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An electrochemical biosensor for direct detection of hepatitis C virus

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ABSTRACT

This paper is aimed at the development of a biosensor for direct detection of Hepatitis C virus (HCV) surface antigen: envelope protein (E2). A recombinant LEL fragment of biological cell receptor CD81 and two short synthetic peptides imitating the fragment of LEL sequence of CD81 (linear and loop-like peptides) capable of specific binding to E2 were tested as molecular recognition elements of the biosensor. For this purpose the selected ligands were immobilized to the surface of a screen-printed electrode utilized as an electrochemical sensor platform. The immobilization parameters such as the ligand concentration and the immobilization time were carefully optimized for each ligand. Differential pulse voltammetry used to evaluate quantitatively binding of E2 to the ligands revealed their similar binding affinity towards E2. Thus, the linear peptide was selected as a less expensive and easily prepared ligand for the HCV biosensor preparation. The resulting HCV biosensor demonstrated selectivity towards E2 in the presence of interfering protein, conalbumin. Moreover, it was found that the prepared biosensor effectively detected E2 bound to hepatitis C virus-mimetic particles (HC VMPs) at LOD value of $2.1 \cdot 10^{-5}$ mg/mL both in 0.01 M PBS solution (pH 7.4) and in simulated blood plasma.

1. Introduction

Hepatitis C is one of the most common liver diseases, affecting more than 130 million people worldwide [1,2]. Most often, hepatitis C is almost asymptomatic and develops for several years. In 75% cases the disease becomes chronic. Hepatitis C is caused by hepatitis C virus (HCV) and often patients do not even suspect that they have been infected with HCV for decades, and when the first symptoms begin to appear, the liver has already been destroyed [3,4].

HCV is usually diagnosed using methods based on the determination of antibodies to the virus (anti-HCV) or viral RNA in blood [4,5]. Due to the fact that anti-HCVs are produced not earlier than in 2–4 weeks after the manifestation of the clinical symptoms at acute course, in months at asymptomatic course, and are not produced at all at cyclic form due to the low concentration of elements of the virus in the blood, the first diagnostic method is not sufficiently effective at the early stages of infection [5]. In addition, the testing for anti-HCVs couldn't differentiate between the current or past infection. Unlike this method, the determination of viral RNA in blood is more accurate. However, both of these detection techniques require extensive technical steps, laboratory-based procedures, technical experts, and long assay protocols, which limits their implementation for inexpensive and fast analysis procedure [6].

The latest breakthrough in the diagnosis of early HCV infection is the detection of the HCV core antigen present at an early stage or before seroconversion [7–9]. Recently, the HCV core antigen assay became commercially available as a cost-effective alternative to PCR-tests [9]. Although the correlation between HCV core antigen tests and PCR assays was demonstrated, suggesting their potential to replace PCR in settings with high HCV prevalence, it might not be adequate in monitoring the treatment response [9,10]. An alternative method for HCV diagnostics can be a detection of HCV surface antigen, i.e an envelope protein E2 that is known to be associated with the infectious particles and could represent a promising approach to estimate the real infectious dose [11–13]. Thus, an assay suitable for quantifying infectious HCV particles using aptamers against HCV E2, where the readout value of HCV linearly correlates with the infectious dose of HCV sample was reported [11,12]. Another approach for detection of HCV E2 protein has been successfully realized by 3D biochips prepared by the covalent immobilization of fragments of cell receptor CD81 spotted onto the surface of macroporous monolithic platforms [14].

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Nevertheless, the development of a highly effective and simple method for early diagnosis of HCV still remains an urgent task to date not only because HCV associated morbidity and mortality, but also due to the importance of early diagnosis for HCV successful treatment. One of the promising bioanalytical tools for rapid and sensitive detection of target components in biological fluids is a biosensor-based technique.

Development of biosensors for medical diagnostics is one of the rapidly growing areas of modern science. Biosensors are devices whose operating principle is based on biological recognition, i.e. on the ability of the analyte to form an affinity complex with a ligand immobilized on the surface of a transducer [15]. Among them electrochemical biosensors represent a great analytical tool for real-time diagnostics due to the advantages of being miniaturized and portable, using small sample volumes and having good selectivity [16]. They can be used as point-of-care devices for the detection and monitoring of different disease markers [17–21]. The electrochemical HCV-biosensors reported are mainly aimed to recognize either the viral RNA [22–26] or HCV-specific antibodies generated by a person's immune system [27–30]. Thus, for example, the electrochemical sensor for the determination of bovine leukemia virus envelope glycoprotein gp51 as well as the sensor utilizing this viral protein as recognition layer have been reported [31,32]. There are few reports on the electrochemical immunosensor for detection of HCV core antigen [33,34]. However, to the best of our knowledge, there is no report on the electrochemical sensor for direct detection of HCV surface antigen: envelope protein E2.

In this work, we report on the development of an electrochemical biosensor capable of detecting a HCV surface antigen: envelope protein E2. To select an appropriate antigen-specific ligand that will serve as a molecular recognition element of the electrochemical sensor, the known mechanism of HCV interaction with the host cell receptor (CD81) was taken into account [35,36]. In this process only the large extracellular loop (LEL) of CD81 is responsible for interaction with E2. LEL represents a protein fragment consisting of amino acids 116 to 202 of CD81 in which the peptide sequence 176–189 (Pro-Ser-Gly-Ser-Asn-Ile-Ile-Ser--Asn-Leu-Phe-Lys-Glu-Asp) is known to be a key one (Fig. 1) [37]. Thus, we aimed to utilize the fragments of CD81 cell receptor, namely, recombinant LEL and two short synthetic peptides: 14-amino acid sequence imitating the fragment 176–189 of LEL (linear peptide) and 15-amino acids peptide similar to the linear peptide, but containing two palmitate groups for native configuration obtaining (Palm)Pro-Ser--Gly-Ser-Asn-Ile-Ile-Ser-Asn-Leu-Phe-Lys(Palm)-Glu-Asp-Lys (loop-like peptides), as molecular recognition elements for direct detection of E2 envelope protein of HCV.

