The determination of cystatin C in biological samples via the surface plasmon resonance method

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ABSTRACT

Surface plasmon resonance imaging biosensors have a number of advantages that make them superior to other analytical methods. These include the possibility of label-free detection, speed and high sensitivity to low protein concentrations. The aim of this study was to create and analyze biochips, with the help of which it is possible to test cystatin C in patient urine samples and compare the results with the one-time traditional ELISA method. The main advantage of the surface plasmon resonance imaging method is the possibility of repeated measurements over a long period of time in accordance with clinical practice. The surface of the biochip was spotted with anticystatin C and a negative control of mouse IgG at a ratio of 1:1. The aforementioned biochip was first verified using standard tests and then with patient samples, which clearly confirmed the required sensitivity even for very low concentrations of cystatin C.

METHOD SUMMARY

Surface plasmon resonance is a sensitive optical method that uses the generation of electromagnetic waves (plasmons) for detection. The propagation of surface plasmons at the metal–dielectric interface is very sensitive to changes in the refractive index, which allows for the monitoring of substance properties very close to the interface. The main advantage of the sensor over other currently used techniques is the possibility of label-free analysis and real-time analysis, with high sensitivity and specificity of the optical method.

KEYWORDS:

biochip ● cystatin C ● plasmon ● SPR ● urine

Biosensors are very important in the medical field because they allow for the detection of various biochemical substances [1,2]. Currently, fluorescence-based detection methods are routinely used for their high sensitivity and diverse selection of labeling agents [3].

Surface plasmon resonance imaging (SPRi) is used for the identification of biological substances. It is an unlabeled optical and selective detection technology that enables real-time observation of the response between molecules [4–6]. A number of technologies similar to the SPRi method are used to detect biomolecular interactions, including interferometers and diffraction and quartz crystal microbalance techniques. Of the listed methods, SPRi is the most used [6].

The SPRi method is often used not only in medicine but also to characterize thin layers or surfaces or to study the concentration of nanoparticles [7,8]. SPR is also applied in the study of biomolecular interactions [9]. The measuring principle is based on the evaluation of the refractive index transformation, which is induced by a biomolecular reaction on an optical prism [10,11]. In this way, substances can be detected without the need to label one of the reactants [5]. In 2003, Homola predicted the use of the SPR method for biological substances and further hypothesized that this method had potential in a number of important areas [12]. The predictions have proven to be correct, and this method is now used in patient diagnosis, climate protection and rapid food quality and safety control [13–15]. An example of one of the many uses of the SPRi method is the testing by Sharma *et al.* of monoclonal antibodies (mAbs) mAb1, mAb2 and mAb3 in the Ebola virus using a gold SPR chip modified with 4-sulfanylbenzoic acid, the results of which were confirmed by the ELISA method [16].

Cystatin C is a protein in the family of cysteine protease inhibitors that is produced in constant amount, filtered by glomeruli and fully reabsorbed by the renal tubules [17]. Cystatins are part of the human immune system [18]. Examination of the content of cystatin C in urine is intended to detect and monitor acute and chronic renal dysfunction. It is the elevated value of cystatin C that can reveal kidney disease, but it is not always elevated cystatin C which indicates disease. In the case of plasma, cancer can be detected by decreased levels of cystatin C [19]. Cystatin C concentrations do not depend on sex, age or body composition; they depend only on the glomerular filtration rate. The reference value in urine is 0.03–0.29 μg/ml [20]. Human cystatin C is found in all extracellular body fluids. The highest

