few and easy assay steps of this technology is that it gains the potential to be adopted in key applications where time-to-result is critical. The method was also verified by measuring a certified reference material for the CRP assay and showed intra- and inter-assay CVs below 6%, which makes it suitable for quantitative measurements in the linear range. The assay reached a LOD of less than 0.5 ng mL^{-1} in a competitive format, even in a complex matrix like saliva. The assay is simple to conduct manually because of the easy handling and reduced number of steps, a consequence of no washing being required for this one-step approach. The two-bead principle makes this assay also highly suitable for automated systems that use microfluidic cartridges. As a next step, we intend to demonstrate the simplified integration and automation of the developed assays on a PoC microfluidic system. Overall, the BFPD assay retains the advantages of ELISA and bead-based assays whilst adding the key features of rapidity, wash-free operation, detection in the bound-free phase, and simplified multiplexing capability. This makes it highly suitable for a broad number of applications including integrated analysis, diagnosis and monitoring.

CRediT authorship contribution statement

Benita Johannsen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, preparation, Visualization, Supervision. **Michal Karpíšek:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - review & editing, Project administration, Funding acquisition. **Desirée Baumgartner:** Conceptualization, Methodology, Investigation, Writing - original draft, preparation. **Vanessa Klein:** Conceptualization, Writing - review & editing. **Nagihan Bostanci:** Writing - review & editing, Supervision, Project administration, Funding acquisition. **Nils Paust:** Writing - review & editing, Supervision. **Susanna M. Früh:** Writing - review & editing, Supervision. **Roland Zengerle:** Writing - review & editing, Supervision. **Konstantinos Mitsakakis:** Writing - original draft, preparation, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was funded by the European Union's Horizon 2020 research and innovation programme under grant agreement No 633780 ('DIAGORAS' project) and by the project 'Respiotic' (E! 113595), which is carried out within the framework of the European funding programme 'Eurostars'. The German partner of Respiotic (E!113595) was funded by the Federal Ministry of Education & Research.

The authors acknowledge Dr. Jürgen Klepp for his valuable support during the critical first steps of the development. We also would like to thank the DIAGORAS project consortium for the constructive feedback during our meetings.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.338280.

References

[1] J. Luong, S.K. Vashist (Eds.), Handbook of Immunoassay Technologies: Approaches, Performances, and Applications, Academic Press an imprint of Elsevier, London, 2018.
[2] R. Hnasko (Ed.), ELISA: Methods and Protocols; Springer Protocols 1318, Humana Pr, New York, 2015.
[3] R. O'Kennedy, C. Murphy, Immunoassays: Development, Applications and Future Trends, Pan Stanford Publishing, Milton, 2017.
[4] E.P. Diamandis, T.K. Christopoulos, Immunoassay, Academic Press, San Diego, 1996.
[5] C.T. Lim, Y. Zhang, Bead-based microfluidic immunoassays: the next generation, Biosens. Bioelectron. 22 (2007) 1197–1204.
[6] I.T. Gug, M. Tertis, O. Hosu, C. Cristea, Salivary biomarkers detection: analytical and immunological methods overview, Trac. Trends Anal. Chem. 113 (2019) 301–316.
[7] N. Spielmann, D. T. Saliva Wong, Diagnostics and therapeutic perspectives, Oral Dis. 17 (2011) 345–354.
[8] P.J. Tighe, R.R. Ryder, I. Todd, L.C. Fairclough, ELISA in the multiplex era: potentials and pitfalls, Proteomics Clin. Appl. 9 (2015) 406–422.
[9] S.K. Vashist, G. Czilwik, T. van Oordt, F. von Stetten, R. Zengerle, E. Marion Schneider, J.H.T. Luong, One-step kinetics-based immunoassay for the highly sensitive detection of C-reactive protein in less than 30 min, Anal. Biochem. 456 (2014) 32–37.
[10] M.A.M. Gijs, F. Lacharme, U. Lehmann, Microfluidic applications of magnetic particles for biological analysis and catalysis, Chem. Rev. 110 (2010) 1518–1563.
[11] K.M. Slagle, S.J. Ghosn, Immunoassays: tools for sensitive, specific, and accurate test results, Lab. Med. 27 (1996) 177–183.
[12] M. Schmalenberg, C. Beaudoin, L. Bulst, D. Steubl, P.B. Luppa, Magnetic bead fluorescent immunoassay for the rapid detection of the novel inflammation marker YKL40 at the point-of-care, J. Immunol. Methods 427 (2015) 36–41.
[13] F. Zhao, Q. Shen, H. Wang, X. Han, Z. Yang, Development of a rapid magnetic bead-based immunoassay for sensitive detection of zearalenone, Food Contr. 79 (2017) 227–233.
