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Non-enzymatic cholesterol biosensor: Electrochemical sensing based on peptide-poly(lactic acid) thin film

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ABSTRACT

Cholesterol is a fundamental lipid prevalent in eukaryotic cell membranes and circulating in the bloodstream bound to lipoproteins. It serves as a precursor to steroid hormones and is regarded as a biomarker for cardiovascular disease and other metabolic disorders. Numerous cholesterol detection methods predominantly rely on enzymes, which suffer from instability, leading to non-cost-effective biosensors with low sensitivity and poor reusability. Therefore, monitoring cholesterol levels with a feasible, rapid, and stable biosensor is critical for diagnosing and treating various disorders. This study aimed to develop a non-enzymatic cholesterol biosensor based on a selected cholesterol recognition peptide as the detection element. Screen-printed carbon electrodes (SPEs) modified with biocompatible poly-L-lactic acid (PLLA) porous nanomembranes (NMs) were utilized as support for the covalent immobilization of the peptide. Data obtained from electrochemical impedance spectroscopy (EIS) demonstrated the peptide's effective binding affinity towards cholesterol, paving the way for its implementation. The determination of cholesterol with the proposed biosensor exhibited a low limit of detection of 6.31 μM with linear responses ranging from 2–15 μM and 20–40 μM . These findings present an alternative method for cholesterol sensing by integrating novel peptides as biorecognition motifs with biocompatible polymeric materials, potentially useful as biocompatible and future point-of-care sensors.

1. Introduction

Cholesterol is an amphiphilic lipid sterol-type ubiquitous in eukaryotic cell membrane and crucial for its organization, dynamics, function, and sorting. It is involved in controlling the activity of a wide range of membrane signalling proteins, mostly G-protein-coupled receptors (GPCRs) including neurotransmitter [1], hormone receptors [2] and ion channels [3] through general effects on fluidity membrane

curvature and specifically, by interactions through direct binding to its transmembrane-spanning domains (TMDs) [4–6].

Furthermore, the imbalance of cholesterol homeostasis has been pointed as a hallmark of metabolic disorders and inflammatory activation of immune cells. In fact, the sustained accumulation of cholesterol leads to chronic inflammatory responses, promoting worsens diseases including cancer, atherosclerosis, and cardiovascular diseases; neurodegenerative and neuroinflammatory disorders, such as Parkinson's and

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Alzheimer's disease, as well as the conditions related to fertility and obesity disorders [7,8]. Therefore, the monitoring of cholesterol and fast detection of its levels in biological samples, living cells, or even in food, is a critical point for the analysis and early diagnosis of diverse clinical disorders.

Nowadays, a plethora of biosensor are currently explored as potential tools for a sensitive and selective analytical detection of cholesterol biomolecule focused on clinical diagnosis. In this regard, most of the biosensors for cholesterol quantification are enzyme-based electrochemical devices, where cholesterol oxidase (ChOx) and/or cholesterol esterase (ChE) are employed as the biorecognition element leading to a potential gradient for electron transfer between the enzyme's active site and the electrode [9–14]. Despite enzymatic assays exhibit a high sensitivity and selectivity, enzymes present the lack of long-term stability caused by their inherent ease denaturation and degradation limiting their application in biosensing.

On the other hand, non-enzymatic cholesterol sensing hasn't been fully explored due to the lack of electroactivity of free cholesterol by itself, poor water solubility due to its hydrophobic character, and its low molecular weight and size. Thus, several challenges have to be overcome to successfully achieve a sensitive platform such as the choice of suitable electroactive matrix material like conducting polymers to enhance the electrical signal, achieve an adequate sensitivity for the detection at micromolar concentrations or the stability of the sensing elements for long-life devices and its use with real samples such as blood serum [15–17]. Additionally, redox mediators such as Prussian blue, methylene blue, and ferrocyanide are often employed to facilitate electron transfer and amplify the electrochemical signal [18,19].

As a promising alternative, putative cholesterol binding peptide sequences identified on a plethora of protein transmembrane domains (TMD) offer new insights for developing non-enzyme-based and label free biosensors presumably with enhanced stability and selectivity to detect cholesterol in its different forms [9,20].

Herein, an innovative cholesterol biosensor was designed based on a cholesterol recognition peptide sequence. The well-known murine peripheral-type benzodiazepine receptor (PBR) presents on its carboxyl domain a cholesterol recognition amino acid consensus motif, termed "CRAC", from where the sequence ATVLNYYVWRDNS (herein called C-pept) was selected [21,22]. This behaviour is usually ascribed to the presence of the apolar-aromatic-basic branched sequence which actuate as the binding TMD of several proteins on cholesterol-enriched membrane regions ensuring the correct orientation at the polar/apolar interface where cholesterol is located [23,24].

The novelty of the work relies on the design of a non-enzymatic cholesterol biosensor based on the cholesterol recognition C-pept covalent bonding to a poly-L-lactic acid (PLLA) film for future point of care sensors. Polylactic acid (PLA) and its copolymers are highly employed in biomedical field (e.g. bone screws, sutures, tissue scaffolds, etc.), since they are biocompatible and biodegradable [25–27]. The superior advantages of peptides as biorecognition element, ease functionalization, stability and reusability without denaturalization, are further enhanced with the biocompatible PLA support to address long-lasting biomedical devices [28–30].

Aiming to obtain a miniaturized system, the construction of C-pept/PLA nanoporous film was conducted using screen-printed carbon electrodes (SPE), as support electrode, and chemical reactions to covalent bond both components. This strategy was inspired by our recent work where cholesterol-poly(ethyleneglycol)-amine terminated molecules were investigated as building block molecules in PLA nanomembranes, for the detection of vitamin C and Trolox (a vitamin E derivative) [31]. The success of the biosensor performance was followed by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) with gradual increasing of cholesterol concentration to the electrolyte bath. Binding affinity of C-pept to cholesterol on model membranes and its predominant selectivity has been further probed with non-related lipids and hormones by fluorescent resonance energy

transfer (FRET) measurements. The chemical structure of the assembled hybrid material was proved by microscopy and spectroscopy analyses.

2. Materials and methods

2.1. Materials

Poly-L-lactic acid (PLLA), a Natureworks product, was kindly supplied by Nupik International (Polinyà, Spain) (polymer 2002D) with $M_n = 98,100 \text{ g}\cdot\text{mol}^{-1}$, $M_w = 181,000 \text{ g}\cdot\text{mol}^{-1}$, and polydispersity of 1.85. For simplification reasons in the acronym of the multi-layered system developed here, we will refer to PLLA as PLA. Poly(vinyl alcohol) (PVA) (87–89 % hydrolysed, $M_w 13,000\text{--}23,000 \text{ g}\cdot\text{mol}^{-1}$), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), phosphate buffer saline, potassium hexacyanoferrate (III) ($\text{K}_3\text{Fe}(\text{CN})_6$), Triton X-100 and cholesterol powder were purchased from Sigma-Aldrich (Spain). 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) was purchased from Apollo Scientific Limit (UK). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 18:1 Pyrene PE 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-1-pyrenesulfonfyl ammonium salt (PyPE) were purchased from AVANTI POLAR Lipids Inc. C-pept was synthesized by ProteoGenix S.A. (France). Screen-printed electrodes (SPE), model C110, provided by a working electrode (WE) area of 25.1 mm^2 (4 mm of diameter); a counter electrode (CE), both composed by conductive carbon; and a reference electrode (RE), composed by silver; were purchased from Metrohm-DropSens (Spain).

Cholesterol (Chol) stock solution (10 mM) was prepared in PBS implemented with 10 % (v/v) Triton X-100 and slowly heated until 70°C for 30 min. Cholesterol is highly hydrophobic, thus, Triton X-100, a non-ionic surfactant, was selected for the use of buffers solutions [32]. The solutions were stored in a refrigerator and used until a slight turbidity was observed.