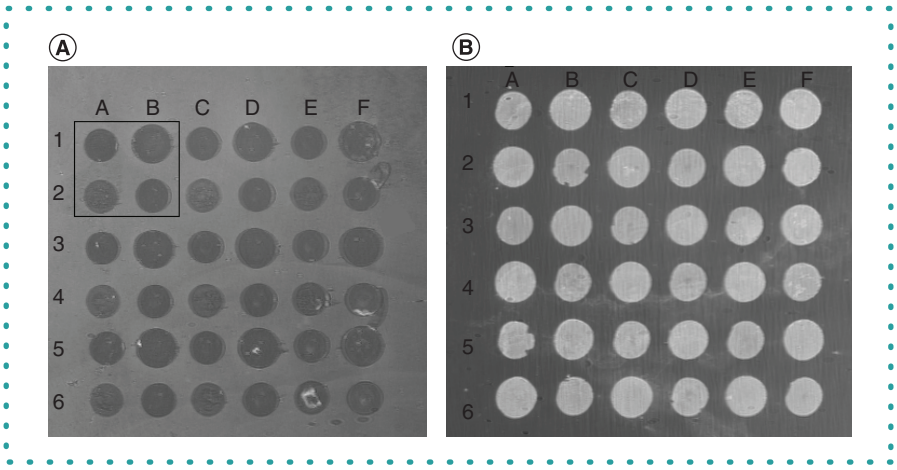

Figure 1. Photographs of biochips for the determination of cystatin C in urine. (A) A biochip with a defective spot. **(B)** A biochip with no defects.

concentration is found in sperm, at 41.2–61.8 μg/ml. It can also be found in plasma, saliva and cerebrospinal fluid. Values for these body fluids range from 0.57 g/ml for plasma to 12.5 μg/ml for cerebrospinal fluid [21]. This protein consists of a single polypeptide chain composed of 120 amino acid residues. The nonglycosylated basic protein has a molecular weight of 13,343 Da [22,23] and is encoded for 146 amino acids. The *CST3* gene is located on chromosome 20 (20p11.2) [24]. The isoelectric point of cystatin C is 9.3, and the form isolated in urine has an isoelectric point of 7.8; the protein is thus positively charged in virtually all body fluids [25,26].

Methods

The experimental device SPRi-Lab+ (Horiba Scientific, Kyoto, Japan) was used to measure the biochemical samples. This device allows real-time monitoring of biomolecule interaction kinetics. It consists of an optical part (light source, conventional differential detector), a part for moving the measured fluid into the cavity and a mechanism for data evaluation (connection to a computer). An optical biochip, which is a glass prism with a deposited layer of gold, is used for the measurement. The thickness of the gold is approximately 44 nm. Surface treatment is applied to this layer to ensure that the biochemical is covalently attached to the surface.

The SPRi-Arrayer (Horiba Scientific) was used to prepare the optical biochip. The necessary biochemicals in the form of spots are applied to the optical prism. This spotting device allows the application of a biomolecule on the surface of the chip with almost zero contamination using a stainless steel or metal–ceramic capillary needle. The application of biological substances is controlled by software, so it is possible to determine exactly which substance is on a given biochip spot. It is possible to choose different numbers and sizes of spots. Up to 500 different ligands can be applied to the chip in duplicate, and the size of the spot can range from 140 to 500 μm.

To prepare the detection of cystatin C in human urine, the authors used a commercial CS biochip from Horiba Scientific with linkers bound to the NHS molecule at the end of the chain on its surface. First, antibody solutions consisting of the antihuman cystatin C mAb (anticystatin C) clone Cyst24 at a concentration of 1 g/l were prepared. Anticystatin C, the biological activity of which has been destroyed by radiation, was used as a negative control.

Antibody spots were applied to the chip surface using the SPRi-Arrayer. The layout of the antibody configuration on the biochip surface was a checkerboard, and a total of 36 spots were created (i.e., 18 anticystatin C spots and 18 mouse IgG negative control spots). The diameter of individual spots was 300 μm. Excess antibodies were neutralized with 0.1 M glycine solution, pH 2.0, for 5 min without exposure to light. The chip was then rinsed with distilled water, and the excess reactive groups on the chip's surface were deactivated by immersing it in a solution of 1 M ethanolamine, pH 9, for 15 min without exposure to light. The chip was rinsed again with distilled water. In the final phase, a 1% solution of bovine serum albumin was added to the chip, with the addition of 0.05% Tween 20 (Sigma-Aldrich, Czech Republic), which was rinsed with distilled water after 10 min. By using a solution of ethanolamine and bovine serum albumin, it was possible to prevent nonspecific interactions on the surface of the biosensor. The biochip with cystatin C therefore contained 36 spots, which were applied using a so-called four-checkerboard layout – that is, A1 (IgG) to B1 (anticystatin C) to A2 (anticystatin C) to B2 (IgG) – as can be seen in Figure 1, where two photographs of prepared biochips are shown. A total of four biochips were prepared for testing using the same technological procedure.