[14] A. Fan, C. Lau, J. Lu, Magnetic bead-based chemiluminescent metal immunoassay with a colloidal gold label, Anal. Chem. 77 (2005) 3238–3242.
[15] M. Ben Ismail, E. de La Serna, G. Ruiz-Vega, T. García-Berrocoso, J. Montaner, M. Zourob, A. Othmane, E. Baldrich, Using magnetic beads and signal amplifiers to produce short and simple immunoassays: application to MMP-9 detection in plasma samples, Anal. Chim. Acta 999 (2018) 144–154.
[16] H. Liu, P. Rong, H. Jia, J. Yang, B. Dong, Q. Dong, C. Yang, P. Hu, W. Wang, H. Liu, et al., A wash-free homogeneous colorimetric immunoassay method, Theranostics 6 (2016) 54–64.
[17] L. Liu, M. Shao, X. Dong, X. Yu, Z. Liu, Z. He, Q. Wang, Homogeneous immunoassay based on two-photon excitation fluorescence resonance energy transfer, Anal. Chem. 80 (2008) 7735–7741.
[18] X. Liu, Q. Dai, L. Austin, J. Coutts, G. Knowles, J. Zou, H. Chen, Q. Huo, A one-step homogeneous immunoassay for cancer biomarker detection using gold nanoparticle probes coupled with dynamic light scattering, J. Am. Chem. Soc. 130 (2008) 2780–2782.
[19] F.J. van der Wal, J.H.W. Bergervoet, R.P. Achterberg, W. Haasnoot, Bead-based immunoassays, in: H. Koistinen, U.-H. Stenman (Eds.), Novel Approaches in Immunoassays, Future Medicine Ltd, Unitec House, 2 Albert Place, London N3 1QB, UK, 2014, pp. 52–71.
[20] Y. Xiong, Y. Leng, X. Li, X. Huang, Y. Xiong, Emerging strategies to enhance the sensitivity of competitive ELISA for detection of chemical contaminants in food samples, Trac. Trends Anal. Chem. 126 (2020) 115861.
[21] L. Beaudet, R. Rodriguez-Suarez, M.-H. Venne, M. Caron, J. Bédard, V. Brechler, S. Parent, M. Bielefeld-Sévigny, AlphaLISA immunoassays: the no-wash alternative to ELISAs for research and drug discovery, Nat. Methods 5 (2008) an8–an9.
[22] G. Lassabe, K. Kramer, B.D. Hammock, G. González-Sapienza, A. González-Techera, Noncompetitive homogeneous detection of small molecules using synthetic nanopeptamer-based luminescent oxygen channeling, Anal. Chem. 90 (2018) 6187–6192.
[23] B. Houser, Bio-Rad's Bio-Plex® suspension array system, xMAP technology overview, Arch. Physiol. Biochem. 118 (2012) 192–196.
[24] Thermo Fisher Scientific Inc, How xMAP® technology works. https://www.thermofisher.com/de/de/home/references/protein-analysis-guide/multiplex-assays-luminex-assays/how-xmap-technology-works.html. (Accessed 22 June 2020).
[25] I. PerkinElmer, Human C-reactive protein (CRP) kit: product No.: al233 C/F. https://www.perkinelmer.com/lab-solutions/resources/docs/TDS_AlphaLISA_

AL233.pdf. (Accessed 29 April 2020).
[26] I. PerkinElmer, Human matrix metalloproteinase-9 (MMP9) kit: product No.: al243 C/F. https://www.perkinelmer.com/lab-solutions/resources/docs/TDS_AlphaLISA_AL243.pdf. (Accessed 29 April 2020).
[27] N. Reslova, V. Michna, M. Kasny, P. Mikel, P. Kralik, xMAP technology: applications in detection of pathogens, Front. Microbiol. 8 (2017) 55.
[28] AlphaLISA and AlphaScreen No-wash assays. https://www.perkinelmer.com/de/lab-products-and-services/application-support-knowledgebase/alphalisa-alphascreen-no-wash-assays/alphalisa-alphascreen-no-washassays-main.html. (Accessed 30 April 2020).
[29] L. Marnell, C. Mold, T.W. Du Clos, C-reactive protein: ligands, receptors and role in inflammation, Clin. Immunol. 117 (2005) 104–111.
[30] J. Volanakis, Human C-reactive protein: expression, structure, and function, Mol. Immunol. 38 (2001) 189–197.
[31] H. Nagase, R. Visse, G. Murphy, Structure and function of matrix metalloproteinases and TIMPs, Cardiovasc. Res. 69 (2006) 562–573.
[32] L.S.J. Lahdentausta, S. Paju, P. Mäntylä, K. Buhlin, T. Tervahartiala, M. Pietiäinen, H. Alfthan, M.S. Nieminen, J. Sinisalo, T. Sorsa, et al., Saliva and serum biomarkers in periodontitis and coronary artery disease, J. Clin. Periodontol. 45 (2018) 1045–1055.
