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Surface plasmon resonance imaging biosensor for cathepsin G based on a potent inhibitor: Development and applications

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ABSTRACT

A specific surface plasmon resonance imaging (SPRI) array biosensor for the determination of the enzymatically active cathepsin G (CatG) has been developed. For this purpose, a specific interaction between an inhibitor immobilized onto a chip surface and CatG in an analyzed solution was used. The MARS-115 CatG peptidyl inhibitor containing the 1-aminoalkylphosphonate diaryl ester moiety at the C terminus and *N*-succinamide with a free carboxylic function was synthesized and covalently immobilized onto the gold chip surface via the thiol group (cysteamine). Atomic force microscopy was used for the observation of surface changes during the subsequent steps of chip manufacture. Optimal detection conditions were chosen. High specificity of synthesized inhibitor to CatG was proved. The precision, as well as the accuracy, was found to be well suited to enzyme determination. The sensor application for the determination of CatG in white blood cells and saliva was shown for potential diagnosis of leukemia and oral cavity diseases during the early stages of those pathological states.

Cathepsin G (CatG,[1] EC 3.4.21.20) belongs to the serine endopeptidases family. The physiological role of CatG is mainly associated with early stages of the immune response. It is also involved in several physiological processes such as tissue degradation, platelet activation, monocyte and neutrophil chemotaxis, and proteolysis of blood coagulation factors [1]. Although a precise diagnostic of the CatG active form is necessary, it has been unsatisfactory. The activity of CatG, as well as other serine proteases, in the organisms is strictly controlled by one or more members of the serine proteinase inhibitor (serpin) protein superfamily. Uncontrolled proteolysis accompanies several diseases, with cancer, chronic obstructive pulmonary disease, and emphysema being just a few examples [2–4].

The surface plasmon resonance imaging (SPRI) technique is a promising tool for the determination of biologically active species. SPRI has been applied for the determination of β-2-microglobulin [5], mucin [6], novel protein immunostimulating factor [7], functional multiprotein complexes [7], microRNAs [8], DNA [5], cystatin C [5], cysteine cathepsins [9], cathepsins D [10,11] and E [11], and papain [9]. SPRI determination of these species is performed by the application of sensitive and specific biosensors. A strategy for SPRI biosensor development is the immobilization of the species specifically reacting with a species to be determined onto a gold chip surface. Interaction between an enzyme to be determined and its inhibitor immobilized onto the gold chip surface is frequently used for the biosensor construction. The enzyme specifically captured from the analyzed solution is subsequently determined by the SPRI.

The aim of this work was the development of a biosensor for specific CatG determination in white blood cells and saliva. MARS-115 was used as a CatG inhibitor covalently immobilized onto a chip surface. MARS-115 is a newly synthesized CatG inhibitor tailored only for this purpose. The heterobifunctional character of MARS-115 allowed for the construction of the SPRI biosensor for CatG determination; the succinamide carboxylic group was used for the covalent attachment of the inhibitor onto the gold chip surface via a thiol linker, whereas the C-terminal phosphonic warhead reacted irreversibly with the CatG active site. To verify the selectivity of the developed sensor, cathepsin B (CatB) was used. CatB belongs to

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[1] *Abbreviations used:* CatG, cathepsin G; SPRI, surface plasmon resonance imaging; CatB, cathepsin B; Tween 20, polyoxyethylenesorbitan monolaurate; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide; NHS, *N*-hydroxysuccinimide; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt; EDTA, ethylenediaminetetraacetic acid disodium salt; PBS, phosphate-buffered saline; CCD, charge-coupled device; SD, standard deviation.

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the cysteine proteinases and is the most abundant -representative of the cathepsin family.