Screen-printed electrode (SPE) was chosen as an electrochemical sensor transducer for immobilization of ligands: recombinant LEL, linear peptide and loop-like peptide. The performance of the prepared HCV-biosensors was tested against the binding of free E2 protein as well as hepatitis C virus-mimetic particles (HC VMPs).

2. Experimental

2.1. Chemicals and materials

All chemicals (3-mercaptopropionic acid, 99% (MPA), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), bovine serum albumin (BSA, 66 kDa), NaCl, lactic acid (LA), methyl ether poly(ethylene glycol)-5000 (PEG-5000)) were obtained from Sigma-Aldrich. Sulfuric acid was provided by Lach-ner (Czech Republic). The chemicals were of analytical standard and were used without further purification. All aqueous solutions were prepared in ultrapure water (Millipore, USA). Phosphate buffered saline (PBS) solution (0.01 M, pH 7.4) and 2-(N-morpholino)ethanesulfonic acid (MES) solution (0.01 M, pH 6.0) were used to prepare the immobilization and analyte solutions.

Gold-coated glass slides (Au-slides) of 76 × 26 mm and gold layer thickness of 200 nm were purchased from Ssens bv (Netherlands). SPEs consisting of a circular (diam. 1 mm) gold working electrode, Ag/AgCl reference and the gold counter electrodes, were purchased from BVT technologies, a.s, (Czech Republic).

Recombinant fragment LEL was purchased from ServiceGen (St. Petersburg, Russia). The amino acid derivatives used for solid-phase peptide synthesis were the products of Iris Biotech (Marktredwitz, Germany). Trifluoromethanesulfonic acid (TFMSA), diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), thioanisole,

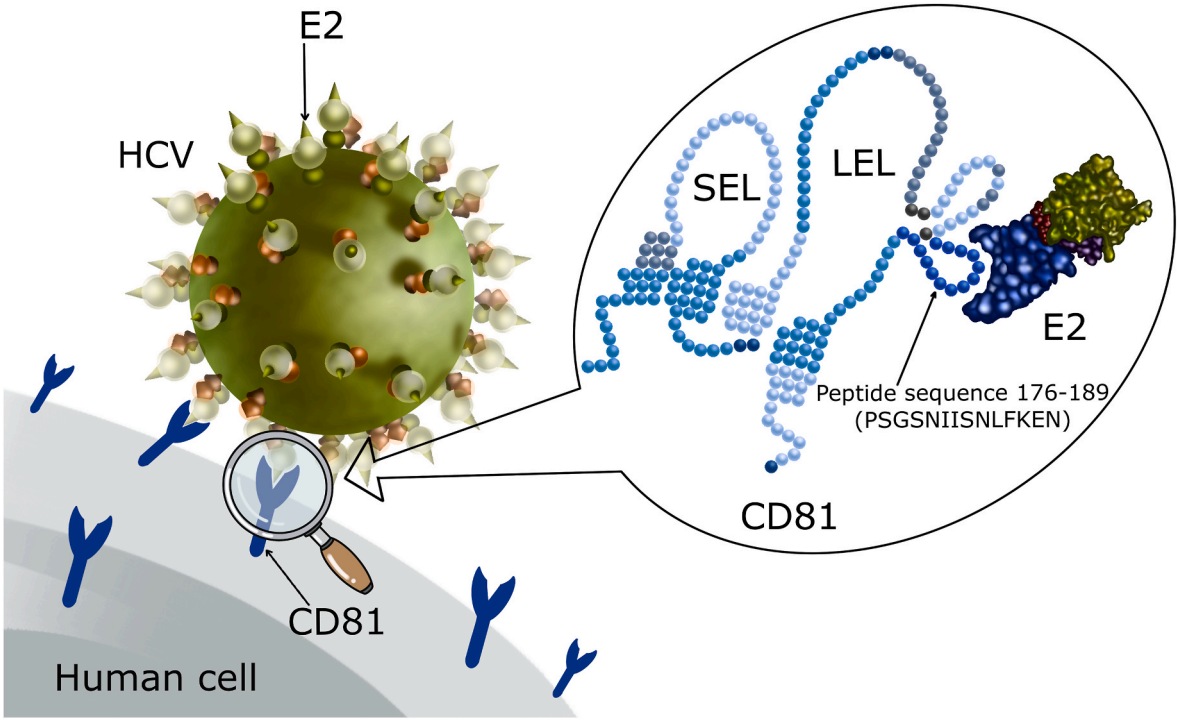

Fig. 1. Schematic representation of interaction of HCV with a host cell receptor CD81.

ethanedithiol were purchased from Sigma–Aldrich (Taufkirchen, Germany) and used as received. Trifluoroacetic acid (TFA) was the product of Panreac (Barcelona, Spain). All solvents used for the synthesis were purchased from Vecton (St. Petersburg, Russia) and redistilled prior to use according standard procedures for their purification. 4-methylbenzhydrylamine resin (200–400 mesh, 0.70–1.30 mmol/g) received from Bachem (Bubendorf, Switzerland) was used as a solid support for peptide synthesis. For prepurification of peptides Sephadex G-15 (Merck, Darmstadt, Germany) was applied. HPLC analysis of peptides was carried out with use of Phenomenex C18 column (4.6 × 150 mm, 5 μm) (Phenomenex, Torrance, CA, USA) for analytical mode and Nucleosil C8 (21 × 250 mm, 5 μm) (Macherey-Nagel, Düren, Germany) for preparative chromatography.

