Analyst

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d0an00308e

Label-free analytical performances of a peptide-based QCM biosensor for trypsin†

Zong-Mu Dong, *[a,b] Lu Cheng,[a] Ping Zhang[c] and Guang-Chao Zhao*[a,b]

Received 12th February 2020,
Accepted 25th February 2020

DOI: 10.1039/d0an00308e

rsc.li/analyst

A label-free biosensor was fabricated for the detection of trypsin by using a peptide-functionalized quartz crystal microbalance gold electrode. The synthetized peptide chains were immobilized tightly on the QCM electrode *via* a self-assembly method, which formed a thin and approximate rigid layer of peptides. The detection signal was achieved by calculating the mass changes on the QCM electrode because the peptide chains could be specifically cleaved in the carboxyl terminuses of arginine and lysine by trypsin. When gold nanoparticles were coupled to the peptide chains, the sensing signal would be amplified 10.9 times. Furthermore, the sensor interface shows a lower resonance resistance change when the peptide chain is immobilized horizontally. Independent detections in parallel on different electrodes have a wide linear range. Under the optimum conditions, the signal-amplified biosensor allowed the measurement of trypsin over the range of 0–750 ng mL^{-1} with a detection limit of 8.6 ng mL^{-1}. Moreover, for screening the inhibitor of trypsin, the IC_{50} values were obtained to be 1.85 μg mL^{-1} for benzamidine hydrochloride and 20.5 ng mL^{-1} for the inhibitor from soybean.

Introduction

Trypsin, with a relative molecular weight of 2.4 kDa, is an extremely important protease mainly found in the digestive system of humans and many other vertebrates, where it selectively hydrolyzes long protein chains *via* high specificity for cleaving peptides at the C-terminal of lysine or arginine amino acid residues.[1–3] As a reliable biological marker, trypsin can promote the pancreatic proenzymes into their active forms and regulates pancreatic exocrine function.[4,5] In general, healthy people have trypsin levels of 0.25 ± 0.1 μg mL^{-1} in serum,[6] and the disorder of the trypsin concentration in the human body and many other vertebrates will lead to many diseases. Therefore, the concentration of trypsin in serum can serve as a clinical diagnostic indicator to distinguish many pancreatic diseases such as acute pancreatitis, meconium ileus, and cystic fibrosis and pancreas transplant patients from healthy people.[7,8] Nowadays, more and more studies have been devoted to developing simple, sensitive and convenient methods toward the detection of trypsin activity and the related protease inhibitor screening which is important for the diagnosis and therapeutics of these diseases.

Conventional enzyme-linked immunosorbent assay (ELISA) is most frequently used for quantitative detection of trypsin with limits of detection ranging from 0.87 ng mL^{-1} to 4.2 ng mL^{-1}.[9] In addition, a variety of techniques have also been established for the measurement of trypsin in biological samples and for screening of protease inhibitors, including photoluminescence,[6] chemiluminescence,[10,11] colorimetric methods,[12] gel electrophoresis,[13] electrochemical sensors,[14–16] and fluorescence methods.[1,7,17] Although the above-mentioned techniques are reliable ways for the detection of trypsin and possess their respective advantages, most of them rely on the reaction between antibodies and antigens for biomolecule recognition. These measurements would require multistep processing, or design of probes with a suitable structure to recognize and interact with trypsin to produce a detectable signal. Of all the methods of analytical research, Wu and Zhen *et al.* demonstrated fluorescence quantum dots coated with a chemically synthesized short peptide for label-free detection of trypsin at a 4–20 ng mL^{-1} level.[18,19] The designed peptide contains arginine and lysine, and it can be hydrolyzed by trypsin in the carboxyl terminuses of arginine and lysine, which will result in the breakage of the peptide chain.[3]

The application of artificial functionalized peptides rather than antibodies as the sensing moiety to detect target proteases is a valuable and versatile approach. Compared to electrochemical and fluorescent probes with more complex affinity scaffolds, artificial short linear peptides have several advantages for biosensor development, such as easy and in-

[a]College of Environmental Science and Engineering, Anhui Normal University, Wuhu 241000, PR China. E-mail: gczhao@mail.ahnu.edu.cn
[b]Anhui Provincial Engineering Laboratory of Water and Soil Pollution Control and Remediation, Anhui Normal University, Wuhu 241000, PR China
[c]Wuhu Institute of Food and Drug, Wuhu 241000, PR China

†Electronic supplementary information (ESI) available. See DOI: 10.1039/d0an00308e

expensive synthesis, being more stable and resistant to harsh environments, and being more amenable than antibodies to engineering at the molecular level.[20–22] Therefore, studies have been conducted with the artificial peptide coupled with quantum dots to create label-free fluorescence or photoelectrochemical biosensors for protease detection.[18,23] While the detection limits were low by using the above-mentioned label-free methods, synthesis of the fluorescence quantum dots and immobilization of the peptide substrate may be prohibitively labor intensive and time consuming.[24]

Among the various label-free signal transduction platforms that have been reported, quartz crystal microbalance (QCM) is promising due to its simple instrumentation, ease of device assembly, adaptability to real-time and *in situ* signal responses.[25] Aptamer based label-free QCM biosensors have been constructed for proteins.[26,27] However, as a mass-sensitive analytical technique, achieving the higher sensitivity of QCM remains a challenge especially for small biomolecule targets. Generally, signal amplification would be one of the major technologies for designing and developing highly sensitive label-free QCM biosensors. Au nanoparticles (Au NPs) are frequently used for signal-amplification probes in small biomolecule target analysis because the synthesis of Au NPs is simple and controllable, and the Au NP has a large mass compared with biomolecules and is easy to functionalize with thiol compared to other metal nanoparticles.[28]

Here, we reported a label-free QCM biosensor based on the immobilization of an artificial peptide on the QCM electrode and Au NPs were used to enhance the frequency signals for the sensitive and selective detection of trypsin. The peptide chain (denoted as P_{CC}), which contains arginine and lysine, was firstly designed and synthesized. Then, the P_{CC} was immobilized on the QCM gold electrode *via* a self-assembly reaction between the gold electrode and the dual-terminal cysteine of P_{CC}. According to the fact that the P_{CC} could be hydrolyzed in the carboxyl terminuses of arginine and lysine by trypsin,[3] a sensing system was constructed. Of particular interest are the QCM biosensors that combine the natural specificity of the biological recognition reaction with selectivity and label-free frequency response readout. When Au NPs functionalized by mercaptoacetic acid (MCA) were coupled with P_{CC}, a signal amplified sensing interface (Au NP-MCA-P_{CC}/QCM) was obtained. The entire construction process was characterized by QCM measurements and electrochemical methods (CV and EIS). The capabilities of Au NP-MCA-P_{CC} to amplify the frequency signals and screen the trypsin inhibitor were evaluated in detail. Furthermore, the trypsin standard spiked serum sample was determined to explore the potential of the Au NP-MCA-P_{CC}/QCM biosensor in practical application.