Materials and methods

Reagents

CatG from human sputum, 2-aminoethanethiol hydrochloride (cysteamine hydrochloride), polyoxyethylenesorbitan monolaurate (Tween 20), *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (Hepes) were obtained from Aldrich (Steinheim, Germany). CatB from human liver was obtained from Calbiochem (Merck group, Warsaw, Poland). Absolute ethanol, acetic acid, ethylenediaminetetraacetic acid disodium salt (EDTA), magnesium chloride, potassium chloride, potassium phosphate, sodium acetate, sodium carbonate, sodium chloride, sodium hydroxide, and sodium phosphate were obtained from POCh (Gliwice, Poland). HBS–ES solution (0.01 M Hepes, 0.15 M sodium chloride, 0.005% Tween 20, and 3 mM EDTA, pH 7.4), acetic buffer (pHs 3.79–5.57), phosphate-buffered saline (PBS, pH 7.2), phosphate buffer (pHs 7.17–8.04), and carbonate buffer (pHs 8.50–9.86) were obtained from Biomed (Lublin, Poland). Photopolimer ELPEMER SD 2054 and hydrophobic protective paint SD 2368 UV SG-DG were obtained from Peters (Kempen, Germany). All of the reagents for buffer preparation that were used in enzyme kinetic assays were purchased from Merck. The enzymes used for inhibition studies were chymotrypsin and subtilisin (Sigma–Aldrich, Poznan, Poland), human neutrophil elastase and CatG (Biocentrum, Cracow, Poland), porcine pancreatic elastase (Serva, Polgen, Lodz, Poland), trypsin (AppliChem, Polgen, Lodz, Poland), and proteinase 3 (Elastin Products, Owensville, MO, USA). The commercially available enzyme substrates (Calbiochem) were as follows: MeO-Suc-Ala-Ala-Pro-Val-AMC, MeO-Suc-Ala-Ala-Pro-Phe-AMC, Cbz-Arg-AFC, and Suc-Ala-Ala-Pro-Phe-AMC. Substrates of proteinase 3 and CatG were prepared as described previously [12,13]. All reagents and solvents used for the solid phase peptide synthesis were purchased from IRIS Biotech (Marktredwitz, Germany). All reagents and solvents used for the synthesis of α-aminoalkylphosphonate di-(4-methylthio)phenyl ester were purchased from Sigma–Aldrich or Alfa Aesar (Warsaw, Poland). Aqueous solutions were prepared with MilliQ water (Simplicity, Millipore, Billerica, MA, USA). Whole citrated blood was provided by the Haematology Clinic of the Medical University in Bialystok, Poland, and saliva samples were provided by volunteers. All samples were provided after obtaining the consent of the bioethical commission.

Enzyme kinetics: the inhibition assays

All of the assays were performed at 37 °C. The inhibitor was freshly dissolved in dimethyl sulfoxide (DMSO) prior to use. Each tested enzyme was incubated for 10 min with the tested compound (inhibitor final concentration ranging from 5 nM to 10 μM), and then the measurement was taken for 15 min. The rate of inhibition of CatG (100 nM) was measured in 0.1 M Tris–HCl and 0.5 M NaCl (pH 7.5), inhibition of human neutrophil elastase (0.0025 U/ml) and chymotrypsin (3 nM) was measured in 0.1 M Hepes, 0.5 M NaCl, and 0.03% Triton X-100 (pH 7.5), inhibition of porcine pancreatic elastase (60 nM) was measured in 0.01 M Tris–HCl and 0.05% Triton X-100 (pH 8.5), inhibition of trypsin (25 nM) was measured in 0.1 M Hepes and 0.01 M $CaCl_2$ (pH 7.5), and inhibition of subtilisin (5 nM) was measured in 0.05 M Tris–HCl, 1 M NaCl, and 0.01% Triton X-100 (pH 7.5). All enzymes were assayed using fluorogenic substrates: MCA-Phe-Val-Thr-Gnf-Ser-Trp-Anb-NH_2 (2.5 μM, CatG), Abz-Tyr-Tyr-Abu-Anb-NH_2 (30 μM, proteinase 3), MeO-Suc-Ala-Ala-Pro-Val-AMC (60 μM, human neutrophil elastase; 100 μM, porcine pancreatic elastase), Cbz-Arg-AFC (100 μM, trypsin), and Suc-Ala-Ala-Pro-Phe-AMC (20 μM, chymotrypsin; 5 μM, subtilisin).