The copolymer of poly(D,L-lactic acid)-*b*-poly((ethylene glycol)-5000 methyl ether) (PLA-*b*-PEG, $MW = 133000$, $Đ = 1.4$) was prepared in the Institute of Macromolecular Compounds of Russian Academy of Sciences.

2.2. Preparation of synthetic peptides and virus-mimetic particles

2.2.1. E2 preparation

HCV viral envelope protein E2 was prepared according to the protocol described earlier [14]. In order to avoid the agglomeration of protein and stabilize its structure E2 was modified with a superfolder green fluorescent protein (GFP). The molecular mass of the prepared E2-GFP was 54 kDa as determined by SDS-PAAG electrophoresis and confirmed by mass spectrometry analysis showed the presence of the peptides of the E2 sequence.

2.2.2. Synthesis of peptides

Linear and loop-like peptides were synthesized by the solid phase method using the Boc/Bzl-strategy. For condensation, the activated ester method was used. Amino acid hydroxybenzotriazolyl esters were prepared using DIC and HOBt. To obtain a loop-like peptide, a pre-synthesized Palm-e-Lys derivative was introduced into the peptide chain. To condensate the amino acids, a 3-fold excess of 1-hydroxybenzotriazolyl ester of corresponding amino acid obtained in situ was applied. The acylation was carried out in DMF for 2×1.5 h. BOC-deprotection was performed with the use of 20% TFA/dichloromethane solution (2 × 20 min).

The cleavage of peptides from the polymer resin was carried out using trifluoromethanesulfonic acid (1 mL) in TFA (10 mL) in the presence of scavengers: 1 mL of thioanisole and 0.5 mL of ethanedithiol (all amounts per 1 g of peptidyl polymer) for 1 h under ice cooling and 1.5 h without cooling under vigorous stirring. Then, 100 mL of diethyl ether was added and the precipitate formed was filtered off. The resulting peptides were dissolved in 10 mL of TFA, filtered to remove the resin and precipitated in 100 mL of dry diethyl ether. The precipitated products were filtered off, washed with diethyl ether and dried in vacuo. Peptides were pre-purified by gel-filtration on the Sephadex G-15 packed column with the use of 50% aqueous solution of acetic acid. Purification of peptides were carried out by reverse-phase HPLC using a linear gradient of acetonitrile in 0.1% TFA (water/acetonitrile mixture from 10 to 60% for 50 min) at a flow rate of 1 mL/min. The purified peptides were lyophilized and analyzed by mass-spectrometry method (detected as $[M+2H]^{2+}$). Molecular masses of both peptides were found to be 1519.79 and 2124.97 that coincided with theoretically calculated values (1519.78 and 2124.96, respectively).

The HPLC analysis and purification of peptides were performed with the use of following chromatographic systems: Shimadzu LC-20 supplied with a diode-matrix detector (Kyoto, Japan) and ÄKTA Pure 25 system with a spectrophotometric detector (GE Healthcare, Chicago, IL, USA).

2.2.3. Preparation of hepatitis C virus-mimetic particles

Hepatitis C virus-mimetic particles (HC VMPs) were prepared according to the protocols described earlier [14]. Firstly, the polymer particles were synthesized by the nanoprecipitation method of 0.5 mg/mL of poly(lactic acid) - poly(ethylene glycol) (PLA-PEG 500) copolymer solution in the mixture of acetonitrile:water = 1:5. As a result, polymer particles with size of 55 nm and polydispersity index of 0.07 were obtained.

For covalent attachment of E2 to the polymer particles the following modification of the particle surface was performed: (1) generation of carboxyl groups by the method of alkaline hydrolysis in 0.01 M NaOH, (2) activation the group by 50 mM NHS and 50 mM EDC in 0.1 M MES (pH 6.0) for 30 min and (3) covalent immobilization of E2 in concentration of 30 μg per 1 mg of particles. After the modification average particle size was equal to 76 nm, and the polydispersity index of 0.237 was found.

2.3. Preparation of HCV-biosensors

Three different ligands: recombinant LEL fragment and two synthetic peptides (linear and loop-like), were immobilized on the surface of SPEs resulting in three types of sensors: HCV-S1, HCV-S2 and HCV-S3, respectively.

Each ligand was immobilized on the working electrode of SPE according to the following procedure. Preliminary, the working electrode was electrochemically activated in 0.1 M sulfuric acid solution by imposing the potential between 0.1 and 1.15 V vs Ag/AgCl at a scan rate of 100 mV/s for 15 cycles. The ligand was covalently immobilized by accomplishing the following steps (Fig. 2): (1) attachment of MPA linker by applying 220 mM MPA ethanolic solution for overnight; (2) activation of free carboxylic groups (-COOH) of the linker with 50 mM EDC and 50 mM NHS in 0.1 M MES (pH 6.0) for 30 min to obtain an amine-reactive NHS-ester moiety; (3) incubation in a solution containing a fixed concentration of the ligand.

To preserve the active conformation of the ligands the immobilization of LEL and linear peptide was performed in 0.01 M PBS (pH 7.4) at 25 °C, while the loop-like peptide was anchored in tetrahydrofuran (THF) containing 10% 0.01 M PBS (pH 8.0). The incubation time varied in the range of 20 and 120 min, respectively. Finally, the ligand-modified SPEs were washed in 0.01 M hydrochloric acid to remove the non-covalently attached peptides, followed by the incubation in 0.1% BSA solution in 0.01 M PBS (pH 7.4) for 30 min to prevent subsequent non-specific adsorption of proteins.