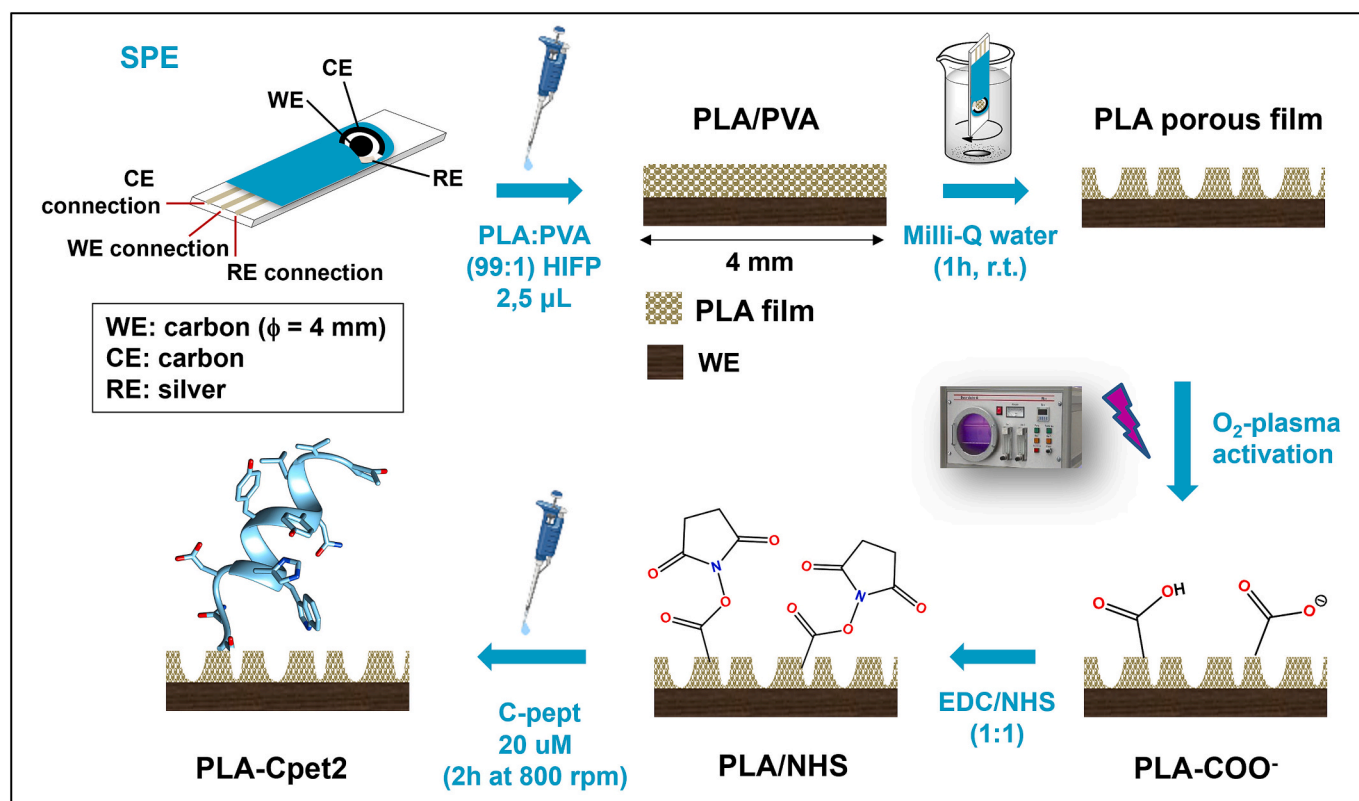
2.2. Modification of screen-printed electrodes with PLA porous films and subsequent covalent bonding with the C-peptide sequence: biosensor preparation

2.2.1. Electrode pre-treatment

Before biosensor build-up process and electrochemical measurements, bare SPE (Metrohm-DropSens C-110, diameter = 4 mm) were washed with milli-Q water under sonication for 5 min and dried under air. In this procedure, carbon working electrodes (WE) were activated by chronoamperometry (CA), applying a constant potential of 1.5 V for 200 s, within an interval time of 0.01 s in 50 mM PBS buffer. After the electrochemical activation step, the SPEs were rinsed with distilled water and dried under nitrogen gas [33,34].

2.2.2. PLA perforated nanomembranes fabrication

The PLA porous films were fabricated over the pre-treated SPE substrates, by drop-casting deposition [35], and selective solvent etching technique by adapting the method previously described by Puiggalí-Jou et al. [36]. For it, separated stock solutions of 10 mg mL^{-1} of PLA and 10 mg mL^{-1} of PVA in HFIP were prepared. PLA and PVA solutions were blended in a 99:1 v/v (PLA:PVA) ratio, to induce phase segregation. Then, filtered blended polymer solution was vortexed for 60s at 1000 rpm and 2.5 μL were drop casted carefully onto the carbon working electrode (WE) of the SPE and were left to dry in the hood, under a flow of air, before use. Finally, the films adhered to the WE were immersed in milli-Q water for 1 h to dissolve the PVA polymer and to create the porous PLA films (hereafter called PLA NM). The WEs were washed three times with 0.01 M of PBS solution and dried. In the whole process, the counter (CE) and the reference (RE) electrodes were protected with Kapton tape to avoid material deposition and oxidation. A simplified illustration of the whole process is depicted in Scheme 1.



Scheme 1. Synthetic route for the deposition of the PLA:PVA solution on the screen printed electrodes (SPE), used as working electrode, and the subsequent covalent immobilization of C-pept compound, with surface activation (carbodiimide strategy), for the further electrochemical monitoring of cholesterol molecules.

2.2.3. Activation of PLA NM with cold-plasma

Afterwards, the PLA NM film was activated by using oxygen-plasma (O₂-plasma) to create polar reactive groups (-COOH, -COO⁻, and radicals). For this step, a low-pressure radio frequency (RF) plasma (80 MHz), LFG generator 1000 W (Diener Electronic GmbH Co., Germany), equipped with a chamber of 2.6 dm³ of volume, was employed. The chamber is vacuum-purged and filled with oxygen gas, once the material has been placed. The specified pumping down pressure was 0.2 mbar, prior to applying the electric discharge. The maximum power supply was fixed to 30 W, maintaining the oxygen pressure at 0.8 mbar, with a minimum gas flux of ~15 sccm. For this process, the power supply was fixed to 100 W and 33 W, with a maximum time of exposure of 2 min each.

2.2.4. Covalent attachment of C-pept to PLA NM films

Thereafter, the PLA NM-SPEs were immediately transferred to a vial containing *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) with concentrations of 0.1 M and 0.2 M, respectively, in milliQ water in a ratio 1:1, to create the amine reactive coupling groups for the subsequent bonding of C-pept. The sequence of reactions and steps can be seen in Scheme 1. The PLA/NHS groups, by transamidation reaction, further react with the amine groups of C-pept, giving rise to the amide linkages, symbolically represented by the 3D model of C-pept above the PLA NM-modified SPE. For this last step, the modified WEs were cleaned with milliQ water to remove any unreacted NHS and EDC molecules, before the drop-casting deposition of 20 μ M solution of C-pept dissolved in 0.1 M PBS. The samples were left to react for 2 h at 800 rpm before being rinsed to eliminate any unreactive molecules (hereafter denoted as PLA-C-pept, for simplification) [34].

2.3. Fluorescence Resonance Energy Transfer for binding analysis of C-pept towards cholesterol

Binding affinity analysis of C-pept to free cholesterol molecules embedded on model membranes was characterized by Fluorescent Resonance Energy Transfer (FRET) [37]. FRET technique is used to evaluate protein-lipid interaction by monitoring the quenching fluorescent emission signal of the donor, the peptide's intrinsic fluorophore, tryptophan (Trp), by an acceptor fluorophore, a synthetic lipid derivative 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(1-pyrenesulfonyl) (PyPE), when are proximal within a distance range of 10–100 Å (8–10 nm).