The standard deviation for presented values is the mean of three independent experiments and does not exceed 10%. All measurements were performed using a SpectraMax Gemini XPS spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA). Inhibition rate constants were measured using the incubation method under pseudo-first-order reaction conditions. For first-order reactions, the pseudo-first-order inhibition rate constants k_{obs} were calculated from the equation $t_{1/2} = 0.693/k_{obs}$. The $t_{1/2}$ for the inhibition reaction is obtained from plots of $\ln(v_t/v_0)$ versus time. The apparent second-order inhibition rate constants $k_{obs}/[I]$ ($M^{-1}\ s^{-1}$) are reported in Table 1. Control curves in the absence of the inhibitor were linear [14].

Chip preparation

Gold chips were manufactured as described in a previous article [15]. The gold surface of the chip was covered with photopolymer and hydrophobic paint as described previously [11]. An array of 9×12 free gold surfaces was obtained (see Fig. 1A). Using this chip, nine different solutions can be simultaneously measured without mixing the tested samples, and 12 single SPRI measurements can be performed from one solution.

SPRI measurements

SPRI measurements were performed as described in previous articles [9,11,16]. A schematic diagram of the apparatus is given in Fig. 2. The SPRI setup consists of HeNe laser (JDS Uniphase, Edmund Industrial Optics, Barrington, NJ, USA), two glass lenses (L1: $f = 3$ mm; L2: $f = 300$ mm), two polarizers (P1 and P2), mirror, prism, and charge-coupled device (CCD) camera. The chip consists of nine measuring fields, each containing an array of 12 spots. Spots are separated with photopolymer, whereas measuring fields are separated by hydrophobic paint. A picture of the chip is shown in Fig. 1A, and an image of the chip obtained by the CCD camera is shown in Fig. 1B. Briefly, the measurements were performed at a fixed angle of incident light, and the reflectivity was simultaneously measured across an entire chip surface. The contrast values obtained for all pixels across a particular sample single spot were integrated. Thus, the SPRI signal was integrated over the single spot area. A background correction was applied. National Institutes of Health (NIH) Image J software (version 1.42) was used to evaluate the SPRI images in two-dimensional form. The signal was measured twice on the basis of registered images: after immobilization of the MARS-115 inhibitor and then after interaction with CatG. The SPRI signal, which is proportional to coupled biomolecules, was obtained from subtraction between the signal before and after interaction with biomolecule for each spot separately.

MARS-115 immobilization

Chips were rinsed with ethanol and water and dried under a stream of nitrogen. They were then immersed in 20 mM cysteamine ethanolic solutions for at least 2 h. The chips were then rinsed with ethanol and water and dried under a stream of nitrogen. MARS-115 inhibitor solution, activated with NHS (50 mM) and EDC (200 mM), was placed on the amine-modified surface and incubated at 37 °C for 1 h. The prepared sensor was used only once [11,15].

Table 1
Potency of MARS-115 action toward tested serine proteases.

Enzyme	CatG	HNE	PPE	Sub	ChTry	Try	PR3
$k_{obs}/[I]$ (M^{-1} s^{-1})	52,500 ± 2500	NI	NI	4000 ± 200	NI	3100 ± 33	1000 ± 40
IC_{50} [nM]	21 ± 5	NI	NI	262 ± 13	NI	366 ± 8	1091 ± 78

Note. HNE, human neutrophil elastase; PPE, porcine pancreatic elastase; Sub, subtilisin; ChTry, chymotrypsin; Try, trypsin; PR3, proteinase 3; NI, no inhibition observed after 10 min of incubation of enzyme with MARS-115.

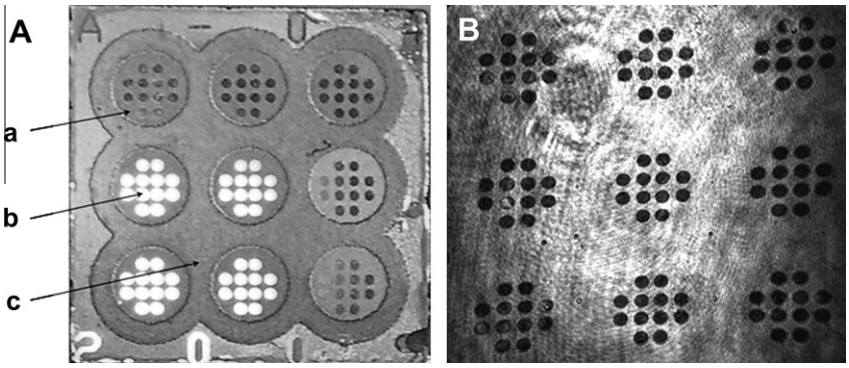

Fig.1. (A) Picture of chip (a: photopolymer; b: free gold surface; c: hydrophobic paint). (B) Image of chip obtained by CCD camera.