To ascertain the ligand immobilization, the ligand-free sensor was prepared and tested as a reference. For this purpose MPA was immobilized on the surface of SPE, activated via EDC/NHS-chemistry and incubated in 0.01 M PBS (pH 7.4) containing 0.1% BSA for 30 min. Then the ligand-free and HCV-S1 biosensors were incubated in the E2 solution in 0.01 M PBS (pH 7.4) with concentrations of 0.01, 0.1 and 0.3 mg/mL for the evaluation of ligand attachment.

Differential pulse voltammetry (DPV) was used to characterize each step of the biosensor preparation. The DPVs were performed on an electrochemical workstation (Reference 600, Gamry Instruments, USA) with help of three-electrode configuration of SPEs. A drop of a solution containing 1 M of KCl and 4 mM of the redox probe ($K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$) was placed on SPE in that way to cover all its electrodes and DPVs were recorded in the potential window between 0 V and 0.4 V with a pulse amplitude of 0.025 V, a pulse width of 0.05 s, a step potential of 0.005 V and sample period 0.1 s. Every measurement was performed in triplicates to confirm its reproducibility.

2.4. Binding and selectivity studies

The prepared biosensors HCV-S1, HCV-S2 and HCV-S3 were characterized in terms of their affinity and selectivity towards E2 and HC VMPs by DPV measurements. First, the optimal incubation time to interact with E2 protein and HC VMPs was determined. For this purpose 5 μL of 0.1 mg/mL of analyte solutions in 0.01 M PBS (pH 7.4) was spotted on the sensing surface of SPE and incubated for 10, 20, 40, 60,

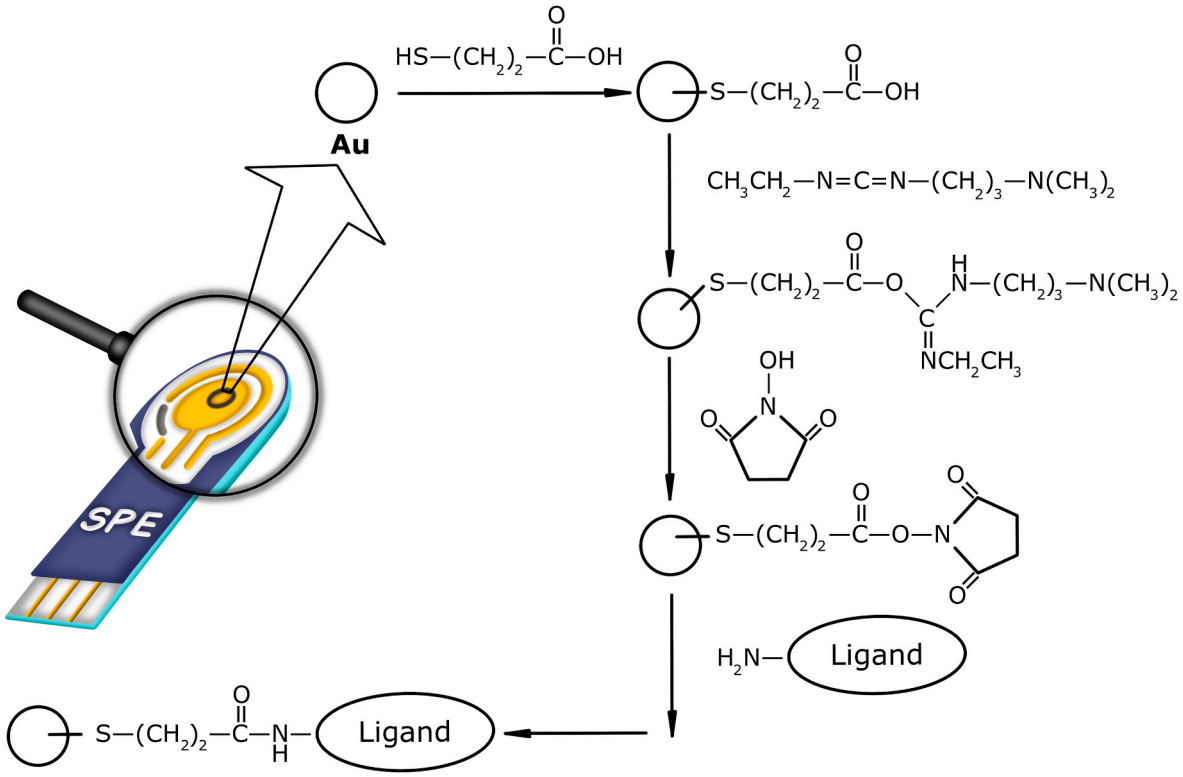

Fig. 2. Schematic protocol for HCV biosensor preparation on SPE. The ligand is either LEL, the linear or loop peptides.

90, 120 and 180 min in the dark at 25 °C. Then the biosensors were incubated in the analyte solutions containing increasing concentrations of E2 protein from 0.001 to 0.3 mg/mL under physiological conditions (0.01 M PBS (pH 7.4)), as well as in simulated blood plasma (8% BSA in a physiological solution). The incubation was carried out in the dark at room temperature for optimal time previously determined. Finally, the biosensors were washed with a working buffer (0.01 M PBS (pH 7.4) or simulated blood plasma), dried under air flow and then analyzed using DPV.

The DPV measurements were performed similarly to that in section 2.3. The DPV peak currents were normalized according to Eq. (1) to obtain response signals (I_n) of the biosensor:

$$I_n = (I_0 – I)/I_0 \quad (1)$$

where, I_0 and I are the peak currents at zero analyte concentration and at any E2 concentration, respectively.