Large Unilamellar Vesicles (LUVs) made up by POPC and Chol at a ratio of 1:0.3 mM in 50 mM Tris-HCl buffer, pH 7.5, were prepared via thin film and extrusion method using Liposofast extruder (AVESTIN Europe GmbH, Mannheim, Germany) equipped with 100 nm pore-size polycarbonate membranes and gas tight syringes (500 μ L volume) as previously described by Angelucci et al. [38]. POPC LUVs of a homogeneous diameter size of 100 nm were prepared in presence and in absence of PyPE at 2 % (m/m) embedded on its membrane for the FRET signal analysis.

FRET data points were measured from the fluorescence intensities of C-pept (emission: 307–355 nm) and POPC-Chol-PyPE (absorption: 351 nm) in a concentration range from 0 to 320 μ M. Half saturation value ([L]_{1/2}) of the peptide-LUVs binding constant is obtained to calculate the membrane binding affinity through a nonlinear regression analysis [38,39].

The measurements were performed in a LS50b spectrofluorometer (Perkin Elmer Inc., Waltham, MA), and further adapted on the EnSpire® Multimode Plate Reader (Perkin Elmer Inc., Waltham, MA) using 96-wells black polystyrene microplates for a high-throughput screening (HTS).

2.4. Molecular modelling and docking analysis

Peptide generation and docking analysis: the final peptide sequence was obtained with AlphaFold2 webserver (<https://alphafold.ebi.ac.uk/>), starting from the amino acid sequence [40]. Among the 5 possible conformations, the best ranked one according to the webserver was picked. Docking analysis was performed with Autodock Vina software (<https://vina.scripps.edu/>) and visualized with Chimera 13.50.53 (<http://www.cgl.ucsf.edu/chimera/>) [41,42]. The 3D structure of cholesterol was obtained from PubChem Compound database (<https://pubchem.ncbi.nlm.nih.gov>). The peptide was converted from pdb to pdbqt with openbabel software (<https://openbabel.org/>), then the two molecules were prepared for docking analysis by running the dockprep command with default parameters in Chimera. Finally, the docking analysis was performed and revealed six different binding poses with a binding free energy ranging from -5.6 to -6.1 Kcal/mol.

2.5. Electrochemical detection of cholesterol with PLA-C-pept biosensor

2.5.1. Electrochemical impedance spectroscopy (EIS) measurements

Experiments were performed by using the carbon WE of SPEs functionalized as described in the previous sections and corresponding to the following samples: PLA-PVA, PLA NM and PLA-C-pept. The electrodes were immersed in a glass cell, filled with the electrolyte solution, and were connected by a cable connector for SPE (Metrohm-DropSens, DRP-CAC) to a potentiostat (Autolab PGSTAT204), controlled by the Nova 2.1.5 software. The counter and reference electrodes were carbon and silver, respectively. For EIS experiments, a sinusoidal voltage of 1 mV of amplitude, within a frequency range between 10^5 Hz and 0.1 Hz, was fixed. All experiments were performed at room temperature. The selected electrolyte solution was PBS 0.1 M pH 7.4 (Dulbecco's) containing 1 % (v/v) Triton X-100 (PBST) with 5 mM $K_3Fe(CN)_6$ in PBS 0.1 M as redox probe. Cholesterol (Chol) stock solutions of 10 mM in PBS with 10 % (v/v) Triton-X100 were prepared and dropped into the cell by sequential additions on the electrolyte solution [43,44]. Therefore, after the dilutions, the Chol concentrations in the electrolyte solution ranged from 2 μ M to 15 μ M. After data collection, EIS results were processed and fitted to an electrical equivalent circuit (EEC) by using Nova software. The experiments were repeated twice with different sets of samples to ensure reproducibility.

2.5.2. Cyclic voltammetry (CV) measurements

The experiments were conducted to monitor the success of the different steps of the biosensor construction and surface modification following the signal of the redox probe $Fe(CN)_6^{3-/4-}$ electrolyte solution. The analysis was carried out in the same equipment as described by EIS analyses and using the glass cell specially designed for SPEs. New samples were prepared on the SPE, used as WE, the counter and reference electrodes were carbon and silver, respectively. The initial and final potential were settled at -0.40 V, and the reverse potential was $+0.50$ V with a scan rate of $50\text{ mV}\cdot\text{s}^{-1}$ for 5 cycles with the electrochemical analyzer operating between 10 mA and 100 nA. Ag/AgCl. The easy electron-transfer kinetics of $Fe(CN)_6^{3-/4-}$ at the SPE validates its potential for sensing application. All experiments were performed at room temperature.

2.6. Physical-chemical characterization

The chemical structures of pristine PLA film and PLA-C-pept films were confirmed by FTIR and XPS spectroscopies. A Jasco 4700 FTIR-ATR spectrometer, equipped with ATR accessory and Spectra Manager software, was used for the IR analyses. All spectra were recorded in a wavenumber range of $4000\text{--}600\text{ cm}^{-1}$ at a resolution of 4 cm^{-1} and 64 accumulation scans. To confirm the previous results, fresh-prepared PLA and PLA-C-pept films, deposited on indium-tin-oxide glass wafers (ITO-glass), were evaluated by X-ray photoelectron spectroscopy (XPS) with

an XPS equipment from SPECS XPS System. The instrument operates with a XR50 Al anode (150 W, 10 kV), with a hemispherical analyzer Phoibos 150 and an electron detector MCD-9. The working pressure was maintained below 5×10^{-8} mbar. High-resolution XPS spectra were acquired by Gaussian/Lorentzian curve, fitting after S shape background subtraction, for the following elements: C 1s, O 1s, N 1s and S 2p. The data were analysed with Casa XPS software (Version 2.3.19PR1.0) and all the spectra were calibrated with respect to the binding energy of the carbon C1s peak (284.8 eV).

Topographical data were analysed by atomic force microscopy (AFM). The images were conducted with silicon TAP 150-G probes (Budget Sensors, Bulgaria), with a frequency of 150 kHz, and a nominal force constant of 5 N m^{-1} , in tapping mode. Images were obtained with an AFM Dimension microscope (Bruker) using a NanoScope IV controller, under ambient conditions. The row scanning frequency was set at 1 Hz and the tip motion speed was $10\text{ mm}\cdot\text{s}^{-1}$. The average of pore's diameters and the standard deviations were calculated from 3 independent zones in the porous PLA surface. The specific profile sections of the 2D and 3D AFM images were determined using the statistics application tools of the NanoScope Analysis software. The scan window size was $10 \times 10\text{ }\mu\text{m}^2$.

Detailed inspection of morphology of the WE, before and after PLA functionalization, was conducted by scanning electron microscopy (SEM). A Focus Ion Beam Zeiss Neon 40 instrument (Carl Zeiss, Germany) equipped with an energy dispersive X-ray (EDX) spectroscopy system and operating at 5 kV was employed. Samples were mounted on a double-sided adhesive carbon disc and were sputter-coated to prevent sample charging. The Image J software was employed to process the micrographs.

2.7. LOD method validation

The value of the limit of detection (LOD) of the method was determined by linear regression analysis following Eq. (1):

$$LOD = \frac{3S_b}{m} \quad (1)$$

where S_b is the standard deviation of the response and m is the slope of the corresponding calibration curve [45]. For each cholesterol concentration EIS was run three times ($n = 3$).

3. Results and discussion

3.1. Rational study and characterization of C-pept used as building block molecule for the biosensor design

In the present study, a 13 amino acid (aa) synthesized peptide from mouse PBR CRAC sequence (147-ATVLNYYVWRDNS-159) was selected as the biorecognition element for the fabrication of the electrochemical cholesterol biosensor. C-pept sequence has been previously identified as the specific region responsible for the binding and interaction of the PBR receptor with cholesterol, which is primarily located in the outer membrane of mitochondria organelles. Molecular modelling and in vitro studies have reported the high affinity of the C-terminal sequence of PBR to free cholesterol molecules by the specific interactions of the putative CRAC residues located on its alpha-helix secondary structure [21,46].