Experimental

Materials

Trypsin was purchased from Biodee Biotechnology Co., Ltd (Beijing, China). The designed terminal cysteine-tagged peptide chains, CRWEKIRLRWIKQC (denoted as P_{CC}) and CRWEKIRLRWIKQ (denoted as P_{CQ}), were obtained from APeptide Biotechnology Co., Ltd (Shanghai, China). The peptide structure has been confirmed by mass spectroscopy. Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was purchased from Sigma-Aldrich and was used as a reducing agent to reduce the disulfide bonds formed in the peptide chain solution.[28] The peptide stock solution (P_{CC} or P_{CQ}) was prepared by dissolving the lyophilized powders into phosphate buffered saline (PBS) consisting of 1.0 mM TCEP and 0.1 mM EDTA (pH 7.4). Trypsin inhibitors (from soybean), lysozyme, thrombin and collagenase were obtained from Sigma-Aldrich. Bovine serum albumin (BSA) was purchased from Solarbio Technology Co., Ltd (Beijing, China). 6-Mercapto-1-hexanol (MCH), mercaptoacetic acid (MCA), and $HAuCl_4$ were obtained from Shanghai Chemical Reagents Co., Ltd (Shanghai, China).

Apparatus

A 7.995 MHz AT-cut quartz crystal (147.3 mm^2 area) with gold electrodes (19.6 mm^2 area) on both sides was mounted levelly in a homemade Teflon cell. All QCM experiments were performed on a quartz crystal microbalance (CHI 440A, Chenhua Co., Ltd, Shanghai, China) with a homemade detector flow cell. The detector flow cell was placed in a temperature-controlled box, and the injection port was assembled with a six-channel peristaltic pump (BT100K, Baoding, China). Electrochemical experiments (EIS and CV) were carried out with an electrochemical workstation (CHI660C, Chenhua Co., Ltd, Shanghai, China). The data of electrochemical impedance spectra can be simulated with an equivalent model using commercially available software (EvolCRT, version 6.0). The experiment temperature was controlled at 37 °C for trypsin detection and screening the trypsin inhibitor.

Preparation of the Au NP-MCA-P_{CC}/QCM biosensor

According to a previously reported method,[29] Au NPs were synthesized with an average diameter of 13.5 nm in this experiment. The as-prepared Au NPs were immersed in 10 mM MCA solution. The Au NP-MCA particles would carry negative charges according to our previous work,[30] subsequently. The functional Au NP-MCA particles were dispersed finally in PBS (pH 7.4) containing 10 mM NaCl for further use (Au NPs-MCA, 20 nM).

Prior to the preparation of the Au NP-MCA-P_{CC}/QCM biosensor, the QCM gold electrode was cleaned with piranha solution (H_2O_2/H_2SO_4 1 : 3 (v/v)) for 10 s, and then rinsed thoroughly with water. The cleaned QCM electrode was subsequently dried with nitrogen flow in a homemade Teflon cell. For the immobilization of P_{CC}, 250 μg mL^{-1} P_{CC} in PBS (adjusting the pH to 10.32 with 0.1 M NaOH) was injected into the QCM sensing cell and incubated with the QCM gold electrode for 12 h under dark and static conditions. The immobilization of P_{CC} to the QCM electrode surface is through the reaction between the thiol groups present in cysteine residues and gold.[31] The cysteine residues can be synthetically introduced at a specific dual-terminal site of the P_{CC} to form a near-horizon-

tally oriented recognition layer.[32] 10 nM MCA functionalized Au NP solution was then injected to the QCM sensing cell until a saturated link between the positively charged P_{CC} and negatively charged functionalized gold nanoparticles was observed. After each step, the QCM sensing electrode was rinsed with PBS solution (pH 7.4) at a flow rate of 0.5 mL min^{-1} for 15 min to remove the unbound compounds. A signal amplified QCM sensing chip (Au NPs-MCA-P_{CC}/QCM) for trypsin detection was obtained. To avoid non-specific links, a solution of 0.02 mM MCH was injected into the Au NP-MCA-P_{CC}/QCM sensing cell for 0.5 h to passivate the QCM biosensor surface.

For comparison, a P_{CC}/QCM electrode was also prepared as a signal unamplified QCM sensing chip for trypsin detection under the same conditions. All mentioned modified QCM sensing chips were stored at −20 °C for further use and have shown peptide activity persisting for at least one month.

QCM measurement processes

The QCM sensing chip was installed on a flow cell (1.0 mL, volume). A six-channel peristaltic pump pumped solutions through the flow cell. 10 mM PBS buffer (pH 7.4) containing 0.1 M NaCl was injected firstly into the QCM cell with a flow rate of 2.0 mL min^{-1}, and the background signal (frequency change, ΔF) was measured. When the background signal was less than 1.0 Hz of frequency drifted in 60 s, further experiment would be carried out. In all parts of processes (rinsing of the electrode, immobilization of peptides, linking of Au NPs and detection of trypsin), the signals (frequency change, ΔF) of the QCM were recorded to evaluate the performances of the sensor. For the measurement of trypsin, the trypsin standard solution was injected into the QCM cell by using a micro-injector and the solution in the QCM cell was kept still until a stable frequency change (ΔF) was obtained. Control experiments were carried out with a similar process.

Electrochemical measurement processes

An Au NP-MCA-P_{CC}/Au electrode was fabricated to evaluate the electrochemical properties of the peptide-based biosensor. Prior to the electrochemical measurement, the gold electrode was subjected to potential cycling between −0.2 and 1.8 V in 0.05 M H_2SO_4 aqueous solution until reproducible scan results were obtained. To characterize the immobilization of the peptide chains, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were performed in the presence of 5 mM $[Fe(CN)_6]^{4-/3-}$ with the following parameters: scan rate 0.1 V s^{-1}, initial potential 0.43 V, frequency 100 kHz to 0.001 Hz, amplitude 5 mV. Typically, prepared Au NP-MCA-P_{CC}/Au electrodes were immersed in trypsin standard samples with an incubation time of 5 min for each. After that, the electrode surface was rinsed using phosphate buffer solution (pH 7.4) and transferred to $[Fe(CN)_6]^{4-/3-}$ solution (5 mM) for the electrochemical impedance spectroscopy measurements. Nyquist plots were plotted for all impedance spectra.