Langmuir and Langmuir-Freundlich equilibrium binding model was applied to fit the adsorption isotherms in order to determine the binding parameters such as K_D, I_{max} and m [38]:

$$I_n = I_{n.max}C/(K_D + C) \quad (2)$$

$$I_n = I_{n.max}C^m/(K_D + C^m) \quad (3)$$

where, I_n and $I_{n.max}$ are sensor responses at concentration C and maximum adsorption, respectively, m is the heterogeneity index and K_D is the equilibrium dissociation constant.

The limit of detection (LOD) and limit of quantification (LOQ) were determined from the linear regression of the calibration plot (Fig. 7) according to Eqs. (4) and (5):

$$LOD = 3S_{y/x}/b \quad (4)$$

$$LOQ = 10S_{y/x}/b \quad (5)$$

where, $S_{y/x}$ is the standard deviation of the regression residuals and b is the slope of the regression line.

To assess the selectivity of the biosensors, their responses against the target analyte (E2) and a non-target analyte (conalbumin) were compared after incubation in 0.01 M PBS (pH 7.4) containing the different concentration of the mentioned proteins (0.01, 0.1 and 0.3 mg/mL). The reproducibility of analytical results was estimated with performing all the measurements in three-fold repetition, that was taken into account when calculating the error in the parameters derived from fitting the binding isotherms.

3. Results and discussion

3.1. Preparation of HCV-biosensors

The sensing concept used in this study relies on a redox marker present in the solution, whose access to the electrode surface becomes hindered upon analyte binding to the sensor surface. However, the amount of a ligand, immobilized beforehand, can also hinder access of a redox marker to the electrode surface, leading to a situation where following analyte binding would not produce well discernible signal inhibition to provide a reliable correlation with concentration of the analyte. Therefore, for such biosensors to achieve their optimal analytical performance, balance between the amount of surface-immobilized ligand and the resulting signal inhibition is crucial.

For that purpose a study on stability of MPA SAM on the surface of SPE was performed (see section S1 for details). It was found that the holding of MPA-modified SPE in the redox probe solution caused partial electrochemical desorption of the thiolate layers, but this process was stabilized around 60 min and thus, this time was selected for further ligand immobilization.

The amount of the ligand on the sensor surface needed for optimal sensing of the analyte was determined by varying incubation time of MPA-modified SPE in 0.01 M PBS (pH 7.4) containing 0.1 mg/mL recombinant LEL fragment. Fig. 3A shows that the sensor response (I_n) increases along with incubation time indicating that the ligand attaches the SPE surface. The most significant gain of I_n (25%) occurred after 20

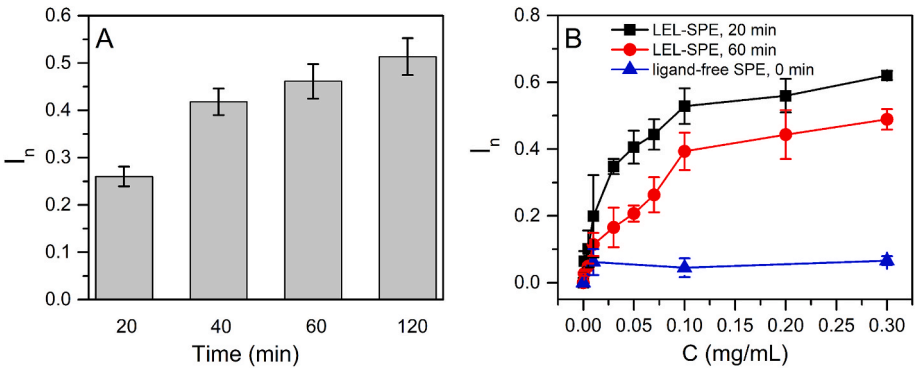

Fig. 3. Optimization of immobilization of LEL on MPA-modified SPE. (A) Normalized DPV responses of MPA-modified SPE with immobilized LEL (0.1 mg/mL) for 20, 40, 60 and 120 min and (B) adsorption isotherms of MPA-modified SPE and ligand-free SPE incubated for 20 and 60 min in target E2 in 0.01 M PBS (pH 7.4) with concentration range 0.01–0.3 mg/mL.

min of incubation followed by gradual saturation.

Fig. 3B confirms that the incubation process leads to attachment of LEL. This is indicated by the significant response of LEL-SPEs against target analyte (E2) as compared with the LEL-free SPEs showing almost no activity in the same matrix. Moreover, incubation up to 20 min made the SPE the most responsive towards E2. Obviously, the presence of extra LELs on the sensing surface does not provide additional advantages in subsequent sensing of the analyte since the activity of $[Fe(CN)_6]^{3-/4-}$ redox system on the SPE/solution interface is suppressed. Therefore, 20 min was selected as the optimal incubation time for attachment of the LEL from 0.1 mg/mL.

The very same optimizations were carried out for the other ligands, linear and loop-like peptides. It was found that for immobilization of the linear peptide the optimal conditions are the same with that for LEL. However, the loop-like peptide in these conditions tended to form micelles. Indeed the peptide contains only one amino group and two hydrophobic residues of palmitic acid, thus favouring self-assembly in water media. This side-process affects negatively on the reaction of the amino group of peptide with the activated ester groups localized on the electrode surface. Therefore, to ensure a sufficient amount of the loop-like peptide on the chip the immobilization was carried out from 0.1 mg/mL peptide solution, but prepared in mixture of THF:0.01 M PBS (pH 8.0) = 9:1 where tetrahydrofuran was a particle destroying agent. At this condition the formation of micelles was minimized.

As a result, three types of biosensors (HCV-S1, HCV-S2 and HCV-S3) having three different immobilized ligands, namely recombinant LEL fragment, linear peptide and loop-like peptide, respectively, were prepared for the following study of their interaction with E2 and HC VMPs.