Complementary modelling and docking analysis with cholesterol were conducted with the selected C-pept sequence. Such interaction can be visualized in the video included in the supporting information file (movie SI.2). The peptide structure was obtained from AlphaFold2 and, out of the 5 potential conformations, the best ranked one resulted model kept the expected alpha-helix conformation [40]. We then conducted a molecular docking analysis on the resulting C-peptide with cholesterol molecule with Autodock Vina software [47]. Among the generated poses, the one with lowest energy, accounting for a binding free energy

of -6.1 Kcal/mol, was chosen (Fig. S1). In this pose, C-pept interacts to cholesterol through ASN-5, TYR-6, VAL-8 and TRP-9 residues belonging to the CRAC sequence, supporting our speculation [46].

In vitro assays were conducted to confirm the behaviour of the synthesized peptide in solution and model membranes. Membrane binding properties of C-pept were studied by measuring FRET signal between C-pept with LUVs made of POPC and Chol with a molar ratio of 1:0.3. The energy is transferred non-radiatively from the donor (Trp) to the acceptor (PyPE) when they are in proximity leading to a decrease in the donor's fluorescence intensity and an increase in the acceptor's emission. The synthetic lipid derivate acceptor PyPE, embedded on LUVs membranes, acts as quencher of the intrinsic Trp fluorescence via FRET when the C-pept binds to the membrane-bound cholesterol within the effective FRET distance. The resulting fluorescent emission spectra (Fig. 1a) perfectly demonstrate the quenching of the Trp fluorescence at 354 nm of the peptide at increasing concentrations of LUVs and the corresponding increment of the PyPE signal at 380 nm and 400 nm provided as the acceptor fluorescent molecule. This event is the subsequent effect of being Trp one of the amino acids responsible for the interaction with cholesterol on CRAC sequence.

The FRET signal can be derived from the total emission at 357 nm in the absence of acceptor by subtracting the emission at donor wavelength in the presence of acceptor as follow: $\Delta F = \Delta F(\text{POPC:Chol/PyPe}) - (\Delta F(\text{POPC:Chol}) - \Delta F(\text{peptide}))$. Thus, by monitoring the quenched FRET signal of the Trp, binding isotherms are obtained and used to calculate the membrane affinity. It has been demonstrated that the dissociation constant, K_d , thus, the affinity of proteins binding to membranes can be calculated from the half-saturation value of protein binding to membranes [48,49].

After data processing with a non-linear fitting of FRET signal, plotted on Fig. 1b, half saturation constant of $L_{1/2} = 17.2 \pm 1.9 \mu\text{M}$ was obtained [38]. This result indicates a high affinity, as expected, and the recognition of cholesterol molecules also once embedded in model membranes, where is its common environment [48].

In order to discern the binding affinity and specificity of C-pept, a series of experiments were performed with LUVs synthesized with varying cholesterol concentrations and different steroids following the same technique as previously described. Experiments tested that the presence and increasing content of cholesterol in LUVs, remarkably enhance the binding of C-pept to liposomes (Table 1), as lower $L_{1/2}$ values rely on a higher affinity of interaction [50,51]. These results confirm that the binding of C-pept to membranes is not merely due to nonspecific interactions with the lipid components (such as POPC), but

Table 1

Half saturation binding values for C-pept to LUVs made of POPC/steroids.

Liposomes	$L_{1/2}$ (μM)
POPC	62.4 ± 5.8
POPC/Cholesterol (1:0.1 mM)	18.9 ± 1.5
POPC/Cholesterol (1:0.3 mM)	17.2 ± 1.9
POPC/Cholesterol free (1:0.3 mM)	41.4 ± 7.5
POPC/Cholesterol (1:0.5 mM)	10.9 ± 1.8
POPC/Hydrocortisone (20 nM)	32.15 ± 3.9
POPC/Progesterone (0.2 μM)	50.2 ± 3.3
POPC/Pregnenolone (20 nM)	62.7 ± 8.3
POPC/Estradiol (2 nM)	74.6 ± 7.5
POPC/Cortisone (0.2 μM)	119.3 ± 19.6
POPC/Testosterone (2 μM)	166 ± 40

rather, it demonstrates a specific recognition and preference for free cholesterol within the membranes (Fig. 2a).

On the other hand, experiments with different steroids elucidate whether the binding of C-pept is mediated through a general affinity for amphiphilic structures and the common 4-ring steroid structure or by an effective cholesterol recognition (Fig. 2b). The low values of half saturation obtained for hydrocortisone and progesterone can be attributed to the amphiphilic nature of those steroids, which enables them to interact with both hydrophobic and hydrophilic regions of the membrane, similar to cholesterol [52]. Even though, the approach to test C-pept's interaction with cholesterol and steroids using FRET in model membranes effectively highlights the specificity of the peptide for cholesterol.

Infrared spectroscopy has been used in a large number of studies to identify amide groups present in proteins, peptides and liposomes, as well as their interactions with other molecules [53,54]. At least, nine amide absorption bands can appear in the spectra of peptides and proteins, from the amino acid side chains: amide A, amide B and amides I-VII. Some of their characteristic peaks can be used to discriminate amino acids, as for example, that containing benzene rings, such as arginine or phenylalanine; and others containing charges, such as lysine and acid derivatives [53]. Based on such knowledge, valine and leucine amino acids are clearly identified by the presence of CH_3 absorption bands, corresponding to two methyl groups on their chemical structures. However, valine methyl band appears at approximately 1450 cm^{-1} , whereas leucine CH_3 's is identified at lower wavenumbers (1375 cm^{-1}). Considering the sequence of C-pept, ATVLNYYVWRDNS, such bands were clearly evidenced in Fig. S2. Moreover, the most intense vibrations were found for N-H ($3265, 3070 \text{ cm}^{-1}$) and O=C-N (1624 cm^{-1})

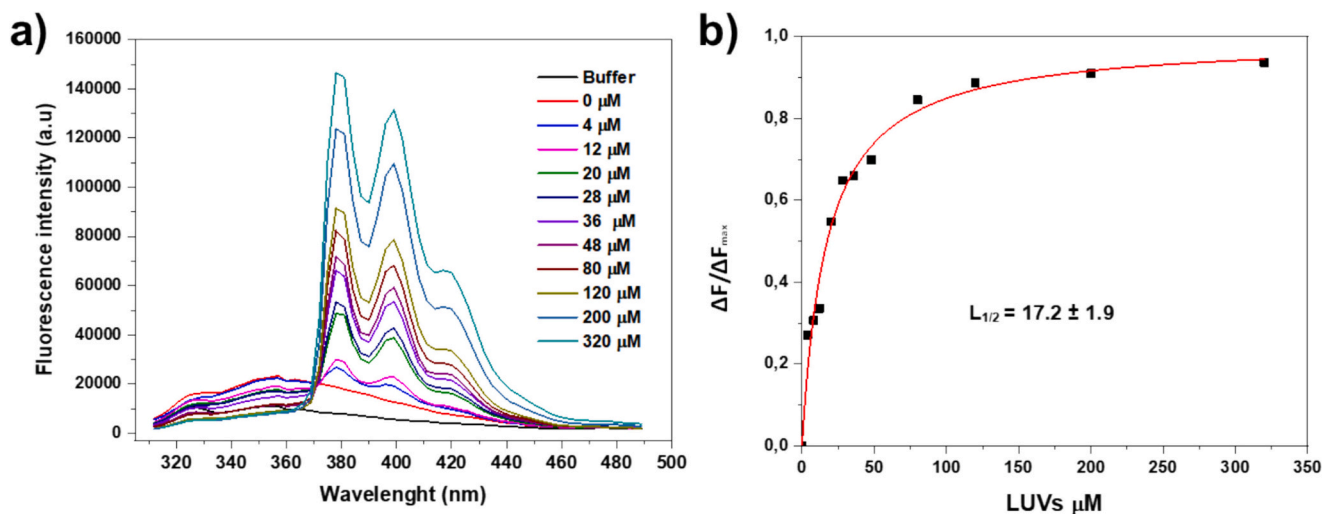


Fig. 1. a) Fluorescence emission spectra of FRET assays between C-Pept (Trp) and POPC:Chol(1:0.3)-PyPE 2 % LUVs, b) binding curve and half saturation value from FRET analysis.