Procedure of trypsin inhibitor screening

For trypsin inhibitor screening, different amounts of the trypsin inhibitor in PBS solution (pH 7.4, containing 10 mM EDTA) were injected to the Au NP-MCA-P_{CC}/QCM sensing system, and then 0.08 μg mL^{-1} trypsin was added. The frequency signal was recorded with the incubation temperature maintained at 37 °C for 30 min. Furthermore, in order to evaluate the specificity and selectivity of the method for trypsin, similar procedures were also performed for other enzymes such as lysozyme, thrombin, collagenase, and pepsin.

Results and discussion

Construction and characterization of the Au NP-MCA-P_{CC}/QCM biosensor

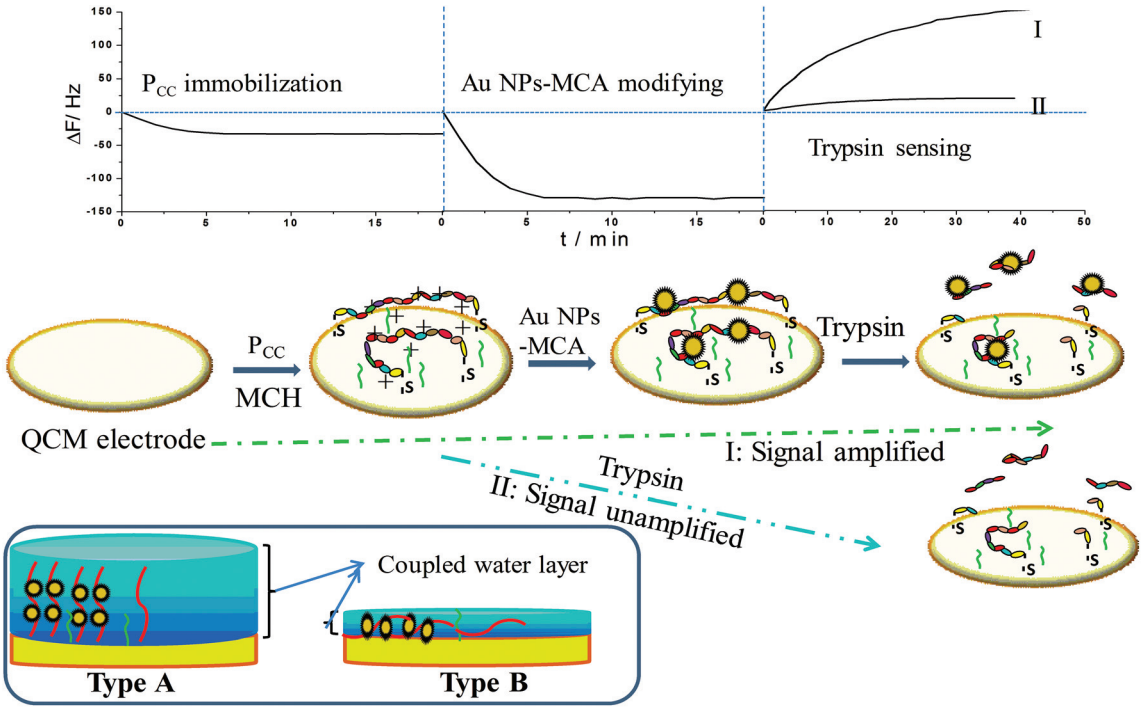
Usually, the peptide chain was single-end immobilized to fabricate a sensing interface in various studies.[33,34] A cysteine anchor was attached at the C-terminal of the peptide to immobilize the peptide on the sensing interface. The other tail end of the peptide chain extends vertically into solution, which resulted in a thick sensing layer in a range of tens to hundreds of nanometers.[20] As shown in the bottom left corner of Scheme 1 type A (see the diagrammatic figure in Fig. S1 in the ESI† for detailed description), the formed layer of peptides (P_{CQ}) is a soft film (*i.e.* water rich; the outer part of adsorbed layers does not follow the oscillation of the sensor surface). Therefore, the linear Sauerbrey relation ($\Delta F = -k\Delta m$) underestimates the changes in the mass of the soft film upon peptide binding.[25] Lots of coupled water in the thick sensing layer not only influence the measurement result of the Sauerbrey equation for trypsin, but also it can cause an increase in the series resonance resistance (ΔR_v) of the crystal.[20] The total frequency changes (ΔF) of the QCM crystal immersed in a liquid containing the binding peptide include the frequency changes (ΔF_m and ΔF_l) due to mass loading and liquid viscosity/density change as given by eqn (1):

$$\Delta F = \Delta F_m + \Delta F_l \quad (1)$$

According to Kanazawa's solution,[35] when the QCM chip comes into contact with a liquid flow, there is a frequency change (ΔF_l) that is dependent on the viscosity (η_l) and density (ρ_l) of the solution. Based on Butterworth-Van Dyke equivalent circuit model,[36] both ΔF_l and ΔR_v are proportional to $(\eta_l\rho_l)^{1/2}$.[37] Furthermore, as far as the peptide modified QCM chip is concerned, the Banta research group gave an experimentally derived relationship between ΔF_l and ΔR_v as shown in eqn (2):

$$\Delta R_v = -k\Delta F_l \quad (2)$$

where k = 0.5 Ω Hz^{-1}.[19] In here, the sensing performances with different immobilization methods of peptide chains (types A and B in Scheme 1) were compared. The electrodes with different modes of immobilized peptides were incubated in the trypsin standard solution (concentration range of 0–100.0 μg mL^{-1}) for 20 min, and were then rinsed and transferred to 0.5 mM solution of $[Fe(CN)_6]^{4-/3-}$ in 0.1 M NaCl for



Scheme 1 The schematic representations of the fabrication of the QCM biosensor and the determination process for trypsin. Also shown is a schematic of the sensing interface with the different peptide immobilization methods in the left corner (A: single-end immobilized P_{CQ}; B: double-end immobilized P_{CC}).