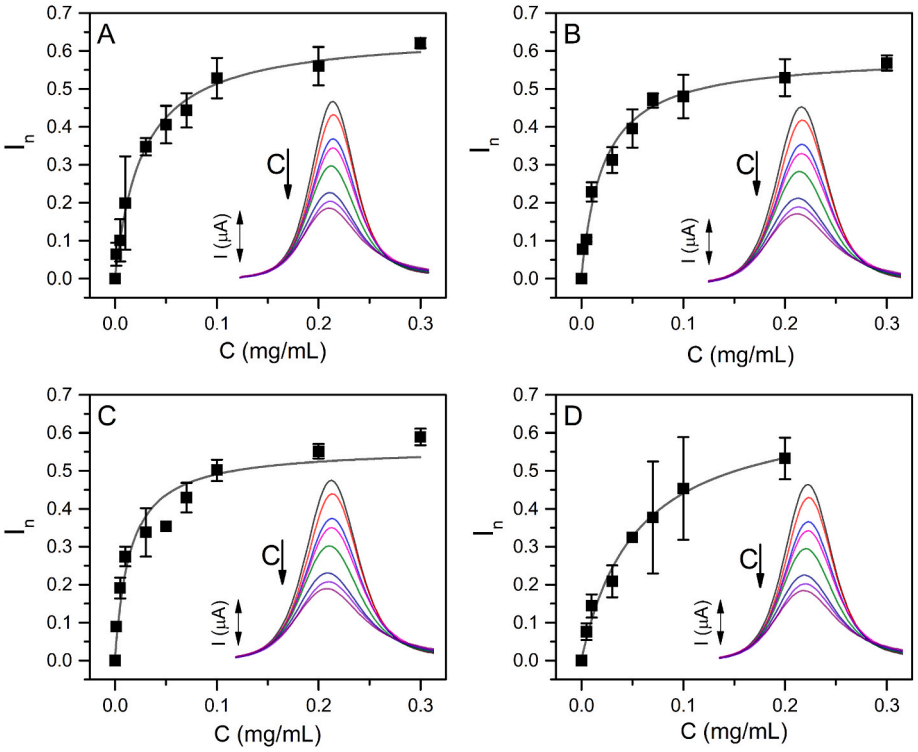

Fig. 4. Adsorption isotherms of E2 on (A) HCV-S1, (B) HCV-S2 and (C) HCV-S3 in 0.01 M PBS (pH 7.4) and on (D) HCV-S2 in the simulated blood plasma. The solid lines represent the fit of the adsorption isotherms to the Langmuir model. The inserts show DPV responses (I, μA) of the corresponding sensor to the increasing concentration of E2.

3.2. Characterization of biosensors

3.2.1. Interaction with E2

Kinetics models are vital to study bimolecular interactions between an analyte and a ligand immobilized on a sensor surface [39,40], however, in current research, to measure these interactions the sensors were preliminary transferred to the redox probe solution making it impossible to follow the process continuously. Thus, to study the interaction of E2 with the ligands the equilibrium affinity assay was employed. At the optimized conditions (see section S2 for details) all sensors demonstrated a saturated binding isotherm (Fig. 4). The isotherms were fitted to Langmuir model of adsorption and the parameters derived from the fitting are presented in Table 1.

As it is seen from the R^2 values, the Langmuir models give an accurate fit to the experimental data. The calculated K_D and I_{max} values for three biosensors were found to be approximately the same, indicating the similar binding affinities of three types of immobilized ligands: recombinant LEL fragment and its synthetic analogues (linear peptide and loop-like peptide), towards E2.

The high affinity of some short peptides and CD81 to E2 has been reported to be comparable [41–43]. The cyclic peptides demonstrate sometimes higher binding affinity than linear analogues [44]. However, this study did not reveal differences against E2 between HCV-S2 and HCV-S3 biosensors bearing linear and loop-like peptides, respectively.

The similarity of the binding affinities of the HCV-S1 and HCV-S2 biosensors towards E2 allowed us to conclude that the use of the linear peptide as a cheaper and easily prepared ligand is a more prospective approach to the preparation of a HCV biosensor. Thus, all further experiments in this study were performed with HCV-S2 biosensor.

The human blood plasma represents a complex biological fluid containing a number of proteins and admixtures of low-molecular compounds. Thus, the real samples for viruses detection may contain other molecules different from the target. These interfering molecules can affect the performance of the biosensor through cross-reactivity with the binding sites. To assess the behavior of the HCV biosensor in real conditions, binding experiments were repeated in simulated blood plasma (Fig. 4D). As it can be seen, saturated response I_{max} were similar and equal 0.59 ± 0.02 and 0.68 ± 0.05 for interaction of HCV-S2 with E2 in 0.01 M PBS (pH 7.4) and artificial blood plasma, respectively (Table 1). Moreover, the sensor had similar binding affinities toward E2 in both media (KD $= 0.02 \pm 0.01$ in 0.01 M PBS (pH 7.4) and 0.05 ± 0.01 mg/mL in simulated blood plasma). Thus, one may deduce that the presence of other additional substances does not significantly affect the E2 binding to HCV-S2, and the biosensor displays potential suitability for use in blood plasma.

The selectivity of the HCV biosensor was examined through a comparison of sensor DPV responses towards E2 (54 kDa) and a non-target protein - conalbumin (76 kDa) at the same concentrations (Fig. 5). As it can be seen the biosensor response was negligible after interaction with non-target protein at concentrations of 0.01, 0.1 and 0.3 mg/mL. At the same time, the incubation of the HCV-S2 in E2 solutions was followed by the increase in normalized DPV signal with the growing of E2 concentration. This indicated that the obtained sensor discriminates E2 from a non-target protein (conalbumin) having bigger molecular weight.