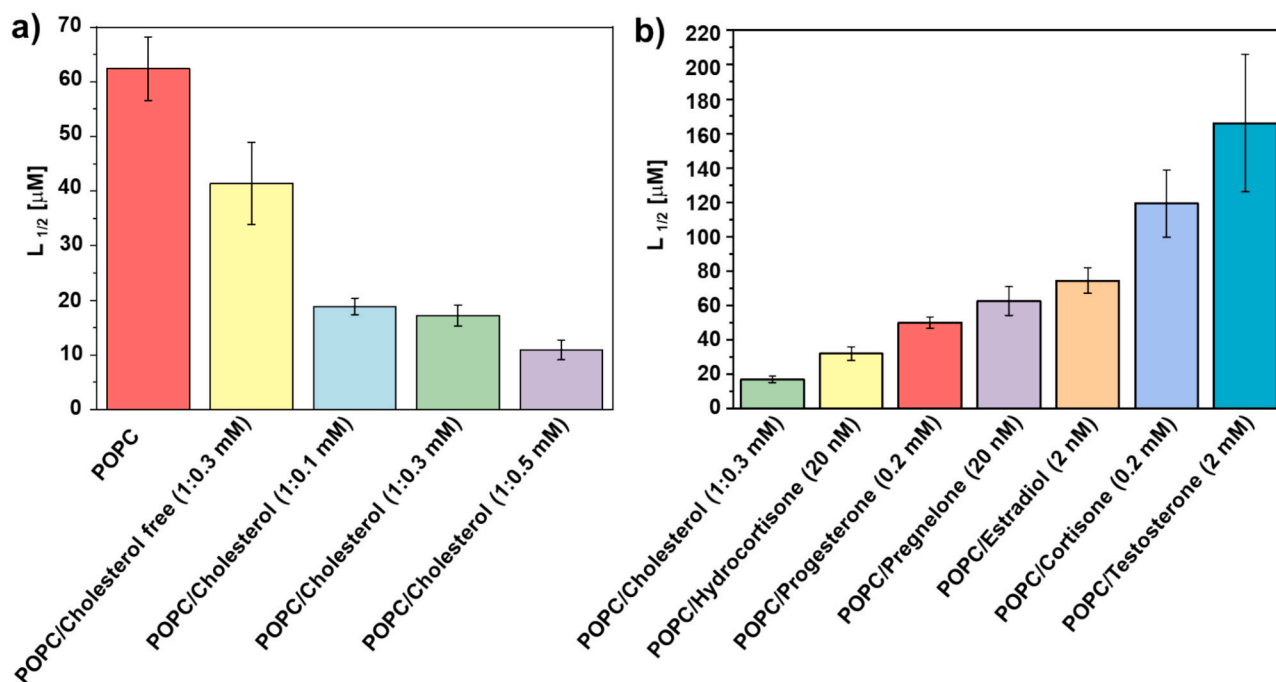


Fig. 2. Half saturation concentration values ($L_{1/2}$) for the binding of C-pept to POPC liposomes: a) at different cholesterol ratios, and b) with different steroid molecules. Data reported are the mean $\pm$ SEM (Standard error of the mean). ($n = 3$) Statistical analysis was performed by ONE-Way ANOVA test, followed by Dunnett's Multiple Comparison Test.

stretching vibrations. The two N—H bands appear due to the hydrogen bonds formation in resonance with overtone of Amide II bands (1540 , 1514 cm^{-1}). The splitting of these bands is mainly due to the contribution of N—H bending and stretching vibrations. Amide III (1198 , 1138 cm^{-1}) also appears as strong and sharp bands, however, with less intensity than Amide II. And, finally, another N—H overtone bending vibration could be identified at 700 cm^{-1} .

3.2. Fabrication of porous PLA NM films with C-pept covalently bonded to their surfaces

The critical step of the biosensor fabrication was the covalent immobilization of C-pept to the hydrophobic PLA surface. For this reason, a surface modification is necessary in order to convert it to more hydrophilic material, able to react with polar groups [55]. Therefore, following the sequence presented in Scheme 1, the pristine material (PLA:PVA 99:1), deposited on SPE, was placed in a water bath to remove the PVA polymer and create pores, enhancing the ionic conductivity through the PLA film. The pore sizes were measured from different topographic images obtained by AFM (Fig. 3a). Thus, by controlling the concentration of PVA, nanometric pores homogeneously distributed on PLA film, could be achieved following the method described elsewhere [31,56]. Hence, the major population of pores has diameters at around 335 nm , as can be seen in Fig. S3. Moreover, the height image in Fig. 3a evidences such uniform pore distribution, with R_q and R_a parameters very low (41.2 nm and 34.8 nm , respectively). The R_q is the root mean square average of height deviation taken from the mean 2D-image data plane. R_a is the arithmetic mean of absolute height values in a roughness profile along a sampling profile. Considering the 3D-image of the porous PLA film, a maximum height of PLA hill was found to be approximately 212 nm . After the removal of PVA blended polymer, cold-plasma activation with oxygen allowed the creation of radical and charged moieties with enhanced affinity to other molecules. This method was extensively reported by ours, with different substrates, proving high efficacy for the subsequent reaction with other materials [31,57–59].

The morphology of porous PLA films, without and with C-pept is

presented in Fig. 3b. In contrast to the AFM topography, which was taken with PLA adhered to ITO-glass substrates, well-known for its very smooth surface and very low roughness [60,61], SEM images revealed a non-smooth morphology, due to the film deposition on carbon WEs (Fig. 3-b1, b2, inset). After C-pept attachment, promoted by the surface activation route, the morphology did not show any substantial differences respect to the pristine PLA film (Fig. 3-b3, inset).

Additionally, we tried to identify the amino acids from C-pept molecules (showed in Fig. S1), covalently bounded to PLA NM, with FTIR-small angle grazing accessory (SAGA), i.e. with the film adhered to a mirror gold substrate. However, it was unsuccessful due to the nanometric assemble of the amino acids of the 13-peptide segment. In our recent work [62], we used FTIR-SEIRAS and quartz-microbalance to evidence the anchoring of a sparsely tethered bilayer lipid membrane connected to a PLA film, demonstrating the facility of the biodegradable polymer functionalization. In the present work, the covalent bonding between PLA and C-pept building block molecules was investigated and demonstrated by XPS analysis (Fig. 3c). The survey spectra revealed the presence of carbon (C1s peak at 285 eV) and oxygen (O1s peak at 533 eV) atoms in both samples, as expected. Additionally, an important contribution from N atoms (N1s peak at 400 eV) arises only in the peptide-modified PLA films, belonging from the amide bonds in the peptide sequence. S2p also appeared in samples with C-pept, which was associated to residual molecules from EDC/NHS transamidation reaction, used as the covalent promoter in the synthetic route for the preparation of our electrochemical sensor (Scheme 1).