EIS measurements. The Nyquist plots of these samples were obtained for data analysis. The electrode system can be represented by an equivalent circuit composed of an electron-transfer resistance (R_{et}), a series resonance resistance (R_v), a capacitance which is represented by a constant phase element (CEP) and a solution resistance (R_s),[37,38] as shown in the inset of Fig. 1B. These electrical parameters were obtained by fitting the Nyquist plots (not shown) with commercially available software (EvolCRT, version 0.6) using the equivalent circuit model and the results are shown in Table 1. For type A, the series resonance resistance change (ΔR_v) in a range of 1.4 to 4.1 Ω, corresponding to ΔF_1 in a range of 2.8 to 8.2 Hz based on eqn (2), was observed in Table 1. The results indicate that a thicker sensing layer of the type A peptide-based sensor was reached as shown in Scheme 1. Moreover, the local viscosity and density of the solution in the sensing layer would be likely to obviously change along with the sensor working process, which leads to a non-linear relationship between the total frequency change (ΔF) and mass change (Δm) by eqn (1). In contrast, only minor and stable changes of the series resonance resistance (ΔR_v, less than 0.4 Ω corresponding to ΔF_1 less than 0.8 Hz) were observed on the type B sensing interface as shown in Table 1, which indicates a rigid packing mode of the immobilized peptide and higher sensitivity for target analyte

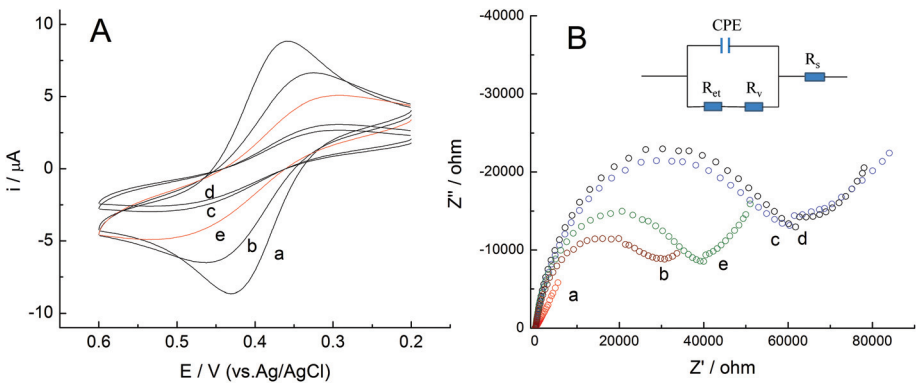


Fig. 1 Characterization of the sensing interface by CV (A) and EIS (B). (a) Bare electrode, (b) after immobilization of P_{CC}, (c) incubation with 0.1 mM MCH solution, (d) after Au NP modification, and (e) after incubation with 0.5 μg mL^{-1} trypsin solution for 10 min.

Table 1 Fitting values of the equivalent circuit elements taken by EvolCRT software for trypsin detection with two types of peptide-based sensors (types A and B in Scheme 1)

	Type A				Type B			
Trypsin concen. (μg mL^{-1})	R_s (Ω)	CPE (μF cm^{-2})	R_{et} (kΩ)	R_v (Ω)	R_s (Ω)	CPE (μF cm^{-2})	R_{et} (kΩ)	R_v (Ω)
0	89.87	1.277	89.65	326.7	72.3	2.213	73.63	163.3
0.05	237.6	1.832	75.29	325.2	192.1	2.116	63.14	163.2
0.5	228.1	1.723	54.37	321.7	229.2	2.321	41.22	162.5
5	231.9	1.765	41.11	217.6	221.4	2.343	34.80	162.1
25	252.6	1.802	35.42	215.1	248.7	2.416	29.52	161.8
100	246.5	1.795	33.25	213.7	257.5	2.456	27.32	161.7

detection.[34] Under the same conditions, the comparison of the signals of type A and type B sensors is shown in the graph of Fig. S1.† During this process, it seems that almost all frequency change is due to the changes in the near-surface mass loading/losing of the sensing interface, *i.e.* ΔF is approximately equal to $\Delta F_m + \Delta F_l$. In order to use this peptide in the development of a sensitive and convenient trypsin label-free biosensor, the type B mode of the immobilized peptide was used to fabricate the sensor for trypsin detection in this work. The peptide chain (P_{CC}) was double-end immobilized horizontally onto the QCM electrode surface, and it formed a thin and approximate rigid layer of peptide chains.

The construction rationales of the QCM biosensor and the determination processes (signal amplified (I) and signal unamplified (II)) for trypsin are shown in Scheme 1. Firstly, a short peptide (P_{CC}) with an amino acid sequence of CRWEKIRLRWIKQC was double-end immobilized on the QCM electrode to form a peptide monolayer. The immobilization of P_{CC} to the QCM electrode surface is through the reaction between the thiol groups present in cysteine residues and gold to form a near-horizontally oriented peptide chain sensing layer.[31,32] This is because the cysteine residues can be synthetically introduced at a specific dual-terminal site of the P_{CC}. As shown in Fig. S2 in the ESI,† a observed frequency drop (~39.2 Hz) of QCM can indicate that the P_{CC} has been immobilized onto electrode surface when the QCM electrode was immersed in the peptide buffer solution in the QCM cell. According to the Sauerbrey equation,[39]

$$\Delta F_m = -C_f \times \Delta m \quad (3)$$

For a 7.995 MHz AT-cut crystal, where C_f = 0.142 Hz (ng cm^{-2})$^{-1}$ at 20 °C, therefore, the mass loading of immobilized P_{CC} per area is about 276.1 ng cm^{-2}. According to the Sauerbrey equation, that gives a peptide surface coverage of 8.66×10^{13} molecules cm^{-2} with a molecular weight of 1918.35 g mol^{-1} for the P_{CC} single-chain peptide. The surface coverage of the peptide calculated by QCM usually also contains the contributions from the coupled water on the sensor surface. Moreover, in order to avoid non-specific binding, the sensing surface was immersed in 0.02 mM MCH solution to block the empty sites of the P_{CC}/QCM electrode.

To evaluate the signal amplification of gold nanoparticles in the sensing process, a signal unamplified sensing chip (P_{CC}/QCM) was prepared for trypsin detection (process II in Scheme 1). For fabricating the signal amplified sensing chip (Au NP-MCA-P_{CC}/QCM), the P_{CC}/QCM chip was further immersed in the functional Au NP solution. The Au NPs with negative charges would be linked to the positively charged peptide chain (P_{CC}) due to the electrostatic interactions (process I in Scheme 1). All the characterization and measurement processes were monitored by QCM and electrochemical measurements (CV and EIS). As shown in Fig. 1 (CV curve a in A and EIS curve a in B), a couple of reversible redox peaks of $[Fe(CN)_6]^{4-/3-}$ and a low interfacial electron-transfer resistance (R_{et} ~ 5.0 Ω) were obtained from the cyclic voltammograms and Nyquist plots at the bare gold electrode.[40] With the immobilization of peptides and blocking empty sites by MCH on the electrode surface, the electron transfer of $[Fe(CN)_6]^{4-/3-}$ was hindered. As shown in Fig. 1 (CV curves b and c in A and EIS curves b and c in B), the anodic peak currents decreased (5.2 μA and 11.6 μA, respectively) and the interfacial impedance obviously increased (30 kΩ and 58 kΩ, respectively). When the Au NP-MCA was modified onto the peptide chains, a slight change of the electrochemical characteristics was observed in Fig. 1 (CV curve d in A and EIS curve d in B). However, a frequency change (ΔF ~ 136 Hz) was obtained with Au NP-MCA modification (inset of Scheme 1). These results suggest the formation of a dense monolayer of immobilized P_{CC} and MCH on the gold electrode surface, and the Au NP-MCA was also modified onto the peptide chains of the sensing layer successfully. When the electrode with the sensing layer was immersed in the standard solution containing 0.15 μg mL^{-1} trypsin for 10 min, the peptide chains would be cracked and leave from the sensing interface because of the trypsin hydrolytic cleavage reaction. The sensing signal (ΔF) would be obtained (inset of Scheme 1). Meanwhile, the interfacial impedance of the sensing surface would also be changed. These results could also be demonstrated by EIS, which is shown in Fig. 1 (EIS curve e in B). As the electrochemical impedance (R_{et}) was related to the amount of trypsin, it could also be measured by the EIS method (data not shown). Through comparing the two methods, the signal amplifying QCM method is a more simple and rapid approach. Moreover, the reproducibility and sensitivity of the EIS method were also