As can be seen in Figure 1A, although the spots are always created the same way (with the same diameter of the spotter), the resulting diameter is different, which is due to the different physical properties of the applied substances (spots A1, C1 and E1 vs B1, D1 and F1 are analogous to other spot lines). In the case of the F2 spot, it is obvious that anticystatin C was applied defectively during its formation. The preparation of biochips took place on a professional device using a precisely defined process. When preparing biochips, the procedure was always in full accordance with the prescribed procedure and in compliance with all conditions, especially with regard to temperature and humidity. A defective spot on a biochip cannot be predicted in advance and cannot be removed afterward, but such a biochip fulfills

its function nonetheless. Each spot on the biochip was tested individually, and the erroneous spot was excluded from the result. Its presence therefore had no effect on the functionality of the entire biochip. The biochip without a defective spot can be seen in Figure 1B.

The prepared biochip was first tested on a cystatin C standard, which was diluted with a 10-mM phosphate-buffered saline (PBS) solution (NaCl 0.138 M and KCl -0.0027 M), pH 7.4, at 25°C. To determine the concentration of cystatin C, it was necessary to verify that the solution functioned properly with the use of an ELISA test. The test was based on the reaction between a polyclonal antibody against human cystatin C immobilized on a microtiter plate and cystatin C from a sample. Eight different samples in the concentration, ranging from 0.001 $mg \cdot l^{-1}$ to 1000 $mg \cdot l^{-1}$, were used during the test. The preparation of reference samples was based on the method recommended by the manufacturer of the ELISA test for calibration to three specific concentration values (i.e., 500, 100 and 10 mg/l). The manufacturer supplied a reference sample with a cystatin C concentration of 500 mg/l, which was then diluted with PBS to other required levels; namely, 100 and 10 mg/l. For the SPR method, this set of three reference samples was extended by five additional concentrations in the indicated interval.

Results & discussion

The experiments themselves were divided into two groups depending on the concentration of cystatin C in the analyzed samples. Experiments measuring high concentrations of the substance are typical when it is important to determine the current condition of a patient in whom the disease has already developed and who has previously been identified. A lower concentration of the substance is characteristic of the onset of the disease and is used to detect the disease at the very beginning. These different types of measurements were performed separately, as different settings were necessary to achieve optimal sensitivity. In using biochips and the SPR method for very low concentrations of cystatin C in urine, the intent was to prove the high sensitivity of this method and the suitability of its use (it was a 'search for the limit' of this method), and it was demonstrated that even as low a concentration as 0.1 mg/l was measurable, and the SPR method was suitable for its determination.

After the initiation of the device and the initial setup, experimental measurements were performed, which followed each other seamlessly. During calibration, the authors selected the optimal angle of the incidence of light on the biochip, which, because of its structure, is an optical prism. At this fixed angle of incidence, the entire measurement is performed, which can take several hours or days (e.g., in clinical trials). The test substance was injected into the device so that it flowed through the biochip while the change in light intensity at the output of the biochip was recorded. After the injection of the substance, the association phase occurred in which the biological reaction of anticystatin with cystatin C took place. When the maximum reflectivity was reached, the process of dissociation (elution) occured. To achieve the same initial state of the biochip before the next measurement, glycine regeneration was performed. After this process, the biochip was ready for further injection of the measured substance. Three measurements performed in succession are shown in Figure 2 for concentrations of 0.1, 0.5 and 1 $mg.l^{-1}$.