The up and down plots in Fig. 3c show the high-resolution spectra of O 1s, N 1s, C 1s and S 2p of PLA-C-pept and pristine PLA films, respectively. The area under the curves were used to quantify the atomic percentage among such atoms and the deconvolution of the peaks allowed the identification of the neighbourhood covalent bonds of each one. These curves of C 1s were deconvoluted to three Gaussian curves and were assigned to the presence of C—C bonds (284.7 eV), C—O bonds (286.6 eV), and O—C=O bonds (288.7 eV). In the case of pristine PLA, the proportion of various bonds was found to be very similar for C—C (34.8%), C—O (32.6%) and O—C=O (32.6%) (Table S1). Following the

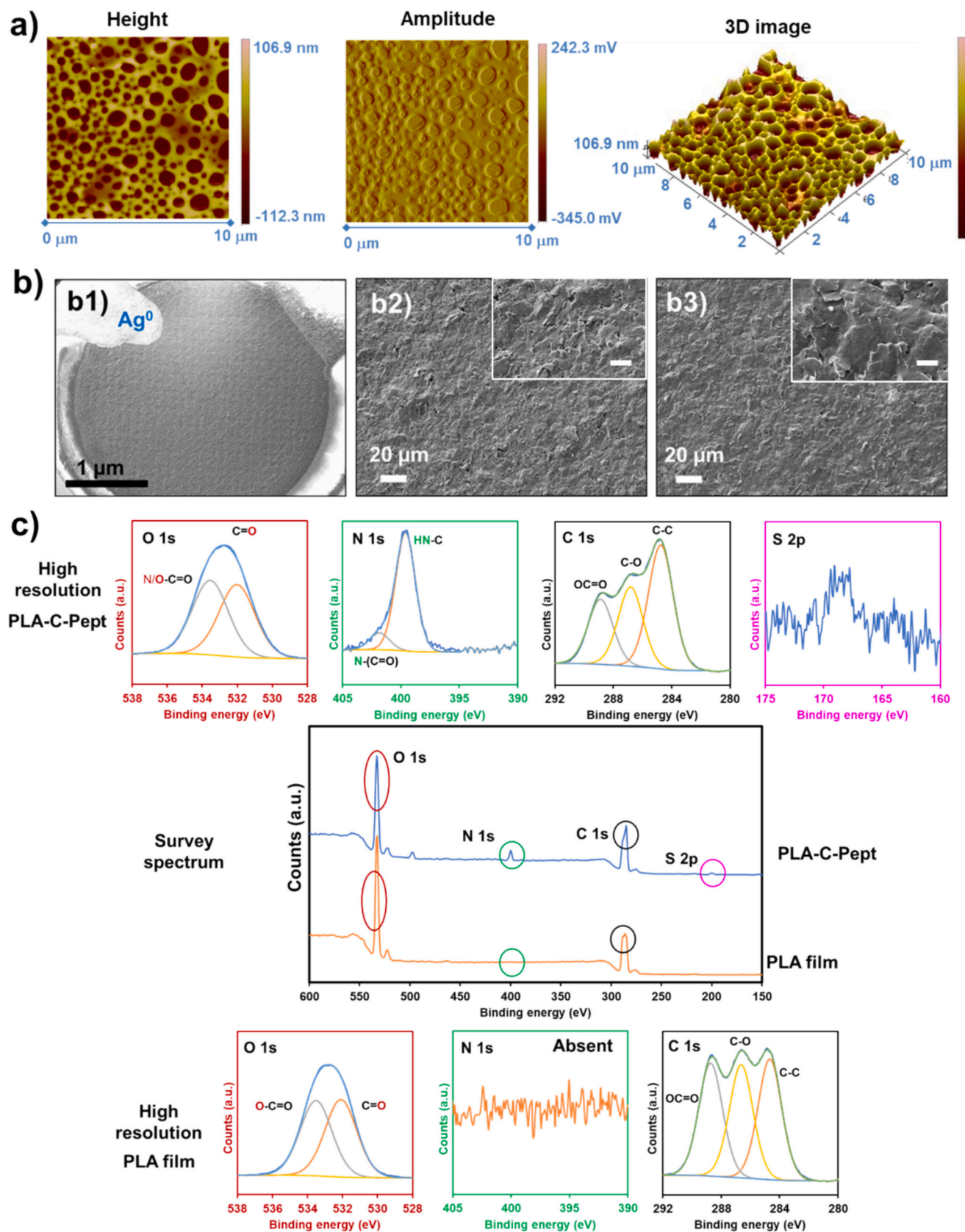


Fig. 3. a) AFM of porous PLA NM film, after etching of PVA polymer. b) SEM images of: b1) SPE-WE with PLA (23 $\times$ magnification), where silver represents the ink used for the electrical contact with the film; b2) higher magnification of micrograph showed in b1 (100 $\times$); b3) PLA-C-pept (100 $\times$). Inset micrographs represent the morphology of films at higher magnification (1000 $\times$); c) XPS survey (middle image) and high-resolution spectra of PLA-C-pept (up) and pristine PLA NM film (bottom) with C 1s, O 1s, N 1s and S 2p atoms.

bonding of C-pept molecules, a slight rise in the percentage of C—C bonds (46.3 %) appeared, accompanied by a modest decrease in the quantity of C=O and O—C=O groups (29.5 % and 24.2 %).

The O 1s peak was also treated with Gaussian curves, resulting in the identification of two curves in both samples, corresponding to C=O and O—C=O groups. An identical pattern is evident in the O1s components, with very similar proportion of 50 %:50 % of C=O (532.0 eV) and O—C=O (533.5 eV) groups (Table S1).

The high-resolution N 1s peak of PLA-C-pept demonstrates the presence of only two Gaussian curves from amine (N—H, highest contribution, 87.7 %) and amide linkages (N—C=O, lowest contribution, 12.3 %). Specifically, it reveals the existence of N—H group at 399.6 eV and a bond of N with a neighbouring carbonyl group at 401.8 eV, which were completely absent in PLA pristine films. The peak of S 2p was not treated due to the very low proportion and high noise accrued in the high-resolution spectrum (Fig. 3c).

The results obtained confirmed that the C-pept molecules were successfully immobilized on the PLA surface to obtain the electrochemical biosensor.

3.3. Electrochemical detection of cholesterol with PLA-C-pept sensor supported on SPE electrodes

Once we have confirmed the success of our biosensor preparation protocol, which resulted in the covalent bonding of the C-pept specific sequence to the perforated PLA NM, we proceeded to study its sensitivity to the addition of the cholesterol target. Despite to popular belief, the lack or excess of such lipids in body is linked not only to heart and coronary artery diseases but also to several other disorders that will undoubtedly affect and exacerbate major health conditions [1,3,7,63]. Therefore, the development of fast non-enzymatic cholesterol biosensors is of upmost relevance in biomedicine [17,64,65].

First, potassium ferrocyanide redox probe electrolyte was used to evaluate the electrochemical behaviour of the SPE-surface assembly with each polymer membrane functionalization and, thereafter, with the peptide immobilization, before the sequential incorporation of cholesterol molecules [32]. Fig. 4 reports the comparison between the experimental EIS results with the EEC simulations (Fig. 4a). PLA:PVA and PLA NM SPEs exhibited similar electrical responses (Fig. 4b), with only small changes in the bulk resistance (R_b) of the films, due to the presence of the pores in the latter (Table 2, R_b values). In the Nyquist plots (Fig. 4b) it can be appreciated that the corresponding semicircles, at high frequencies, are related to the electron charge transfer resistance of the film. The non-ideal capacitances were fitted as CPE_b to the polymer bulk representation and to the double-layer constant phase element (CPE_{dl}) appeared between the SPE, related to the rough carbon activated electrode surface, and the polymeric film. The high insulating character of the non-perforated PLA:PVA film ($R_b \sim 5.11$ k Ω) is reflected in its higher impedance values respect to the nanoporated one ($R_b \sim 1.89$ k Ω) (Table 2). The subsequent creation of the porous on the PLA film substantially reduced the impedance values, as can be also visualized in Bode plots. Fig. 4c shows low real and imaginary impedance values at high frequencies and Fig. 4d evidences the low impedance modulus at high frequency (10^5 Hz) related to the excellent electron transfer through the working electrode in such films. Thus, the EIS curves confirm an improvement in the electrochemical conductivity to faster electron transfer thanks to the creation of the pores by solvent etching of PVA molecules.