not as good as those of QCM were, so the signal amplifying QCM method was utilized to detect trypsin in this work. Furthermore, to investigate the fabrication of the Au NP-MCA-P_{CC}/QCM biosensor and the process of trypsin detection, SEM images were also taken for observing the surface change of bare QCM, P_{CC}/QCM, Au NP-MCA-P_{CC}/QCM and Au NP-MCA-P_{CC}/QCM as shown in Fig. S3.†

Trypsin QCM biosensor operation

According to the earlier report that the carboxyl terminuses of arginine and lysine of the peptide could be hydrolyzed by trypsin,[3] a mass sensing system was constructed. When functionalized Au NPs were coupled with P_{CC}, a signal amplified biosensor (Au NPs-MCA-P_{CC}/QCM) was fabricated for trypsin detection.

Amplification effect of the QCM sensing system

As a piezoelectric quartz mass sensor, the sensitivity depends on the mass change (Δm) on the QCM chip surface. In order to evaluate the amplification effect of Au NPs, the direct detection of trypsin was firstly studied through monitoring the QCM responses induced from the P_{CC} cleavage reaction in the presence of trypsin by using a P_{CC}-modified QCM electrode. As illustrated in Fig. 2 (curve b), with the injection of trypsin solution (150 ng mL^{-1} final concentration), a frequency increase from 0.5 to 11.6 Hz was observed within 10 min. When trypsin concentration was lower than 50 ng mL^{-1}, the frequency change was difficult to discriminate from the frequency response from the baseline noise (ΔF, less than 1 Hz). When the Au NP-MCA-P_{CC}/QCM chip in the same experimental process replaced the P_{CC}/QCM chip, the frequency obviously increased from 0.6 to 126.5 Hz with the same concentration of trypsin, as shown in Fig. 2, curve c, which is 10.9 times compared with the P_{CC}/QCM biosensor (Fig. 2, curve b) under the same conditions. This could be explained by the fact that Au NPs were introduced as a signal enhancement reagent to further improve the sensitivity of the biosensor. This signal amplification effect is superior to that of the biosensor in our early work,[30] which may be due to optimized immobilization mode of the peptide in this work. Simultaneously, a control experiment was carried out using the bare QCM chip as shown in Fig. 2, curve a, which revealed that the frequency change of the biosensor over time was minimal (less than 1 Hz) and did not impact on the measurements.

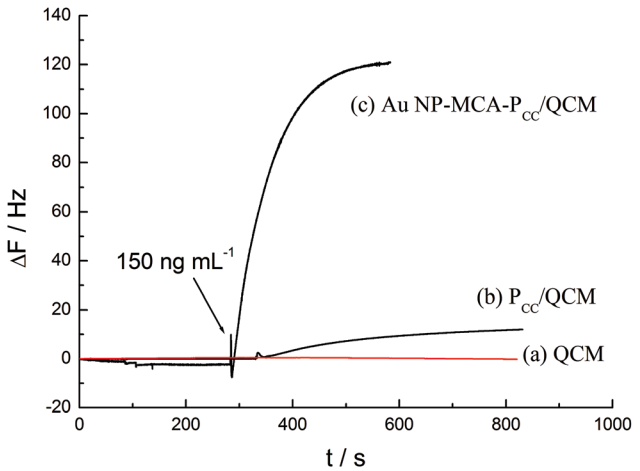


Fig. 2 Signal responses of the bare QCM (a), P_{CC}/QCM (b) and Au NP-MCA-P_{CC}/QCM (c) biosensors in 150 ng mL^{-1} trypsin solution at 37 °C.

Sensitivity of the Au NP-MCA-P_{CC}/QCM biosensor

The quantitative detection of trypsin at different concentrations was performed firstly on the Au NP-MCA-P_{CC}/QCM electrode. There are two methods to carry out a response test: sequential dose injection on one QCM electrode (the signal responses are shown in Fig. 3A) or independent detections in parallel on different QCM electrodes (the signal responses are shown in Fig. 3B), and these electrodes were prepared under the same conditions. An advantage of the former method is that it is convenient due to using one electrode for sequential dose injection, so that its output provides real-time and *in situ* signals along with trypsin concentration changes.

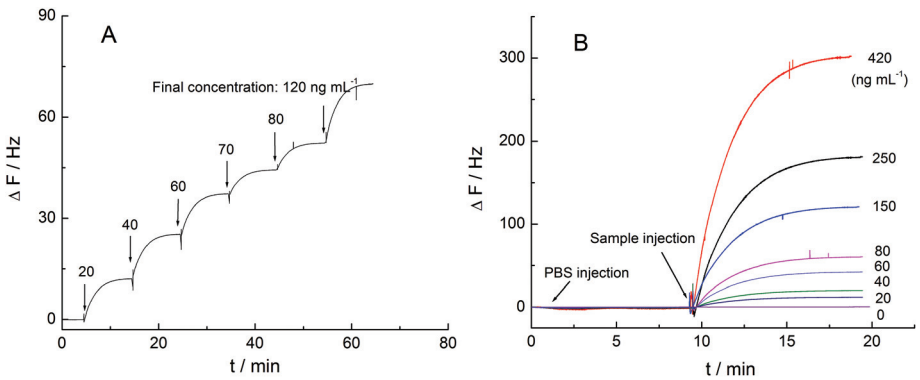


Fig. 3 Signal responses of the Au NP-MCA-P_{CC}/QCM biosensors by using two methods, A: a sequential dose injection on one Au NP-MCA-P_{CC}/QCM electrode and B: independent detections in parallel on different Au NP-MCA-P_{CC}/QCM electrodes.