In the graph in Figure 2, the first increase in the curve itself corresponds to the biochemical interaction of cystatin C and anticystatin on the biochip when testing a sample with a concentration of 1 $mg.l^{-1}$. The termination of the biochemical reaction regardless of physical phenomena is evident at the first extreme (maximum reflectivity), which occured in the tenth minute of the experiment. This is followed by dissociation phases to remove the bonds between cystatin C and anticystatin, including the application of glycine. In the event that the bonds are not completely broken, which occurs at higher concentrations of substances, glycine must be repeatedly applied to the biochip.

After reaching the maximum in the test graph of the respective sample, only PBS flowed through the cells. Thus, the cystatin and anticystatin bonds were dynamically dissociated. Everything was bound to a fixed amount of test substance; namely, 2 μl of urine. Steady state formation was not determined by time but by the amount of sample tested, cystatin concentration and flow rate. Given the maximum number of possible tests, it would seem appropriate to increase the flow rate as much as possible (the authors used a peristaltic pump), but the time to establish a bond might not be sufficient, and the functionality and accuracy of the measurement would not be ensured. For this reason, the flow rate through the cells was adjusted to 50 μl/min in all measurements, which was assessed as the most optimal on the basis of many tests and the manufacturer's recommendations.

An analogous course is characteristic of the other two areas in the graph in Figure 2; however, as a result of lower concentrations of the substance in the sample, the reflectivity occurred at lower values. The biochemical response of the sample with a concentration of 0.5 mg l^{-1} took place in the time interval between 32 and 40 min of measurement. The biochemical reaction can be detected similarly in the case of the sample with the lowest concentration.

The evaluation of the measurements of samples with low concentrations 'over one another' in a single graph is shown in Figure 3 to better compare the dependence of reflectivity on time. The graph always captures only the area characteristic of the biochemical reaction of the sample.

A similar comparison of experimental measurements is shown for higher concentrations in Figure 4. The graph shows the area of biochemical reaction, including the actuation of PBS.

When the SPR method is applied to the testing of biological samples, the test sample is injected from the loop into the cell of the SPR experimental device. The volume of the tested sample was 2 μl. Until the test sample reached the biochip, the biochip was continuously washed with PBS only. At time '0 s,' the test substance was added to the loop, and at a given flow rate, it took approximately 45 s for the substance to reach the test cell and then flow through the PBS again. The flow of the entire test sample therefore took 2.4 min.

