Analytica Chimica Acta xxx (2018) 1–11

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Electrical double layer modulation of hybrid room temperature ionic liquid/aqueous buffer interface for enhanced sweat based biosensing

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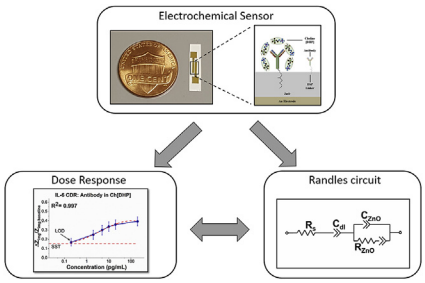
HIGHLIGHTS

- Leveraging kosmotropic and kaotropic behavior of RTILs for electrochemical detection of biomolecules.
- Correlation between surface charge modulation using zeta potential to non-faradaic impedance measurements.
- Enhancement in sensitivity for cortisol and IL-6 using RTIL/sensor interface.
- Identifying and matching the appropriate RTIL to biomolecule for maximizing non-faradaic interfacial response.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 October 2017
Received in revised form
31 January 2018
Accepted 5 February 2018
Available online xxx

Keywords:
Non-faradaic impedance spectroscopy
Electrical double layer
Room temperature ionic liquid
Biosensor

ABSTRACT

We have investigated the role of kosmotropic anionic moieties and chaotropic cationic moieties of room temperature hydrophilic ionic liquids in enhancing the biosensing performance of affinity based immunochemical biosensors in human sweat. Two ionic liquids, 1-butyl-3-methylimidazolium tetrafluoroborate (BMIM[BF_4]) and choline dihydrogen phosphate (Choline[DHP]) were investigated in this study with Choline[DHP] being more kosmotropic in nature having a more protein stabilizing effect based on the hofmeister series. Non-faradaic interfacial charge transfer has been employed as the mechanism for evaluating the formation and the biosensing of capture probe antibodies in room temperature ionic liquids (RTILs)/aqueous human sweat interface. The charge of the ionic moieties were utilized to form compact electrical double layers around the antibodies for enhancing the stability of the antibody capture probes, which was evaluated through zeta potential measurements. The zeta potential measurements indicated stability of antibodies due to electrostatic repulsion of the RTIL charged moieties encompassing the antibodies, thus preventing any aggregation. Here, we report for the first time of non-faradaic electrochemical impedance spectroscopy equivalent circuit model analysis for analyzing and interpreting affinity based biosensing at hybrid electrode/ionic liquid-aqueous sweat buffer interface guided by the choice of the ionic liquid. Interleukin-6 (IL-6) and cortisol two commonly occurring biomarkers in human sweat were evaluated using this method. The limit of detection (LOD) obtained using both ionic liquids for IL-6 was 0.2 pg mL^{-1} with cross-reactivity studies indicating better performance of IL-6 detection using Choline[DHP] and no response to cross-reactive molecule. The LOD of 0.1 ng/mL was achieved for cortisol and the cross-reactivity studies indicated that cortisol antibody in BMIM[BF_4] did

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https://doi.org/10.1016/j.aca.2018.02.013

not show any signal response to cross-reactive molecules. Furthermore, improved sensitivity and LOD was achieved using ionic liquids as compared to capture probes in aqueous buffer.

1. Introduction

Human sweat contains a wide range of ions, proteins such as interleukin-6 (IL-6), and hormones such as cortisol. These biomolecules are biomarkers that can provide health information [1] and therefore, this biological fluid is suitable for rapid non-invasive biosensing. IL-6 is a cytokine secreted by lymphoid and non-lymphoid cells. IL-6 is an important inflammatory biomarker that can be useful in monitoring immune response for cancer treatment [2]. IL-6 also impacts basal glucose intake [3] and increases cortisol secretion during psychological stress [4,5]. The physiological levels of IL-6 in sweat range between 5 and 15 pg mL^{-1} and show a good correlation to plasma levels [6]. Therefore, IL-6 levels can be monitored non-invasively using sweat based biosensors. Cortisol, a hormone, is a generic biomarker for stress and is expressed in sweat in the concentration range 20–140 ng mL^{-1} [7]. The increased physiological stress is a growing concern and monitoring of cortisol serves as the most valuable biomarker [8] for stress-related disorders. Furthermore, cortisol levels vary as per the circadian rhythm and environmental conditions which necessitates continuous monitoring of cortisol [9]. Sweat being a non-invasive fluid would be the most suitable biological fluid for real-time continuous monitoring of these biomarkers. Among the existing methods for non-invasive biosensing from human sweat, electro-analytical techniques, particularly, non-faradaic impedance measurements have high sensitivity, fast response time and can be miniaturized for low cost diagnostic assessments of biomolecules [10].