However, the surface states of the Au NP-MCA-P_{CC}/QCM biosensor have been changed due to the addition of the previous sample in the process of sequential dose injection, and the sensing sensitivity would be affected. The latter strategy is closer to a normal detection protocol and is more stringent because it requires identical electrode surface modifications and morphologies.[20]

The analytical results by the two sampling methods have been compared, and the quantitative relationships between trypsin concentrations and frequency changes (ΔF) are shown in Fig. 4. Compared to the response curves, a larger sensitivity (0.68 Hz (ng $mL^{-1})^{-1}$, defined as the slope of the calibration curve) and a more linear concentration range (0–750 ng mL^{-1}) (R = 0.997) with a detection limit of 8.6 ng mL^{-1} were observed during independent detections in parallel on different QCM electrodes in Fig. 4B. In general, the response curve of any enzyme-biosensor arises from two separate events: the enzyme affinity step and the signal readout step.[41] In terms of the signal readout step, it is the same process in which the frequency change is proportional to the mass change (Δm) of bound Au NP-MCA-P_{CC} due to the trypsin cleavage reaction. However, the enzyme affinity step is different in the two sampling methods. In the latter (the process B in Fig. 3), because the fabrication conditions of the Au NP-MCA-P_{CC}/QCM chips are the same, the amount of P_{CC} of each biosensor surface hardly changes before the sample addition. As for the former (the process A in Fig. 3), the surface states of the Au NP-MCA-P_{CC}/QCM biosensor are changed in the process of sequential dose injection, which will result in a relatively low sensitivity (0.58 Hz (ng $mL^{-1})^{-1}$), narrow linear range (0–250 ng mL^{-1}), and poor precision. As shown in Fig. 4A, larger error bars were observed in higher concentration ranges. Furthermore, in the enzyme-catalyzed reaction, the rate of the enzyme-catalyzed reactions relates to the concentration of the enzyme in the matrix.[42] Therefore, as in our previous research,[30] the rates of frequency changes (ΔF) could also be calculated on the basis of trypsin activity as an internal standard. In Fig. S4,† a linear relationship between log[rate($\Delta F/s$)] and the concentration of trypsin was obtained in a range from 0 to 170 ng mL^{-1} with a detection limit of 20.2 ng mL^{-1}. However, this quantitative method requires strict temperature control in the sensing process, and displays dissatisfactory reproducibility with a relative standard deviation (RSD) of 19.5%, which may be due to the non-linear relationship between the enzyme catalytic rate and the frequency change of the QCM sensor.

Selectivity, practicability and storage capability of the Au NP-MCA-P_{CC}/QCM biosensor

To study the selectivity of the Au NP-MCA-P_{CC}/QCM biosensor for trypsin, homologous enzymes such as lysozyme, thrombin, and collagenase and other biological species proteins, such as BSA, were also tested. The results are shown in Fig. 5. Compared to the Au NP-MCA-P_{CC}/QCM biosensor signal of the blank, none of these homologous enzymes caused obvious frequency changes (less than 1 Hz), even at a 10-fold higher concentration (0.2 μg mL^{-1} final concentration) than that of trypsin. As shown in Fig. 5D, however 20 ng mL^{-1} trypsin solution exhibited an obvious frequency enhancement (13.5 Hz) compared to that of BSA and homologous enzymes. These results indicate that the Au NP-MCA-P_{CC}/QCM biosensor exhibited a good specificity and satisfactory analytical ability towards trypsin in the standard-spiked serum sample.

The present sensor has been evaluated for practicability by analyzing its reliability, which was evaluated in three independent QCM electrodes fabricated with Au NPs-MCA-P_{CC} in 60 ng mL^{-1} trypsin. The present sensor agreeably displays excellent repeatability with a relative standard deviation (RSD) of 0.62% (Fig. S5A†). Further, the long-term storage stability of the Au NP-MCA-P_{CC}/QCM biosensor was investigated by placing it at −20 °C temperature for two weeks, then analyzing it against 60 ng mL^{-1} trypsin and monitoring the frequency responses, which were found to be 92.7 and 90.6% respectively after two and four weeks from the initial frequency response (Fig. S5B†). The specificity, repeatability and long-term stability of the Au NP-MCA-P_{CC}/QCM biosensor were all tolerable; thus, it can be highly suitable for the sensitive quantification of trypsin in complicated trypsin samples.

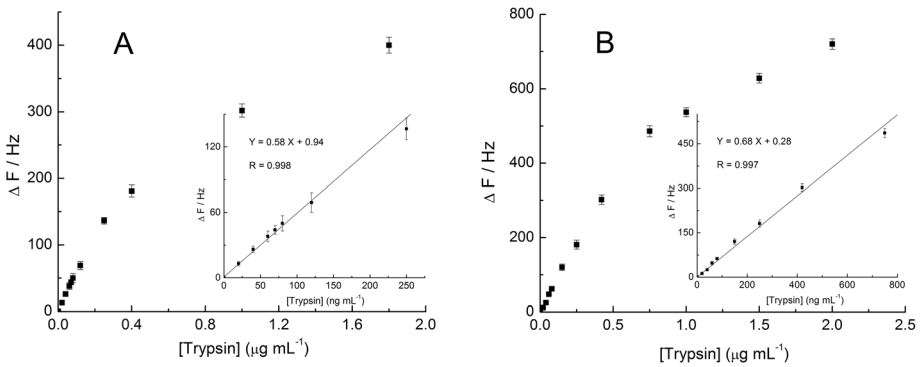


Fig. 4 Calibration curves of the Au NP-MCA-P_{CC}/QCM biosensors by using two methods, A: a sequential dose injection on one Au NP-MCA-P_{CC}/QCM electrode and B: independent detections in parallel on different Au NP-MCA-P_{CC}/QCM electrodes.