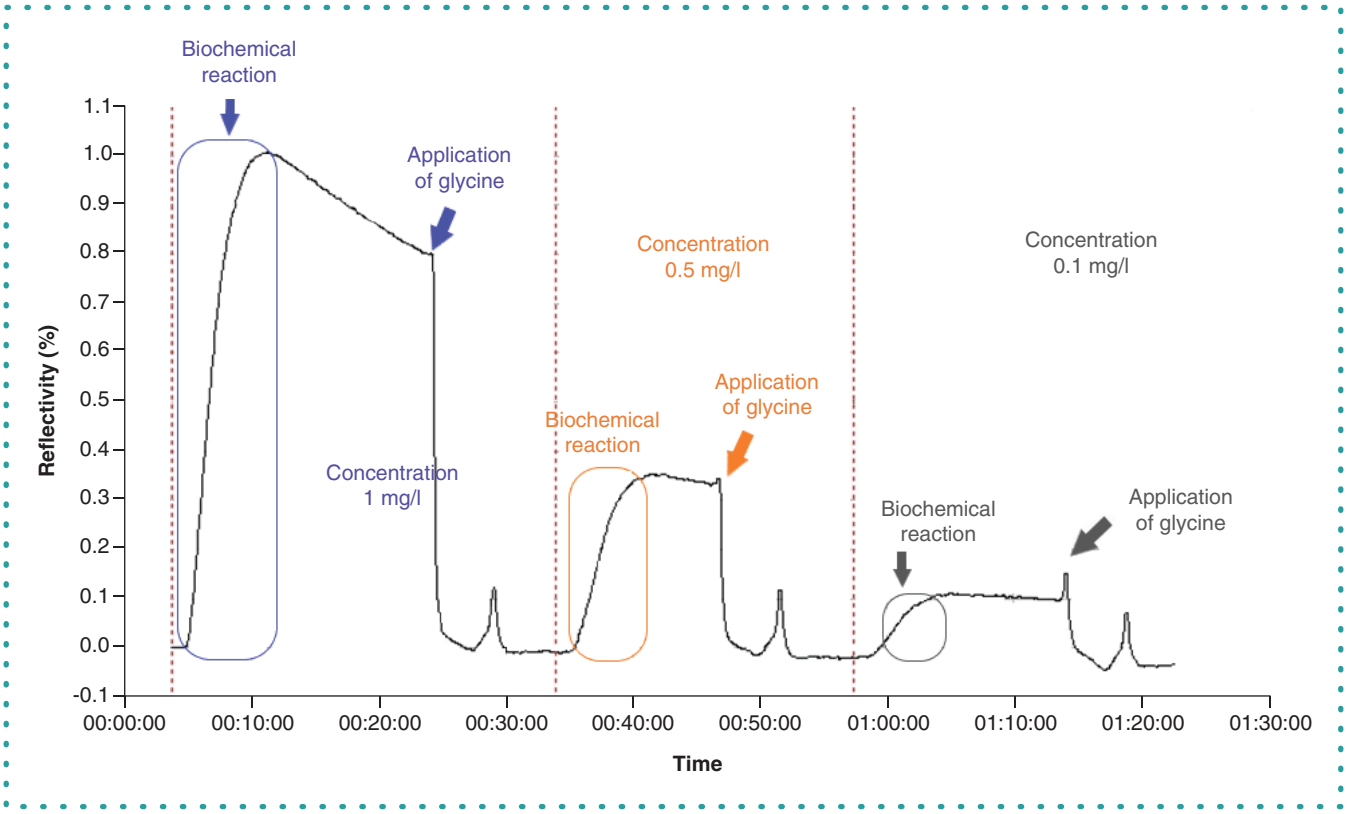

Figure 2. Recording of the entire measurement of low concentrations (1, 0.5 and 0.1 mg l^{-1}).