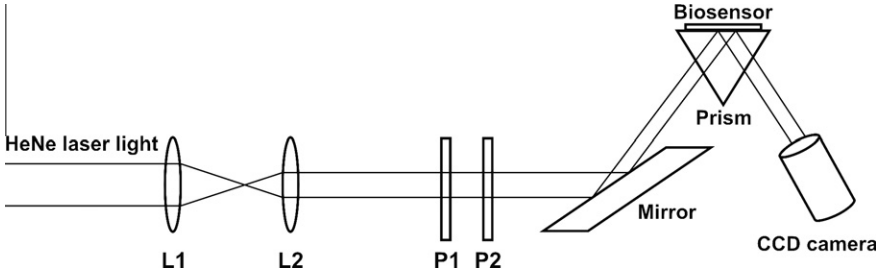

Fig.2. Scheme of SPRI setup. L, lens; P, polarizer.

Preparation of standard curve

The response of the analytical SPRI signal for CatG concentration was measured within a concentration range between 0.25 and 2.5 ng/ml. The chip surface was covered by a monolayer of cysteamine and a layer of immobilized inhibitor MARS-115 (4 μg/ml). The experiments were performed at pH 8.0. The time of interaction was 10 min because it was assessed to be optimal. Application of a longer interaction time did not lead to higher amounts of the bound enzyme.

Preparation of biological samples

White blood cells

TLE reagent was prepared from 4.143 g of ammonium chloride, 0.00175 g of EDTA, and 5 ml of 1 M Hepes in 500 ml. Whole blood (9 ml) was centrifuged at 1000*g* for 10 min. A supernatant was removed. The remaining sediment was transferred to the sterile test tubes, and 15 ml of TLE reagent was added. The whole mixture was incubated for 30 min with stirring and then centrifuged at 1000*g* for 10 min. Supernatant was removed, and 6 ml of TLE reagent was added to the remaining sediment. The whole mixture was transferred to an Eppendorf tube and centrifuged at 1000*g* for 5 min. Lysed red blood cells were removed, and 0.5 ml of TLE reagent was added to the sediment. The mixture was frozen at −80 °C. The solution of white blood cells was diluted twofold with a buffer of pH 8.0 and transferred onto the sensor surface for 10 min. The volume of the sample applied on each measuring field was 3 μl. The surface of the biosensor was then washed six times with the HBS–ES buffer. The SPRI measurement was performed. The concentration of CatG was evaluated using a calibration curve.

Saliva

Saliva (3 ml) in fasting state was centrifuged at 100*g* for 15 min, and supernatant was separated. Finally, the sample was filtered three times through a paper filter of medium density. The prepared saliva was diluted twofold with a buffer of pH 8.0 and transferred onto the sensor surface for 10 min. The volume of the sample applied on each measuring field was 3 μl. The surface of the biosensor was then washed six times with the HBS–ES buffer. The SPRI measurement was performed. The concentration of CatG was evaluated using a calibration curve.

Results and discussion

Influence of inhibitor concentration on analytical signal of CatG

The purpose of this part of the research was optimization of conditions for the CatG determination. The investigation was performed within a MARS-115 inhibitor concentration range between 0.25 and 6.0 μg/ml at a constant CatG concentration (1.0 ng/ml). A series of nine MARS-115 inhibitor solutions of increasing concentration were measured (0.25, 0.75, 1.50, 2.25, 3.00, 3.75, 4.50,

5.25, and 6.00 μg/ml). These solutions were transferred onto different points of the surface of a chip, which had been previously modified with cysteamine. MARS-115 inhibitor was immobilized during this treatment. Next, the chip was treated with the CatG solution (1.0 ng/ml) for 10 min, rinsed with water, and dried, and then the SPRI measurement was performed. The results are given in Fig. 3.