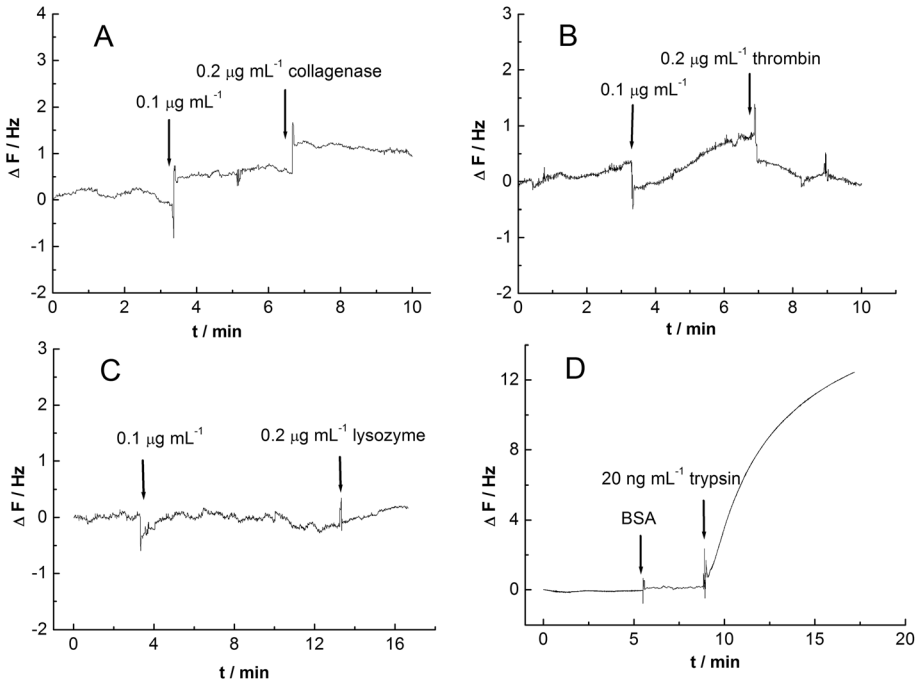


Fig. 5 Selectivity of the Au NP-MCA-P_{CC}/QCM biosensor. The standard solutions of collagenase, thrombin and lysozyme (0.2 μg mL^{-1} final concentration, respectively) were injected instead of trypsin.

Au NP-MCA-P_{CC}/QCM biosensor for trypsin inhibitor screening

In addition to calcium and magnesium ions, a biological protease inhibitor can specifically react with the trypsin molecule and reduce its catalytic activity. The corresponding inhibitor can be used to treat a variety of related diseases.[43,44] Therefore, the development of a convenient and effective method for inhibitor screening is significant for clinical medicine and drug development. Soybean[45] and benzamidine hydrochloride,[46] two potent inhibitors toward trypsin, were selected as the model inhibitors to evaluate the Au NP-MCA-P_{CC}/QCM biosensor for inhibitor screening. As shown in Fig. 6, the results showed that the inhibitor in a concentration-dependent manner suppressed the activity of trypsin. In terms of the inhibitory effect from soybean (Fig. 6A), the inhibition efficiency reached almost 100% at 110 ng mL^{-1} inhibitor concentration. The IC50 value (the concentration corresponding to 50% inhibition) for the trypsin inhibitor from soybean was 20.5 ng mL^{-1}. In terms of benzamidine hydrochloride, the corresponding IC50 value was about 1.85 μg mL^{-1}, which was much larger than that of the trypsin inhibitor from soybean (20.5 ng mL^{-1}). These results demonstrated that the label-free sensing method is feasible in screening of trypsin inhibitors.

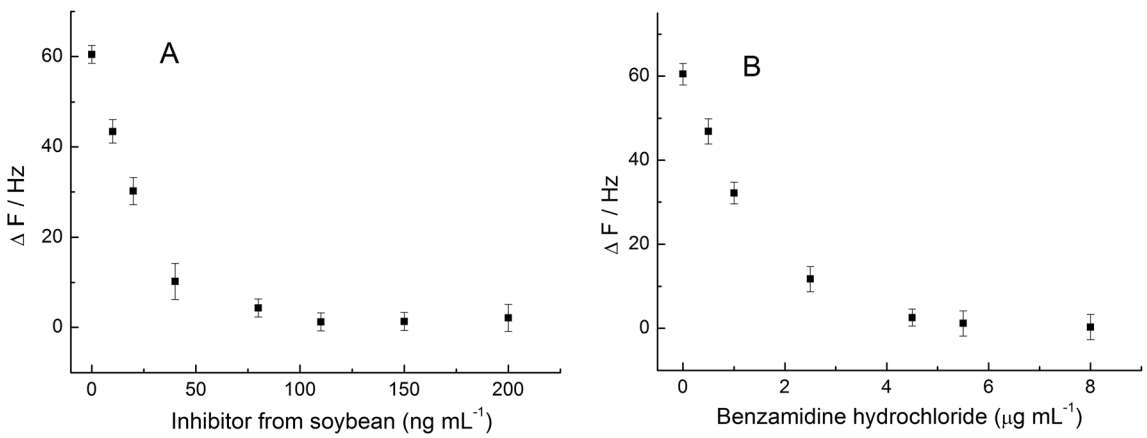


Fig. 6 Inhibitory efficiency of the trypsin inhibitor from soybean (A) and benzamidine hydrochloride (B) on the activity of 80 ng mL^{-1} trypsin by using the Au NP-MCA-P_{CC}/QCM biosensor. The error bar represents the standard deviation of three measurements.

Conclusions

We have developed a label-free QCM method for the assay of trypsin, in which a specific Au NP-MCA-functionalized peptide was employed as the mass-probe. The research shows that the modification of rigid peptide chains on the surface of the QCM electrode determines its superior sensing performance. The Au NP-MCA-PCC/QCM biosensor is simple, label-free, highly sensitive, and highly selective for trypsin detection. What's more, the method was successfully applied for the measurement of trypsin in the standard-spiked serum samples and screening of protease inhibitors, demonstrating its potential for application in drug development and clinical diagnosis. This study opens a new avenue to develop new peptide-based QCM biosensors toward different proteases.

Abbreviations

QCM	Quartz crystal microbalance
P_{CC}	CRWEKIRLRWIKQC
P_{CQ}	CRWEKIRLRWIKQ
Au NP	Gold nanoparticle
MCA	Mercaptoacetic acid
MCH	6-Mercapto-1-hexanol
TCEP	Tris (2-carboxyethyl) phosphine hydrochloride

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Anhui Provincial Natural Science Foundation (No. 1908085 MB 52) supported this work.