After the C-pept sequence attachment, a separation of the bulk resistance (R_b) from the PLA film and that of the charge transfer resistance (R_{ct}) of the electrolyte diffusion through the pores was evidenced at high-to-medium frequencies, in PLA-C-pept biosensor (Table 2). Those separation of electrical charges is reflected in two characteristic time constants (τ_1 and τ_2), with a flattened semi-circle and a broader curve at high and medium frequencies, respectively, better evidenced in Fig. 4d–e (Bode plot), and a bulk capacitance reduction of PLA

counterpart. These time constants are completely different from those obtained for the former films, in which PLA-PVA and PLA NM displayed one well-resolved semi-circle and the second in the process of formation (Fig. 4b). The modulus of impedance versus log of frequency curves (Fig. 4d), clearly confirm the two break pointing changes in the slopes of the curves, corresponding to τ_1 and τ_2 . By contrary, the functionalized PLA NM has an initial τ_1 change at very high frequency domain and τ_2 Change at medium frequency range. It suggests that the more active ionic groups of the peptide facilitate the electron transfer rate of the redox probe on the electrode surface. The other evidence is the lower values of R_b observed, if compared to PLA NM value (Table 2). By the other hand, the C-pept most important contribution of non-polar groups appears as a third-time constant (τ_3) at low frequencies, causing an enhancement of PLA-C-pept resistance, which is ten times higher than that of PLA NM apart (hereafter denoted as R_{pept}).

When comparing the changes occurring in the phase angle curves (Fig. 4e), similar conclusions are extracted. The phase angle tends to zero degree at very high frequency owing the very low resistance of the electrolyte in all cases. Then, the phase angle rises to values of 50–60° due to the contribution of the PLA insulating film. The covalent bonding of the C-pept molecules, with polar amino acid groups, to PLA surface helps the ionic mobility, shifting the phase angle to values similar to that of the porous PLA (50°), at medium frequency zone. Further, the contribution of the non-polar segments in C-pept leads to the enhancement of such values close to 70° in the lowest frequency interval.

The excellent fitting of the EEC, represented in Fig. 4b–e, with the experimental results confirms the mechanism of charge transfer proposed. The values of the electrical elements extracted from the theoretical simulation are shown in Table 2.

Then, having checked the electrical circuits, which provides useful information concerning the systems under investigation, let's move to the interpretation of the biosensor detection results with EIS. PLA-C-pept resistances (R_{pept}) resulted on variations during Chol calibration curve, as can be seen in the Table 2, corroborating the binding affinity demonstrated in FRET results. The fitting and experimental errors found to R_{pept} are less than or equal to 1 %. Consequently, the fitted curves are perfectly able to follow the increase of Chol contents with a remarkable reduction on R_{pept} , whereas obeying a slight increasing with gradual addition of [Chol] aliquots until to reach 15 μ M. Hence, the big macromolecule termed "CRAC" is the responsible for the high R_{pept} obtained (19.3 k Ω), inexistent in PLA NM films. After the addition of only 2 μ M of the lipid, this resistance decreases to 8.7 k Ω , which can be attributed to changes in the C-pept receptor due to the lipophilic nature of cholesterol. When increasing the aliquots, the system starts to saturate the PLA-C-pept film due to few positions of linkage with amino acid groups from CRAC. It is the main hypothesis for the enhancement of the R_{pept} again observed in Nyquist and Bode plots. Moreover, the electrolyte resistance (R_s) was maintained stable during cholesterol incorporation, thus confirming the lipid molecules do not interfere with the electrolyte probe diffusion, as expected. It can be concluded that the proposed EEC circuit is capable of simulating EIS responses of Chol for SPEs accounting PLA-C-pept as sensor, representing a step forward in such biomolecule detection.

In order to confirm the previous hypothesis, we simulated a new dataset of EIS measurements with higher Chol concentrations, aiming to demonstrate the model's predictive capability of PLA-C-pept biosensor response for the non-enzymatic detection of Chol molecules. In Fig. S4, the Bode plot depicts the consecutive additions (titration) of Chol molecules up to 40 μ M, along with the related calibration curve. The calibration curve exhibits two linear ranges: 2–15 μ M and 20–40 μ M. Based on these ranges, a limit of detection (LOD) of 6.31 μ M and a sensitivity of 2.91 Ohm μ M⁻¹ mm⁻² were calculated. Under conditions with a lower concentration of cholesterol, the sensor exhibited a higher degree of sensitivity. The analytical performance of the developed biosensor compared to those reported for cholesterol is shown in Table 3. Enzyme based biosensors report low detection limit and wide linear ranges of

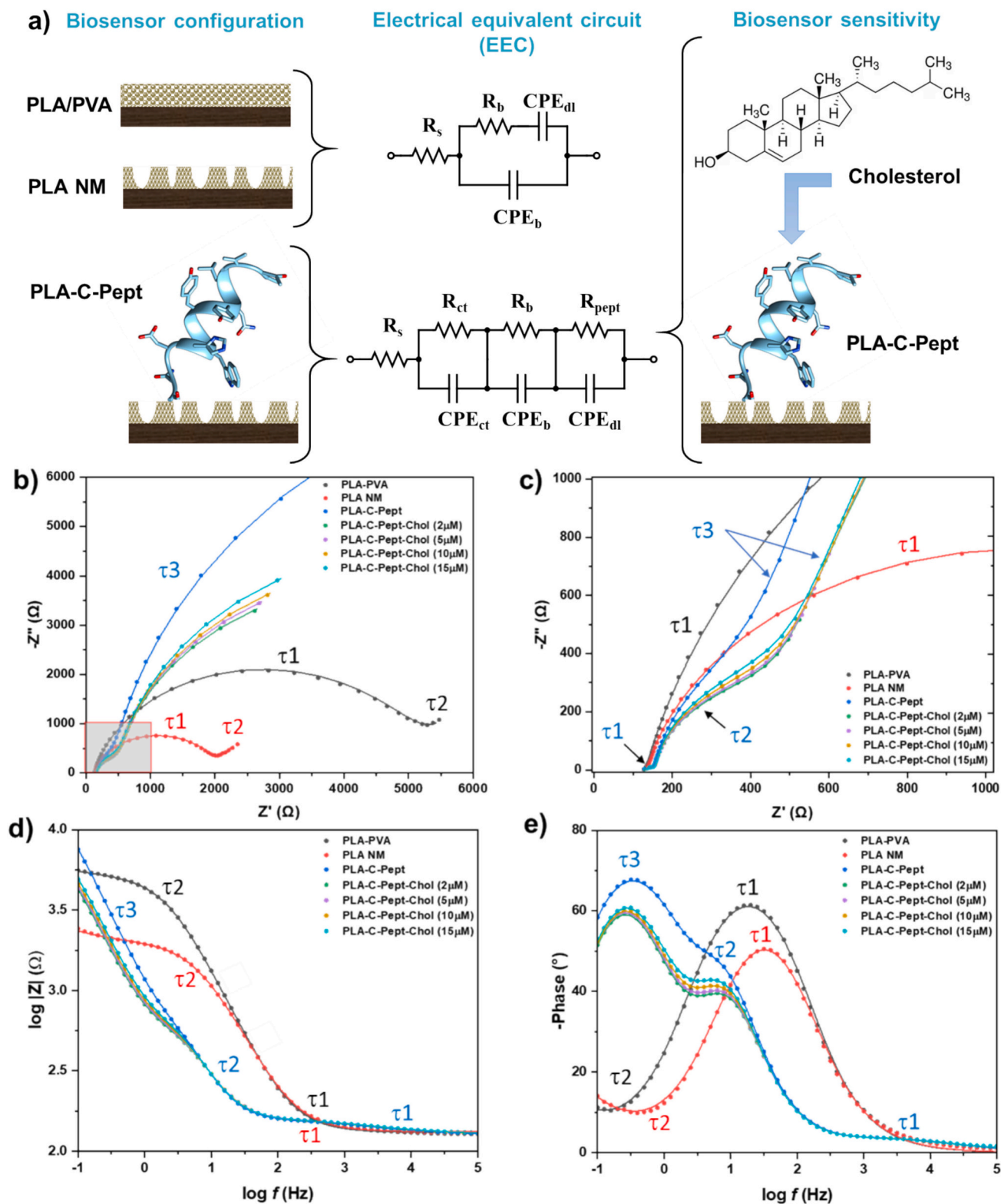


Fig. 4. a) Scheme of the different biosensor electrodes and their respective EECs used to fit the experimental data showed in Nyquist and Bode plots. b) Nyquist plots of PLA:PVA, nanoporated PLA film, PLA-C-pept, and PLA-C-pept with different aliquots of free cholesterol molecules, from 100 mHz to 100 kHz. c) Nyquist plot of high frequency region highlighted in b). d) $\log |Z|$ impedance versus $\log$ of frequency; and e) phase angle versus $\log$ of frequency. Symbols represent the experimental data, solid lines represent the EEC fitting and τ symbolizes the time constant of each serial circuit contribution showed in a). The colour of “ τ ” letter and numbers represent the different systems: black for PLA-PVA insulating film, red for PLA-NM and blue for PLA functionalized with C-peptide, without and with several concentrations of cholesterol target molecule.