The obtained curve is a Langmuir isotherm type, with the plateau of the signal for inhibitor concentration above 3.0 μg/ml. A concentration of inhibitor equal to 4.0 μg/ml was selected as optimal for further investigation.

Influence of solution pH on interaction process

The influence of pH of the CatG solution on the SPRI signal was studied for nine different pH values (within the range of 5.0–10.5) under conditions of constant concentration of CatG (1 ng/ml) and inhibitor (4.0 μg/ml). Solutions of MARS-115 inhibitor were transferred onto different points of a chip that had been previously modified with cysteamine. In this manner, the inhibitor was immobilized onto the chip surface. The chip was then treated with CatG solutions (1 ng/ml) with different pH values for 10 min. The results are shown in Fig. 4.

The maximum of the SPRI signal for the MARS-115 inhibitor–CatG complex is for pH 8.0, and this value was selected as optimal for further investigation. The optimal pH value for SPRI measurements in the case of commercially available CatG inhibitor I turned out to be 8.0 as well, as described previously [16]. It was also described elsewhere [17] that the optimal pH for CatG on most substrates is 8.0.

Analytical response of sensor to CatG concentration: calibration curves

The response of the analytical SPRI signal for CatG concentration was measured within a concentration range between 0.25 and 2.5 ng/ml. The chip surface was covered by a monolayer of cysteamine and a layer of immobilized inhibitor (4.0 μg/ml). The experiments were performed at pH 8.0. The time of interaction was 10 min. The obtained calibration curve is shown in Fig. 5.

The calibration curves represent a Langmuir isotherm type with the dynamic response range between 0.25 and 1.5 ng/ml (0.01–0.06 nM). The plateau of the curve corresponds to saturation of active points of the sensor. The detection limit calculated on the 3-SD (standard deviation) basis is equal to 0.23 ng/ml.

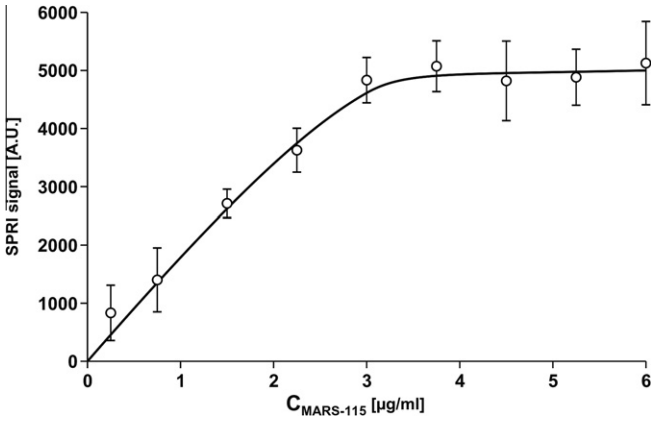

Fig.3. Dependence of SPRI signal (in arbitrary units) of MARS-115 inhibitor–CatG complex on MARS-115–inhibitor concentration. CatG concentration: 1.0 ng/ml. Initial pH of CatG solution: 8.0. Error bars were calculated at a 95% confidence level.

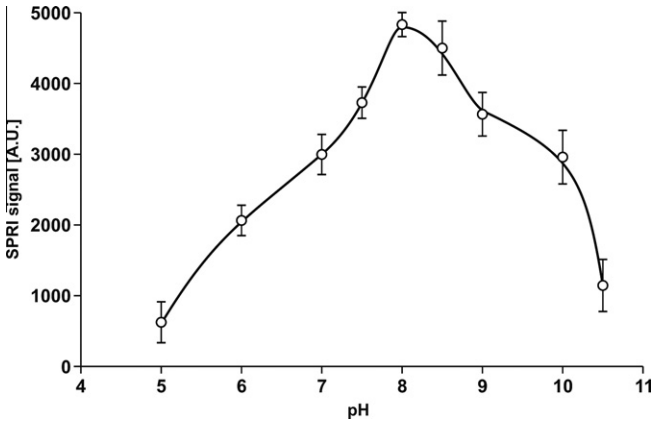

Fig.4. Dependence of SPRI signal (in arbitrary units) of MARS-115 inhibitor–CatG complex on pH. Initial inhibitor concentration: 4.0 μg/ml. Initial CatG concentration: 1.0 ng/ml. Error bars were calculated at a 95% confidence level.