Table 1
Parameters derived from fitting the binding isotherms (Fig. 4) to the Langmuir adsorption model (Eq. (2)).

Sensor	HCV-S1	HCV-S2	HCV-S3	HCV-S2
Measured media	0.01 M PBS (pH 7.4)	0.01 M PBS (pH 7.4)	0.01 M PBS (pH 7.4)	simulated blood plasma
$I_{n.max}$	0.65 ± 0.02	0.59 ± 0.02	0.56 ± 0.04	0.68 ± 0.05
K_D (mg/mL)	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.05 ± 0.01
R^2	0.989	0.983	0.933	0.985

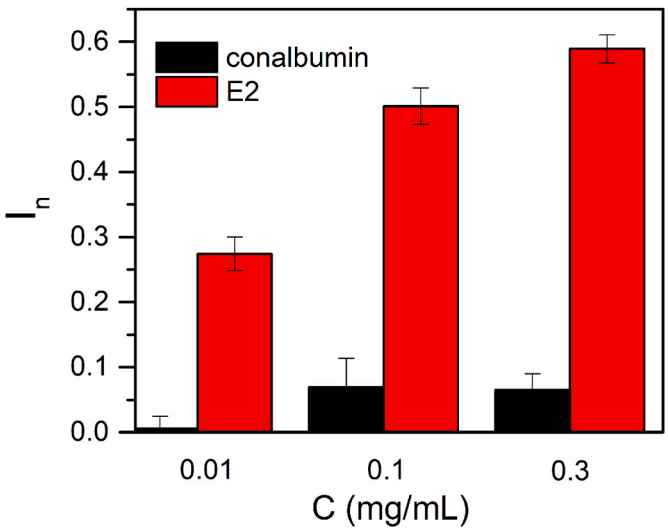

Fig. 5. Normalized responses of HCV-S2 biosensor after incubation in 0.01 M PBS (pH 7.4) containing increasing concentrations (0.01, 0.1 and 0.3 mg/mL) of conalbumin and E2.

Since, according to our knowledge, there is no report on the electrochemical sensor for direct detection of HCV surface antigen (E2), HCV-S2 was compared to the reported electrochemical sensors for detection of HCV core antigen (see section S4 and Table S4). Although HCV-S2 has significantly higher LOD value, it is targeted against HCV envelope protein being thus potentially capable of selective binding of a whole virus particle and providing a possibility of quantitative analysis.

3.2.2. Interaction with HC VMPs

To evaluate the applicability of the HCV-S2 for selective rebinding of HCV particles we performed the binding experiments using VMPs as a model analyte. VMPs are similar to biological viruses in their physicochemical characteristics but they are cheaper and safer objects [38]. The suitability of VMPs as the models reproducing the behavior of real viruses has been testified in many works [41–43].

In this work, HC VMPs containing E2 on its surface were applied in order to interact with the linear peptide immobilized on the sensor. Since the analyte in the form of HC VMPs had a much larger size, and the configuration of E2 bound to polymer particles might differ from that of free E2, this can affect the binding to the ligand immobilized on the sensor. As a result the successful affinity binding can take more time. Thus, the time of interaction between the HC VMPs and HCV-S2 was first optimized. The optimal incubation time of the biosensor with HC VMPs was found and equal to 3 h and subsequently used for all experiments (See section S3 for details). The adsorption isotherms for HC VMPs on HCV-S2 in 0.01 M PBS (pH 7.4) and simulated blood plasma (Fig. 6) showed that the system reached an adsorption saturation after concentrations of 0.1 mg/mL. The analytically relevant concentration range to detect HCV in blood as estimated by PCR method is between 45 and 25,000,000 UI/mL (135–75,000,000 HCV RNA/mL) that corresponds to 10^{-12} and 10^{-6} mg/mL based on the calculation of approximate mass of one viral particle [45]. Thus, these conditions are far away from those that would bring the sensor to saturation, but require its sufficient sensitivity to respond against the small quantities of target analyte.

The Langmuir (L) and Langmuir-Freundlich (LF) binding models both did not describe very well HC VMP adsorption, though the latter provided somewhat better agreement with the binding isotherm data than the former (coefficients of correlation, (R^2) are 0.990 and 0.975, respectively (Table 2).

Presumably, this can be explained either that HC VMPs interact with each other forming a multi-layer or the redox reaction suppression does not directly reflect occupation of the binding sites on the sensing surface

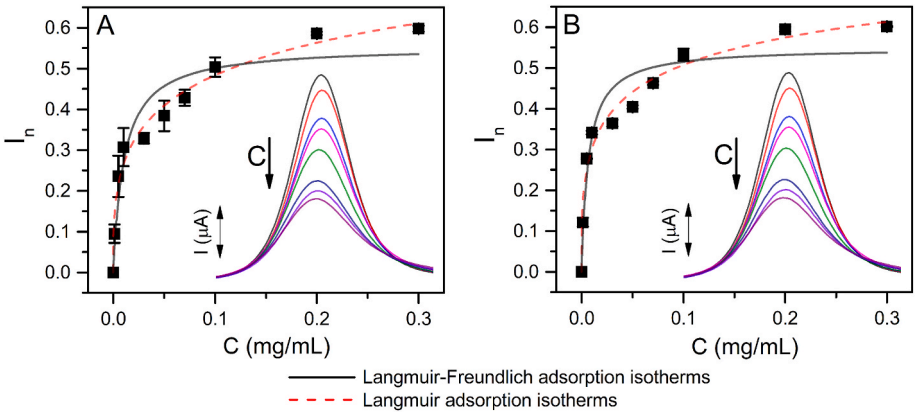

Fig. 6. Adsorption isotherms of HC VMPs on HCV-S2 in (A) 0.01 M PBS (pH 7.4) and (B) simulated blood plasma. The inserts show the respective DPVs measured by HCV-S2.