[33] U.K. Gursoy, E. Könönen, P. Pradhan-Palikhe, T. Tervahartiala, P.J. Pussinen, L. Suominen-Taipale, T. Sorsa, Salivary MMP-8, TIMP-1, and ICTP as markers of advanced periodontitis, J. Clin. Periodontol. 37 (2010) 487–493.
[34] M.W. Seitz, S. Listl, A. Bartols, I. Schubert, K. Blaschke, C. Haux, M.M. van der Zande, Current knowledge on correlations between highly prevalent dental conditions and chronic diseases: an umbrella review, Prev. Chronic Dis. 16 (2019) E132.
[35] G.J. Seymour, P.J. Ford, M.P. Cullinan, S. Leishman, K. Yamazaki, Relationship between periodontal infections and systemic disease, Clin. Microbiol. Infect. 13 (Suppl 4) (2007) 3–10.
[36] M.P. Cullinan, P.J. Ford, G.J. Seymour, Periodontal disease and systemic health: current status, Aust. Dent. J. 54 (Suppl 1) (2009) S62–S69.
[37] K. Mitsakakis, F. Stumpf, O. Strohmeier, V. Klein, D. Mark, F. von Stetten, J.R. Peham, C. Herz, P.N. Tawakoli, F. Wegehaupt, et al., Chair/bedside diagnosis of oral and respiratory tract infections, and identification of antibiotic resistances for personalised monitoring and treatment, Stud. Health Technol. Inf. 224 (2016) 61–66.
[38] N. Bostanci, K. Bao, Contribution of proteomics to our understanding of periodontal inflammation, Proteomics 17 (2017).
[39] N. Bostanci, K. Bao, D. Greenwood, A. Silbereisen, G.N. Belibasakis, Periodontal disease: from the lenses of light microscopy to the specs of proteomics and next-generation sequencing, Adv. Clin. Chem. 93 (2019) 263–290.
[40] P.M. Preshaw, N. Foster, J.J. Taylor, Cross-susceptibility between periodontal disease and type 2 diabetes mellitus: an immunobiological perspective, Periodontol. 2000 45 (2007) 138–157.
[41] B. Kjellström, L. Rydén, B. Klinge, A. Norhammar, Periodontal disease - important to consider in cardiovascular disease prevention, Expert Rev. Cardiovasc Ther. 14 (2016) 987–989.
[42] K. Abbayya, N.Y. Puthanakar, S. Naduwinmani, Y.S. Chidambar, Association between periodontitis and Alzheimer's disease, N. Am. J. Med. Sci. 7 (2015) 241–246.
[43] F.Q. Bui, C.L.C. Almeida-da-Silva, B. Huynh, A. Trinh, J. Liu, J. Woodward, H. Asadi, D.M. Ojcius, Association between periodontal pathogens and systemic disease, Biomed. J. 42 (2019) 27–35.
[44] T. Bansal, A. Pandey, D. D, A.K. Asthana, C-reactive protein (CRP) and its association with periodontal disease: a brief review, J. Clin. Diagn. Res. 8 (2014) ZE21–Z24.
[45] N. Bostanci, V.Ö. Oztürk, G. Emingil, G.N. Belibasakis, Elevated oral and systemic levels of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) in periodontitis, J. Dent. Res. 92 (2013) 161–165.
[46] K.A. Jakobsen, H. Melbye, M.J. Kelly, C. Ceynowa, S. Mölstad, K. Hood, C.C. Butler, Influence of CRP testing and clinical findings on antibiotic prescribing in adults presenting with acute cough in primary care, Scand. J. Prim. Health Care 28 (2010) 229–236.
[47] H.J. Prins, R. Duijkers, P. van der Valk, M. Schoorl, J.M.A. Daniels, T.S. van der Werf, W.G. Boersma, CRP-guided antibiotic treatment in acute exacerbations of COPD in hospital admissions, Eur. Respir. J. 53 (2019) 1802014.
[48] B. Johannsen, D. Mark, N. Boillat-Blanco, A. Fresco, D. Baumgartner, R. Zengerle, K. Mitsakakis, Rapid diagnosis of respiratory tract infections using a point-of-care platform incorporating a clinical decision support algorithm, Stud. Health Technol. Inf. 273 (2020) 237–239.
[49] A. Akcalı, N. Bostanci, Ö. Özçaka, B. Öztürk-Ceyhan, P. Gümüş, T. Tervahartiala, H. Husu, N. Buduneli, T. Sorsa, G.N. Belibasakis, Elevated matrix metalloproteinase-8 in saliva and serum in polycystic ovary syndrome and association with gingival inflammation, Innate Immun. 21 (2015) 619–625.