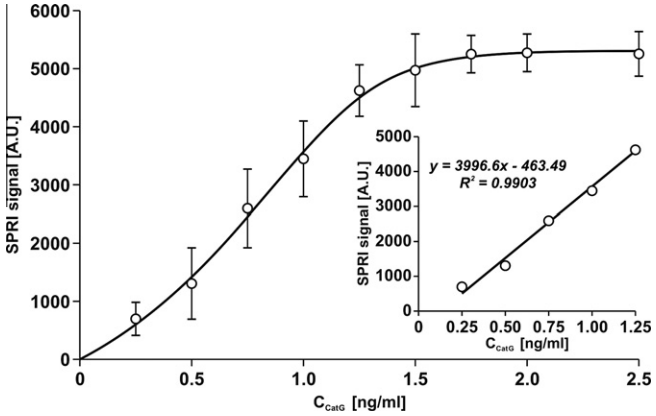

Fig.5. Dependence of SPRI signal (in arbitrary units) on CatG concentration with the application of the developed biosensor with the MARS-155 inhibitor. Initial pH value of CatG solution: 8.0. Error bars were calculated at a 95% confidence level.

Table 2
Influence of CatB on SPRI determination of CatG with developed biosensor.

CatG/CatB	Added CatG (ng/ml)	Found CatG (ng/ml)	Recovery (%)
1:1	1.00	1.01 ± 0.01	101
1:10	1.00	1.02 ± 0.01	102
1:100	1.00	1.02 ± 0.02	102
1:1000	1.00	1.02 ± 0.01	102

Note. The concentration of CatG is 1 ng/ml.

MARS-115 selectivity and potency of action: enzymatic assay

The inhibitory properties of MARS-115 against CatG and selected serine proteases are presented in Table 1. The obtained data show that MARS-115 displayed the highest potential of action toward CatG ($k_{obs}/[I]$ at 52,500 ± 2500 M^{-1} s^{-1}, IC_{50} = 21 ± 5) among all tested proteases. MARS-115 showed no activity against chymotrypsin, human neutrophil elastase, or porcine pancreatic elastase. The $k_{obs}/[I]$ values for subtilisin, proteinase 3, and trypsin were 4000 ± 200 M^{-1} s^{-1}, 1000 ± 40 M^{-1} s^{-1}, and 3100 ± 33 M^{-1} s^{-1}, respectively, whereas IC_{50} values were 262 ± 13, 366 ± 8 nM and 1091 ± 78 nM respectively.

Selectivity of MARS-115 inhibitor–CatG interaction

To be sure that the interaction between the MARS-115 inhibitor and CatG is selective and the sensor does not react with the other

Table 3
Precision of CatG concentration measurement with developed biosensor.

	Number of measurements	Added (ng/ml)	Found (ng/ml)	Recovery (%)	SD (ng/ml)	Confidence (confidence limit, 95%) (ng/ml)
CatG	24	1.00	1.00	100	0.15	0.06

Table 4
Recoveries of CatG spike in white blood cells as determined by SPRI with MARS-115 inhibitor.

	CatG concentration without spike (ng/ml)	Added CatG (ng/ml)	Found CatG (ng/ml)	Recovery (%)
1	0.84 ± 0.02	0.50	1.35 ± 0.02	100.7
2	0.98 ± 0.02	0.50	1.48 ± 0.04	99.7
3	0.79 ± 0.04	0.50	1.27 ± 0.05	98.4
4	0.97 ± 0.03	0.50	1.46 ± 0.04	99.3
5	0.90 ± 0.03	0.50	1.38 ± 0.05	98.6
6	0.94 ± 0.04	0.50	1.42 ± 0.04	98.6

enzymes of the cathepsin family, the chip containing immobilized inhibitor was treated with mixtures of CatG and CatB. Various excesses of CatB within the range between 1:1 and 1:1000 were examined. Results are shown in Table 2.

The results show no influence of CatB on the results of determination of CatG even at a 1000-fold excess. Thus, sensor high selectivity was confirmed.