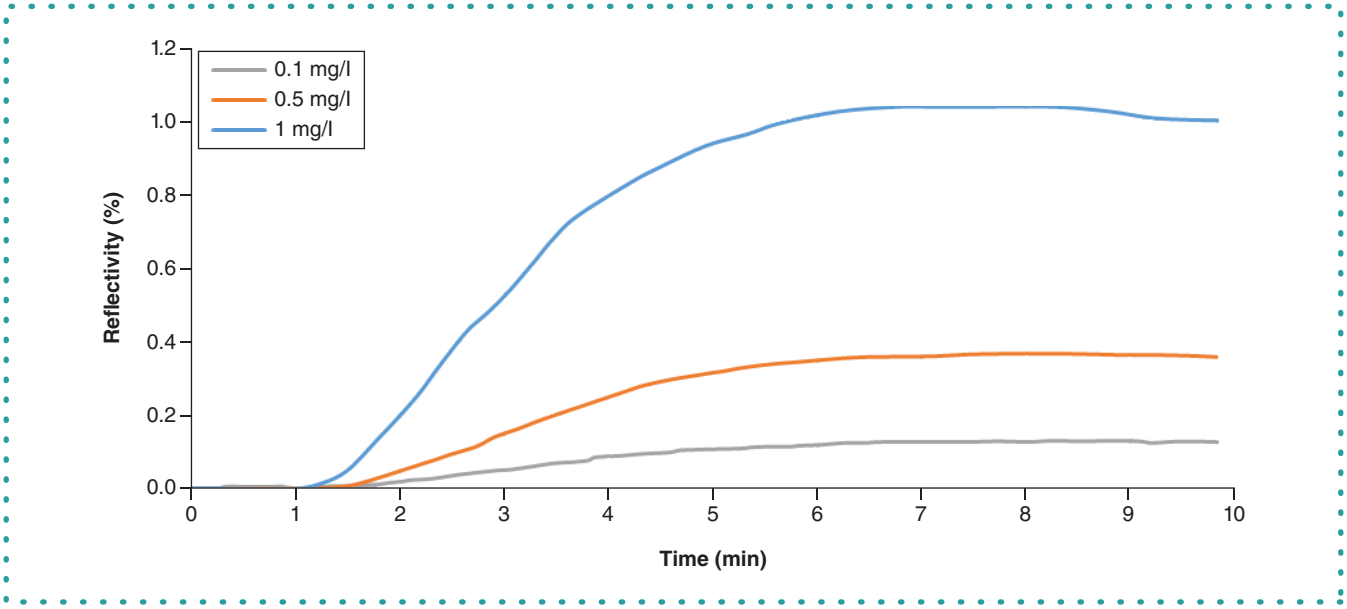

Figure 3. Comparison of low-concentration measurements with imaging of the area of biochemical reaction and dependence of the response on time in samples with lowest concentrations of cystatin C.

Testing was terminated 25 min after the start of the experiment, although to demonstrate the presence of cystatin in the sample, it was possible to terminate the measurement immediately after its binding to the biochip. However, a regeneration phase must always follow so that the biochip can be ready for testing a new sample.

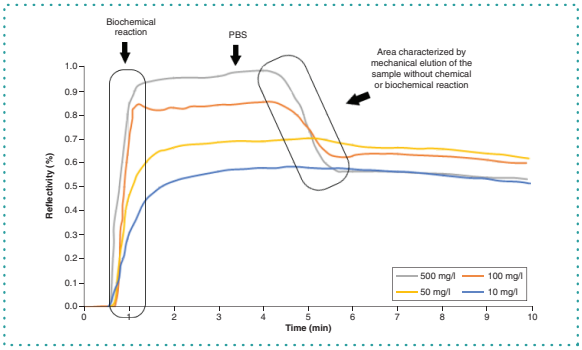

Figure 4. Comparison of the results of measurements of higher concentrations of cystatin C in urine samples.

The testing of biochips in laboratory conditions was followed by a phase of comparison with the results of the ELISA measurement method. To be able to assess the sensitivity of measurements with the help of this biochip, the authors compared the measurements with measurements performed by the University Hospital Ostrava, Czech Republic. The University Hospital Ostrava used a standard method for measurement, a commercial kit (human cystatin C ELISA kit; BioVendor, Brno, Czech Republic) they commonly used to determine cystatin C. In the authors' measurements, control samples from these kits were used for self-calibration. Each measurement needs to be calibrated. The measuring apparatus measures at a fixed angle of incidence, and this angle was set at the beginning of the measurement. Using the three different concentrations mentioned earlier (500, 100 and 10 mg/l), the authors performed a calibration that was used to evaluate the measured data. This calibration must be performed for each measurement; the calibration is dependent on the settings.

The model experiments presented here were performed on a test set of samples with defined concentrations of cystatin C over a large interval. The aim of the experiments was to determine whether the response to different concentrations would be linear; that is, whether there would be false negatives or false positives in the tested samples, which can happen in the case of the ELISA method. In the tests involving extremely high concentrations of cystatin in urine using the standard ELISA method, some samples showed false negatives; therefore, it was necessary to perform control ELISA tests with a significantly lower concentration of the biological sample. In the case of the SPR method, a linear response to the test samples was demonstrated at all concentrations; no sample from the test set or a set of patient samples showed a false response to the test.

After experiments with known cystatin C content in the samples, the testing of unknown patient samples was performed. Thanks to cooperation with the University Hospital Ostrava, experiments were performed on patient samples, and the results were subsequently compared with ELISA measurements performed in the hospital. The measurements were performed in the same way as the test experiments. For the determination of cystatin C, the results of both methods were clinically identical in the concentration range from 0.01 to 5 $mg.l^{-1}$, with an error below 1%. Cystatin C can be detected when measuring up to 1000 $mg.l^{-1}$, whereas error using the ELISA measurement is up to 5%.