Table 2

Values of resistances and capacitances, for each sample analysed by EIS in potassium ferrocyanide redox probe electrolyte solution, after fitting with the EEC displayed in Fig. 4a. The error percentages are indicated between parentheses.

Sample	R_s (Ω)	R_b ($k\Omega$)	CPE_b ($\times 10^{-5} F \cdot s^{n-1}$)	n	CPE_{dl} ($\times 10^{-3} F \cdot s^{n-1}$)	n
PLA-PVA	131.0 (0.36)	5.11 (1.59)	2.00 (1.61)	0.87 (0.34)	1.77 (11.1)	0.66 (14.1)
PLA NM	132.1 (0.34)	1.89 (1.19)	2.23 (2.08)	0.85 (0.42)	2.29 (4.5)	0.65 (5.88)

Sample	R_s (Ω)	R_{ct} (Ω)	CPE_{ct} ($\times 10^{-4} F \cdot s^{n-1}$)	n	R_b (Ω)	CPE_b ($\times 10^{-4} F \cdot s^{n-1}$)	n	R_{pept} ($k\Omega$)	CPE_{dl} ($\times 10^{-4} F \cdot s^{n-1}$)	n
PLA-C-pept	126.6 (0.26)	30.4 (2.38)	1.20 (13.4) (2.57)	0.58 (2.57)	261.3 (2.06)	1.70 (1.48)	0.95 (0.56)	19.3 (0.99)	1.83 (0.14)	0.93 (0.22)
PLA-C-pept-Chol (2 μ M)	127.3 (0.32)	31.7 (2.93)	1.37 (15.8) (3.11)	0.56 (3.11)	323.5 (1.29)	1.20 (1.29)	0.95 (0.45)	8.72 (1.04)	3.27 (0.18)	0.93 (0.28)
PLA-C-pept-Chol (5 μ M)	127.4 (0.31)	32.0 (2.82)	1.43 (15.0) (2.99)	0.56 (2.99)	331.3 (1.25)	1.21 (1.21)	0.95 (0.43)	9.09 (0.99)	3.15 (0.17)	0.93 (0.27)
PLA-C-pept-Chol (10 μ M)	127.2 (0.30)	31.8 (2.73)	1.42 (14.6) (2.91)	0.55 (2.91)	339.2 (1.28)	1.23 (1.17)	0.95 (0.42)	9.50 (0.97)	3.00 (0.16)	0.93 (0.27)
PLA-C-pept-Chol (15 μ M)	127.3 (0.32)	32.0 (2.87)	1.46 (15.3) (3.07)	0.57 (3.07)	345.3 (1.44)	1.27 (1.23)	0.95 (0.45)	10.3 (1.03)	2.79 (0.17)	0.93 (0.28)

Table 3

Comparison of different biosensors for the enzymatic and non-enzymatic detection of cholesterol.

Working electrode	Method	Linear range (μ M)	LOD (μ M)	Ref.
Grp/ β -CD/methylene blue	DPV	1–100	<1	[66]
MMIP ICPTE/m-GEC	SWP	0.51–11.0	0.15	[67]
Si-GO-g-CMNC/GCE	CV	5.18–25.9	7.4	[68]
ChOx/PGE	EIS	1×10^3 – 3×10^3	62.0	[69]
ChOx/Pt/rGO/P3ABA/SPE	CA	250 – 4.00×10^3	40.50	[70]
ChOx/Au/ZnO/MWCNTs	DPV	0.1–100	0.1	[71]
ChOx/NPG/SPE	Amperometry	50 – 6×10^3	8.36	[14]
C-pept/PLA NM/SPE	EIS	2–6	6.31	This work

detection since total cholesterol molecules in samples are converted into cholest-4-en-3-one and hydrogen peroxide due the enzymatic activity of ChOx. Even though, the developed biosensor still presents a clinically relevant detection limit and PLA-C-pept film might be implemented as a valuable system to retain cholesterol molecules due to its outstanding binding properties.

Moreover, it can be deduced that, the increment of cholesterol concentration in the solution, results in the modification of the electrical double layer at the electrode/electrolyte interface and a reduction of PLA-C-pept resistance (R_{pept}), if compared to the PLA-C-pept pristine sample. It traduces in a decrease slope at the lowest frequency in $\log |Z|$ edge (Fig. 4d) and a shift of the phase angle to lower values (Fig. 4e). To compare the results, the $\log |Z|$ experimental impedance values are collected in Table S2 at the select frequency of 0.10 and 0.50 Hz. The choice of such low frequency interval is due to the supposedly low mobility of cholesterol molecules in water and the previous FRET detection evidence that such molecules are trapped by the C-pept biomolecules. This low diffusion of cholesterol in the aqueous solutions is due to its hydrophobic nature and relatively long organic molecule structure. The interaction with PLA-C-pept will occur at slow time constants in the low frequency EIS domain.

Complementary results were obtained with CV studies, with similar concentrations of Chol as that used for the EIS analyses. They are discussed in the supporting file (Fig. S5).

4. Conclusions

In the present study, we investigated the potential of a selected peptide sequence, known as the CRAC-modified peptide and referred to as C-pept, for the development of a non-enzymatic cholesterol biosensor. Our experimental approach elucidated specific interactions at the molecular level within membrane biology, which are essential for understanding metabolic diseases characterized by cholesterol dyshomeostasis and for the future implementation of therapeutic strategies involving membrane components. The use of FRET offered a sensitive and quantitative method for these studies, although careful interpretation of the results is required, considering the distances and orientations between donor and acceptor molecules.

The synthesis strategy proved to be effective for the covalent bonding of the novel C-pept sequence to biodegradable PLLA films. Its easy reproducibility presents a significant advantage for large-scale biosensor fabrication in pharmaceutical production. The success of the synthesis was confirmed through spectroscopy and microscopy analyses.

The EIS results validated the sensitivity of PLA-C-pept film as an efficient electrochemical cholesterol biosensor, with a LOD of 6.31 μ M for free-cholesterol in solution. Additionally, saturation of the PLA pores was observed beyond 30 μ M, where the impedance signal remained constant. Despite this saturation, the biosensor limits established in the present study enable detection at sufficiently low concentrations, facilitating its use as a point-of-care sensor for small sample volumes, without the need for enzyme interactions or complex diagnostic tools.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2024.136337>.

CRedit authorship contribution statement

Ana Lia Bernardo: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ahmed H.M. Mohammed-Sadhakathullah:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Cloilde B. Angelucci:** Validation, Investigation, Formal analysis. **Francesc Estrany:** Formal analysis. **Alessandro Berghella:** Formal analysis. **Juan Torras:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Elaine Armelin:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation. **Sergio Oddi:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Enrico Dainese:** Writing – review & editing, Supervision, Resources, Project administration, Funding

acquisition, Conceptualization.

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Declaration of competing interest

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

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Data availability

Data will be made available on request.

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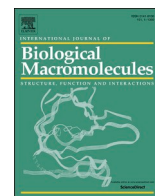
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Corrigendum

Corrigendum to “Non-enzymatic cholesterol biosensor: Electrochemical sensing based on peptide-poly(lactic acid) thin film” [Int. J. Biol. Macromol. 281 (2024) 136337]

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