The problem of the development of a specific sensor for CatG requires a specific inhibitor for this cathepsin. There is only one commercially available inhibitor of CatG, namely CatG inhibitor I, which was used in our previous research [16]. However, there are reports of developments of new potent inhibitors of CatG [18]. Construction of a biosensor with the use of a new selective inhibitor was the subject of the current study.

Precision of developed method for CatG determination

Precision of the developed method was tested under optimal conditions, that is, pH 8.0 and the MARS-115 inhibitor concentration at the chip preparation stage equal to 4.0 μg/ml. The precision of CatG determination was tested for the concentration of this enzyme equal to 1.0 ng/ml. The results are shown in Table 3.

The SD and confidence limit, assuming a confidence level of 95%, are relatively low.

Recovery of the developed method was checked by the addition of spikes of CatG into real samples of white blood cells of leukemic patients. The results are given in Table 4.

CatG determination in human fluid samples

White blood cell samples

Samples of white blood cells from seven patients suffering from different types of leukemia were analyzed for CatG determination using the developed sensor. The obtained results are given in Table 5. The concentration of CatG is given in nanograms per thousand white blood cells because different persons have a divergent number of white blood cells per unit of volume. The concentration of CatG in white blood cells of healthy individuals was below the detection limit.

Obtained results for CatG in white blood cells of patients suffering from leukemia are within the range of 0.74 to 41.65 $\times$ 10^{-5} ng/10^3 white blood cells. A wide range of obtained concentrations in Table 5 is due to different types of leukemia. The concentration of CatG in white blood cells of healthy individuals is below the detection limit of the developed method.

Table 5
Concentration of CatG in white blood cells of patients suffering from leukemia as determined by SPRI with MARS-115 inhibitor.

Patient number	Leukemia type	Mass of CatG in white blood cells $\times$ 10^5 (ng/10^3 white blood cells)
1	AML	34.55 ± 3.10
2	CLL	10.82 ± 1.03
3	CML	0.74 ± 0.27
4	CLL	10.50 ± 1.91
5	AML	41.65 ± 2.26
6	CLL	21.58 ± 1.24
7	CEL	29.32 ± 5.18

Note. AML, acute myelogenous leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; CEL, chronic eosinophilic leukemia.

Table 6
Concentration of CatG in saliva of healthy donors within age range of 19 to 24 years and over age 75 years as determined by SPRI with MARS-115 inhibitor.

Sample number	CatG concentration (ng/ml)	
	Donors within the age range of 19 to 24 years	Donors over age 75 years
1	1.75 ± 0.02	3.54 ± 0.02
2	2.26 ± 0.03	3.28 ± 0.04
3	1.16 ± 0.29	3.36 ± 0.05
4	1.73 ± 0.03	3.39 ± 0.17
5	1.77 ± 0.26	3.27 ± 0.02
6	1.28 ± 0.21	3.31 ± 0.04

Saliva samples

CatG was determined in six samples of saliva to demonstrate the developed sensor's applicability for this purpose. Results are shown in Table 6.

The range of CatG concentrations in the saliva of donors with an age range of 19 to 24 years is between 1.25 and 2.17 ng/ml, whereas donors over the age of 75 years present a concentration between 3.18 and 3.51 ng/ml. The changes of CatG activity in saliva can play an important role in the pathobiochemistry and diagnostics of salivary gland, gingiva, and oral mucosa diseases. It is worth stressing that an elevated CatG concentration in saliva can be a symptom of oral cavity diseases [19].

Conclusions

A specific SPRI array biosensor for the determination of active CatG has been developed. The sensor works on the basis of a highly specific reaction between immobilized inhibitor and CatG in solution. The MARS-115 CatG inhibitor containing the 1-aminoalkylphosphonate diaryl ester moiety joined with the carboxyl end group was synthesized for this purpose.

Under optimized conditions, the sensor exhibits specificity to CatG, and the precision and accuracy are well suited to enzyme determination. The sensor can be used for the determination of CatG in white blood cells and saliva. The concentration of CatG in white blood cells of healthy individuals was below the detection limit of the developed method, whereas patients suffering from leukemia had a CatG concentration within the range between 0.74 $\times$ 10^{-5} and 41.65 $\times$ 10^{-5} nanograms per 10^3 white blood cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2012.01.033.

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