A graphical representation of the comparison of samples tested by both methods is shown in Figure 5 and demonstrates a very good correlation between the obtained experimental data, as specified by the value of Pearson's correlation coefficient (r = 0.99971).

The suitability and advantage of the SPR method are also clear from an economic point of view. In the case of the preparation of a biochip, this is a one-time higher initial investment, but no further investment is required based on the subsequent long-term, repeated usability of such a biochip. The financial demands of biochip production are €160–200 for one biochip and €550 for the necessary biochemicals. If the biochip is handled carefully and all proper storage conditions are observed, a biochip prepared in this way is reusable for at least 3 months. Once prepared, the biochip does not require any additional costs, which, in comparison with a one-time ELISA test (at a price of approximately €380), demonstrates significantly the economic advantage of the SPR method. In the case of the storage of biochips in a hydrated state in PBS solution, response in each subsequent experiment is immediately maximal. In the case of dry storage, the onset of the response is somewhat slower, but increasing hydration quickly increases the biochip's sensitivity and reliability, which was confirmed by the intensive testing of a large number of biochips. The biochip pool available to the authors contains biochips that are several years old and yet continue to show consistently correct results.

Conclusion

The SPR method is a very sensitive optical method for the detection of various substances. It is used in various fields, from laboratories to medicine. Because of the relatively high sensitivity of the method and multifunctional use, SPRi analysis appears to be a very attractive detection method for clinical biochemistry.