Notes and references

1 S. S. Huang, F. F. Li, C. Y. Liao, B. Z. Zheng, J. Du and D. Xiao, *Talanta*, 2017, **170**, 562.
2 B. X. Li, D. Lan and Z. J. Zhang, *Anal. Biochem.*, 2008, **374**, 64.
3 J. V. Olsen, S. E. Ong and M. Mann, *Mol. Cell. Proteomics*, 2004, **3**, 608.
4 P. G. Noone, Z. Q. Zhou, L. M. Silverman, P. S. Jowell, M. R. Knowles and J. A. Cohn, *Gastroenterology*, 2001, **121**, 1310.
5 M. B. Strader, D. L. Tabb, W. J. Hervey, C. L. Pan and G. B. Hurst, *Anal. Chem.*, 2006, **78**, 125.
6 T. T. Xia, Q. Ma, T. Y. Hu and X. G. Su, *Talanta*, 2017, **170**, 286.
7 D. Zhao, C. X. Chen, J. H. Zhao, J. Sun and X. R. Yang, *Sens. Actuators, B*, 2017, **247**, 392.
8 C. Y. Poon, Q. H. Li, J. L. Zhang, Z. P. Li, C. Dong, A. W. M. Lee, *et al.*, *Anal. Chim. Acta*, 2016, **917**, 64.
9 M. A. Seia, P. W. Stege, S. V. Pereira, I. E. DeVito, J. Raba and G. A. Messina, *Anal. Biochem.*, 2014, **463**, 31.
10 H. H. Zhang, D. L. Yu, Y. J. Zhao and A. P. Fan, *Biosens. Bioelectron.*, 2014, **61**, 45.
11 Q. F. Zhang, W. Y. Li, J. Chen, F. Y. Wang, Y. Wang, Y. Chen and C. Yu, *Chem. Commun.*, 2013, **49**, 3137.
12 L. F. Zhang and J. X. Du, *Biosens. Bioelectron.*, 2016, **79**, 347.
13 R. B. Lefkowitz, G. W. Schmid-Schonbein and M. J. Heller, *Electrophoresis*, 2010, **31**, 2442.
14 M. M. Dong, H. L. Qi, S. G. Ding and M. Li, *Microchim. Acta*, 2015, **182**, 43.
15 S. Park and H. Yang, *Analyst*, 2014, **139**, 4051.
16 M. Stoytcheva, R. Zlatev, S. Cosnier and M. Arredondo, *Electrochim. Acta*, 2012, **76**, 43.
17 Y. B. Liu, F. M. Zhang, X. He, P. Y. Ma, Y. B. Huang, S. Tao, Y. Sun, X. H. Wang and D. Q. Song, *Sens. Actuators, B*, 2019, **294**, 263.
18 M. M. Wu, X. Y. Wang, K. Wang and Z. J. Guo, *Talanta*, 2017, **174**, 797.
19 F. F. Zheng, J. F. Wu and G. C. Zhao, *Anal. Methods*, 2012, **4**, 3932.
20 J. Wu, D. M. Cropek, A. C. West and S. Banta, *Anal. Chem.*, 2010, **82**, 8235.
21 J. E. Dover, G. M. Hwang, E. H. Mullen, B. C. Prorok and S. J. Suh, *J. Microbiol. Methods*, 2009, **78**, 10.
22 S. S. Iqbal, M. W. Mayo, J. G. Bruno, B. V. Bronk, C. A. Batt and J. P. Chambers, *Biosens. Bioelectron.*, 2000, **15**, 549.
23 H. Dai, S. H. Chen, Y. L. Li, B. S. Zeng, S. P. Zhang, Z. S. Hong and Y. Y. Lin, *Biosens. Bioelectron.*, 2017, **92**, 687.
24 A. Poturnayova, I. Karpisova, G. Castillo, G. Mező, L. Kocsis, A. Csámpai, Z. Keresztes and T. Hianik, *Sens. Actuators, B*, 2016, **223**, 591.
25 V. C. Ozalp, *Analyst*, 2011, **136**, 5046.
26 K. Min, M. Cho, S. Y. Han, Y. B. Shim, J. Ku and C. Ban, *Biosens. Bioelectron.*, 2008, **23**, 1819.
27 Q. Chen, W. Tang, D. Z. Wang, X. J. Wu, N. Li and F. Liu, *Biosens. Bioelectron.*, 2010, **26**, 575.
28 N. V. Kulagina, K. M. Shaffer, F. S. Ligler and C. R. Taitt, *Sens. Actuators, B*, 2007, **121**, 150.
29 J. Q. Zhang, Y. S. Wang, Y. He, T. Jiang, H. M. Yang, X. Tan, R. H. Kang, Y. K. Yuan and L. F. Shi, *Anal. Biochem.*, 2010, **397**, 212–217.
30 Z. M. Dong, X. Jin and G. C. Zhao, *Biosens. Bioelectron.*, 2018, **106**, 111.
31 M. B. Dickerson, K. H. Sandhage and R. R. Naik, *Chem. Rev.*, 2008, **108**, 493.
32 T. Baas, L. Gamble, K. D. Hauch, D. G. Castner and T. Sasaki, *Langmuir*, 2002, **18**, 4898.
33 G. J. Wegner, H. J. Lee and R. M. Corn, *Anal. Chem.*, 2002, **74**, 5161.
34 J. Wu, D. M. Cropek, A. C. West and S. Banta, *Anal. Chem.*, 2010, **82**, 8235.

35 K. K. Kanazawa and J. G. Gordon, *Anal. Chim. Acta*, 1985, **175**, 99.
36 S. J. Geelhood, C. W. Frank and K. Kanazawa, *J. Electrochem. Soc.*, 2002, **149**, H33.
37 S. J. Martin, J. J. Spates, K. O. Wessendorf, T. W. Schneider and R. J. Huber, *Anal. Chem.*, 1997, **69**, 2050.
38 F. Lisdat and D. Schafer, *Anal. Bioanal. Chem.*, 2008, **391**, 1555.
39 G. Sauerbrey, *Z. Phys.*, 1959, **155**(2), 206–222.
40 C. Y. Deng, J. H. Chen, Z. Nie, M. D. Wang, X. C. Chu, X. L. Chen, X. L. Xiao, C. Y. Lei and S. Z. Yao, *Anal. Chem.*, 2009, **81**, 739.
41 J. S. Daniels and N. Pourmand, *Electroanalysis*, 2007, **19**, 1239.
42 M. Ajmala, G. Fieg and F. Keil, *Chem. Eng. Process.*, 2016, **110**, 106.
43 E. Deu, M. Verdoes and M. Bogyo, *Nat. Struct. Mol. Biol.*, 2012, **19**, 9.
44 S. Y. Zhang, C. Chen, X. F. Qin, Q. C. Zhang, J. H. Liu, J. X. Zhu, Y. Q. Gao, L. Li and W. Huang, *Talanta*, 2018, **189**, 92.
45 D. L. Hwang, D. E. Foard and C. H. Wei, *J. Biol. Chem.*, 1977, **252**, 1099.
46 Q. F. Zhang, W. Y. Li, J. Chen, F. Y. Wang, Y. Wang, Y. Chen and C. Yu, *Chem. Commun.*, 2013, **49**, 3137.