[50] D.-H. Choi, I.-S. Moon, B.-K. Choi, J.-W. Paik, Y.-S. Kim, S.-H. Choi, C.-K. Kim, Effects of sub-antimicrobial dose doxycycline therapy on crevicular fluid MMP-8, and gingival tissue MMP-9, TIMP-1 and IL-6 levels in chronic periodontitis, J. Periodontal. Res. 39 (2004) 20–26.
[51] G.G. Nascimento, V. Baelum, T. Sorsa, T. Tervahartiala, P.D. Skottrup, R. López, Salivary levels of MPO, MMP-8 and TIMP-1 are associated with gingival inflammation response patterns during experimental gingivitis, Cytokine 115 (2019) 135–141.
[52] J. Pum, A practical guide to validation and verification of analytical methods in the clinical laboratory, Adv. Clin. Chem. 90 (2019) 215–281.
[53] H.N. James, Risk management for point-of-care testing, EJIFCC 25 (2014) 154–161.
[54] U. Andreasson, A. Perret-Liaudet, L.J.C. van Waalwijk Doorn, K. Blennow, D. Chiasserini, S. Engelborghs, T. Fladby, S. Genc, N. Kruse, H.B. Kuiperij, et al., A practical guide to immunoassay method validation, Front. Neurol. 6 (2015) 179.
[55] S. Zehnle, M. Rombach, R. Zengerle, F. von Stetten, N. Paust, Network simulation-based optimization of centrifugo-pneumatic blood plasma separation, Biomicrofluidics 11 (2017) 24114.
[56] D.A. Armbruster, T. Pry, Limit of blank, limit of detection and limit of quantitation, Clin. Biochem. Rev. 29 (Suppl 1) (2008) S49–S52.
[57] J.M. Leppilahti, M.-M. Ahonen, M. Hernández, S. Munjal, L. Netuschil, V.-J. Uitto, T. Sorsa, P. Mäntylä, Oral rinse MMP-8 point-of-care immuno test identifies patients with strong periodontal inflammatory burden, Oral Dis. 17 (2011) 115–122.
[58] T. Sorsa, U.K. Gursoy, S. Nwhator, M. Hernandez, T. Tervahartiala, J. Leppilahti, M. Gursoy, E. Könönen, G. Emingil, P.J. Pussinen, et al., Analysis of matrix metalloproteinases, especially MMP-8, in gingival creviclular fluid, mouthrinse and saliva for monitoring periodontal diseases, Periodontol. 2000 70 (2016) 142–163.
[59] N. Bostanci, N. Selevsek, W. Wolski, J. Grossmann, K. Bao, A. Wahlander, C. Trachsel, R. Schlapbach, V.Ö. Öztürk, B. Afacan, et al., Targeted proteomics guided by label-free quantitative proteome analysis in saliva reveal transition signatures from health to periodontal disease, Mol. Cell. Proteomics 17 (2018) 1392–1409.
[60] A.M. Marcaccini, A.B. Novaes, C.A. Meschiari, S.L. Souza, D.B. Palioto, C.A. Sorgi, L.H. Faccioli, J.E. Tanus-Santos, R.F. Gerlach, Circulating matrix metalloproteinase-8 (MMP-8) and MMP-9 are increased in chronic periodontal disease and decrease after non-surgical periodontal therapy, Clin. Chim. Acta 409 (2009) 117–122.
[61] N. Grassl, N.A. Kulak, G. Pichler, P.E. Geyer, J. Jung, S. Schubert, P. Sinitcyn, J. Cox, M. Mann, Ultra-deep and quantitative saliva proteome reveals dynamics of the oral microbiome, Genome Med. 8 (2016) 1194.
[62] E. Williams, K. Bond, B. Zhang, M. Putland, D.A. Williamson, Saliva as a noninvasive specimen for detection of SARS-CoV-2, J. Clin. Microbiol. 58 (2020) e00776-20.
[63] K.K.-W. To, O.T.-Y. Tsang, C. Chik-Yan Yip, K.-H. Chan, T.-C. Wu, J.M.C. Chan, W.-S. Leung, T.S.-H. Chik, C.Y.-C. Choi, D.H. Kandamby, et al., Consistent detection of 2019 novel coronavirus in saliva, Clin. Infect. Dis. 71 (2020) 841–843.
[64] B. Johannsen, L. Müller, D. Baumgartner, L. Karkossa, S.M. Früh, N. Bostanci, M. Karpíšek, R. Zengerle, N. Paust, K. Mitsakakis, Automated pre-analytic processing of whole saliva using magnet-beating for point-of-care protein biomarker analysis, Micromachines 10 (2019).
[65] J. Verstappen, J.W. von den Hoff, Tissue inhibitors of metalloproteinases (TIMPs): their biological functions and involvement in oral disease, J. Dent. Res. 85 (2006) 1074–1084.
[66] R. Visse, H. Nagase, Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry, Circ. Res. 92 (2003) 827–839.