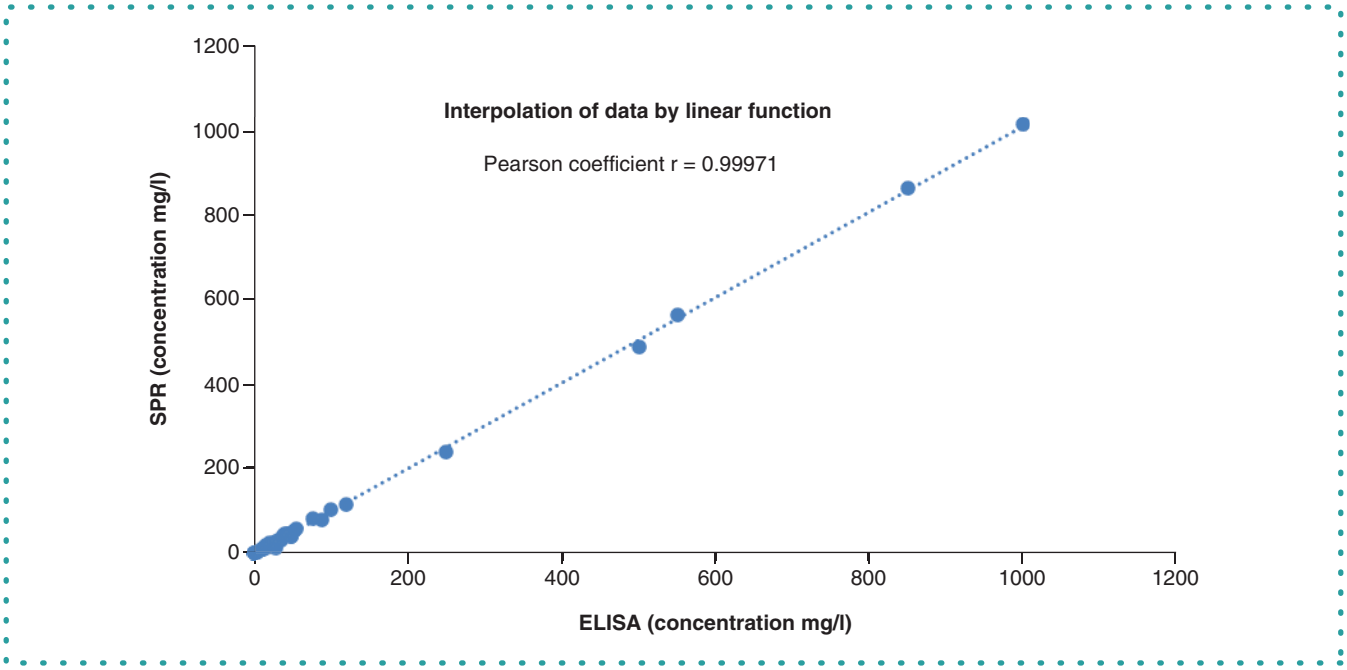

Figure 5. Comparison of SPR and ELISA methods for the determination of concentrations of cystatin C in urine samples.
SPR: Surface plasmon resonance.

The ELISA method is a validated test used in medical facilities, but the test set is single-use only. In the case of the SPR method for testing biological samples, the possibility of long-term use is an indisputable advantage. The created biochips have a higher purchase price, but when sufficient hydration is ensured and mechanical damage is eliminated, their consistent sensitivity is guaranteed for many months. The SPR method is fast, selective and repeatable.

Further research in this area focused on a set of patient urine samples. All conclusions were verified on a total of 115 samples in two phases, with a time interval of 6 months, on an identical biochip. All samples (from the original and continuing series) followed a completely identical procedure; namely, evaluation by the ELISA method in the biochemistry laboratory of the University Hospital Ostrava and subsequent performance of tests using the SPR method. All results of the SPR method showed a completely linear response for all concentrations, and none of the tests showed false negativity or positivity.

Future perspective

The SPR method described here proved to be a very powerful tool and an effective means of determining the concentration of cystatin C in biological samples. The advantage of high sensitivity and simplicity destines this method for wide clinical use in identifying a wide range of chemicals in biological samples. The use of the SPR method with biosensors can reliably replace financially and time-consuming current methods.

Author contributions

All the authors contributed to the content of the manuscript. M Lesnak and K Barcova contributed to the conception of the study, analysis and interpretation of data, drafting of the article and general supervision. D Jursa and M Miskay contributed to the acquisition of data, analysis and interpretation of data and drafting of the article. H Riedlova contributed to the drafting of the article and data interpretation. M Adamek contributed to the conception of the study and critical revision.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

All human and/or animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Executive summary

- The surface plasmon resonance (SPR) method is a very suitable analytical method for the identification of cystatin C in urine samples.
- The use of the SPR method with prepared biochips can be employed to test for a wide range of cystatin C concentrations in patient samples, from 0.1 to 1000 mg/l.
- The surface of the biochip was spotted with anticystatin C and a negative control of mice IgG at a ratio of 1:1. Each biochip prepared in this way was first verified using standard tests and then with patient samples, thus clearly confirming the required sensitivity even for very low concentrations of cystatin C.
- For tests of high concentrations of cystatin in urine via the standard ELISA method, some samples showed false negatives; therefore, it was necessary to perform new control ELISA tests with a significantly lower concentration of the biological sample. In the case of the SPR method, a linear response to the test samples was demonstrated at all concentrations; no sample from the test set or set of patient samples showed a false response to the test.
- The results of the measured urine sample concentrations by the SPR method were verified using a standardized ELISA test, and Pearson's correlation coefficient confirmed consistency (r = 0.99971).
- Preparation of biochips for testing is a one-time activity that takes about 24 h. However, a single biochip can be used repeatedly for at least several months, with use extended to several years with the correct storage process.
- Compared with the ELISA method, the use of biochips is a significantly less costly method, as ELISA tests are single-use only, whereas biochips are reusable and do not require any additional investment.
- When used in clinical practice, it is possible to shorten the test time by stopping the measurement in the initial part of the experiment immediately after the detection of cystatin C in the analyzed sample (i.e., immediately after the biochemical reaction, followed always by the wash/regeneration phase).

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