Table 2
Parameters derived from fitting the binding isotherms (Fig. 6) to the Langmuir and Langmuir-Freundlich adsorption models.

Sensor	HCV-S2	HCV-S2
Measured media	0.01 M PBS (pH 7.4)	Simulated blood plasma
Langmuir adsorption model		
I_{max}	0.55 ± 0.04	0.55 ± 0.03
K_D (mg/mL)	0.01 ± 0.01	0.01 ± 0.01
R^2	0.906	0.91
Langmuir-Freundlich adsorption model		
I_{max}	1.3 ± 0.8	1.1 ± 0.5
K_D (mg/mL)	0.7 ± 0.9	0.47 ± 0.05
m	0.4 ± 0.1	0.4 ± 0.1
R^2	0.977	0.974

of HCV-S2 or both factors at the same time.

Nevertheless, it can be noted that HCV-S2 is capable of effectively binding E2 as a part of HC VMPs since the binding process is similar in both PBS and the simulated blood plasma indicating the absence or very low cross-reactivity with plasma components.

By analyzing the linear portion of the sensor signal at low HC VMPs concentration the LOD and LOQ values were calculated to be $2.1 \cdot 10^{-5}$ mg/mL and $7.1 \cdot 10^{-5}$ mg/mL, respectively (Fig. 7). Taking into account

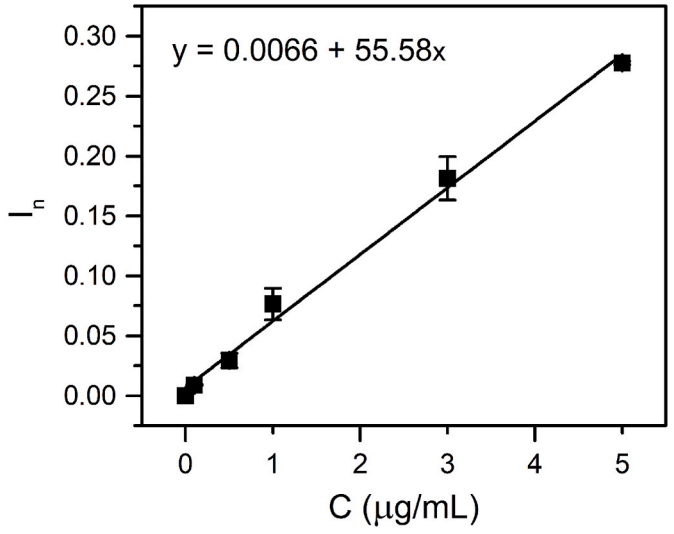

Fig. 7. The calibration plot of HCV-S2 biosensor obtained at low concentration (0.1–5 μg/mL) of HC VMPs in a simulated blood plasma. The solid line shows a linear regression fit.

the roughly estimated mass of one HC VMP ($2.76 \cdot 10^{-7}$ ng) this LOD corresponds to $7 \cdot 10^6$ HC VMPs/mL. However, the LOD is quite high to compete with existing methods that allow determination of dynamic range of quantification between 600 IU/mL and 850,000 IU/mL [46]. According to the European Association for the Study of the Liver (EASL) the serum HCV RNA level of 800,000 IU/mL or 2,000,000 HCV RNA copies/ml should be used as the decision threshold to tailor the duration of the currently approved treatment [46]. Therefore, the performance of HCV-S2 can suppose the use of the sensor to determine only high viral loads occurring during the active state of the illness.

Thus, in contrast to the HCV core antigen detection method, HCV-S2 being targeted against HCV surface antigen is capable of detecting both free E2 proteins and E2 associated with viral particles, i.e. HCV VMPs, providing a possibility for evaluating viral load. Moreover, since as it was previously shown HCV envelope proteins (E1 and E2) were associated with the infectious particles only [13,47], the proposed method will be potentially suitable for assessment of infection dose of HCV.

4. Conclusion

This work demonstrated for the first time the possibility of building electrochemical biosensors on the base of CD81 fragments to detect HCV through its envelope protein (E2). Among the three types of CD81 fragments, the linear peptide was shown to be the most suitable molecular recognition element for HCV biosensor (HCV–S2) being cheaper and easier to prepare but providing similar binding affinities towards E2. The HCV-S2 was able to detect E2 in the simulated blood plasma with very low or without cross-reactivity indicating its potential applicability for analyzing HCV in complex biological samples. Moreover, the sensor capability to selectively bind E2 as a part of HC VMPs could represent an approach that allows quantifying viral particles as well as estimation of the infectious dose of HCV. Since, the calculated LOD value was quite high to compete with existing methods used for diagnostics of hepatitis C, the HCV-S2 could be potentially suitable for monitoring only the acute phase of disease, when the viral load is high. Thus, future research will continue to further improve the ligand immobilization protocol in order to increase the amount of specific binding sites on the sensor surface as well as to validate selectivity of the sensor in clinical samples.

CRediT authorship contribution statement

Mariia Antipchik: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Evgenia Korzhikova-Vlakh:** Conceptualization, Resources, Writing – review & editing. **Dmitry Polyakov:** Resources. **Irina Tarasenko:** Resources. **Jekaterina Reut:** Funding acquisition, Methodology, Resources, Writing – original

draft, Writing – review & editing. **Andres Öpik:** Resources. **Vitali Syritski:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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