Electrochemical impedance spectroscopy (EIS) is a label-free method that utilizes very small voltage perturbations to measure biomolecular interactions and hence, is more robust as compared to other electrochemical techniques in detecting biomolecules without the requirement of a redox label or tag. EIS measures changes in impedance across measurement electrodes in biosensor systems due to biomolecular binding with in the electrical double layer (EDL) at the electrode-solution interface [11]. Furthermore, EIS based measurement is most attractive to human sweat based biosensing as modulation to the EDL associated with biomolecule binding can be captured with low power bioelectronics interfaces thus enabling wearable biosensing. In this article, we have investigated methods of enhancing the modulation of EDL in order to enhance the analytical performance of a human sweat based biosensor.

In an affinity mechanism based biomolecule sensing system measured using EIS, the changes in impedance resulting from the interaction between the capture probe and target analyte within the EDL can be modeled as a Randle's equivalent electrical circuit. The modulation of EDL resulting from bimolecular interactions at the electrode-solution interface may be attributed to changes in terms of charge-transfer resistance (R_{ct}) or double layer capacitance (C_{dl}). Hence, to accurately identify these biomolecular interactions vis-a-vis non-specific interactions, it is necessary to have a stable and compact EDL formed at the electrode-solution interface. Although an EDL is formed upon voltage bias at the electrode-aqueous solution interface, it is primarily dependent on the ionizable content within the buffer which is subject to a number of microscale electro-kinetic mechanisms such as electro-osmosis and electro-convection, thus, resulting in charge screening effects and producing reduced signal response at the electrode interface.

One strategy that our group has adopted to enhance the EDL stability and obtaining resolution in the modulation to the EDL is the use of room temperature ionic liquids (RTILs). These non-aqueous highly polar solvents exhibit excellent capacitive properties and form compact EDLs [12] that enhance the signal response in addition to enhancing protein stability. Effective affinity based biosensing can be achieved when there is a stable bio-functionalization of the affinity based capture probes required for the detection of the target biomolecules. RTILs are known to stabilize proteins and enzymes [13]. We have previously shown a stable and reliable detection of proteins in human sweat for up to 1 week using capture probe antibodies in RTILs as compared to capture probes in aqueous buffers that lack stability over time [12]. This enhanced performance in RTIL is primarily due to the protein stabilization property of RTIL and formation of compact EDL resulting in high charge density. However, no characterization on the modulation of EDL due to biomolecular binding at the electrode/ionic liquid interface has ever been performed. Here, we report for the first time using electrochemical equivalent circuit analysis of the tuning of compact EDL at the hybrid ionic liquid interface that results in enhanced biosensing performance. Most of the biosensing platforms that use RTILs do not leverage these electro-chemical properties of RTILs and rely mostly on amperometric or potentiometric techniques which often require the use redox labels in conjunction with the RTILs. These techniques with redox molecules require higher input voltages and have slower response time, and the measurement mechanism is faradaic in nature. Faradaic impedance spectroscopy requires high concentration of redox labels and is dependent on the electron transfer kinetics between the electrode and redox probe. Whereas, non-faradaic impedance spectroscopy is a label-free technique as it does not involve any redox probe and therefore, eliminates any complexity in the electrochemical system [14]. Non-faradaic EIS measures capacitive changes in the electrical double layer at the electrode-solution interface with a fast response time [14–16].

Table 1 summarizes various biosensing platforms using RTILs and the corresponding method of electrochemical detection. The stable biosensing performance using RTILs may be influenced by the properties of RTILs such as size, hofmeister series or charge density. The interactions between the anionic moiety of the RTIL and protein affect the stability of the immunoassay. 1-butyl-3-methylimidazolium tetrafluoroborate (BMIM[BF_4]) and Choline dihydrogen phosphate (Choline[DHP]) that fall at the center and more kosmotropic region of the hofmeister series respectively have shown to enable biomolecule stabilization [13]. EDL modulation mediated through the use of these two RTILs independently was investigated for biosensing of two well established biomarkers in human sweat, IL-6and cortisol.

In this work, we provide a comprehensive analysis on the gold/ZnO and aqueous/RTIL buffer hybrid EDL interfaces and its impact on the electrochemical biosensing of proteins and metabolites in sweat.

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Table 1
Comparison of biosensing performance using ionic liquids and the electrochemical method used for biosensing.

RTIL	Detection	Method	Limit of detection	C_{DL} Modulation	Reference
1-butyl-3-methylimidazolium hexafluorophosphate	DNA hybridization	Cyclic Voltammetry, Differential Pulse Voltammetry	3.72 μg mL^{-1}	**X**	[17]
1-octyl-3-methylimidazolium trifluoromethylsulfonate ([OMIM][NTF_2])	Cholesterol	Faradaic EIS	4.4 μM	**X**	[18]
1-Decyl-3-methylimidazolium chloride(DMIM-Cl)	Glucose	Cyclic Voltammetry	0.028 mM	**X**	[19]
1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide (BmimNTF_2)	Tri glycerides	Cyclic Voltammetry	0.11 μg mL^{-1}	**X**	[20]
1-butyl-3-methylimidazolium tetrafluoroborate(BMIM[BF_4])	IL-6	Non-Faradaic EIS	0.2 pg mL^{-1}	**Yes**	**This work**
Choline dihydrogen phosphate (choline[DHP])	Cortisol		0.1 ng mL^{-1}		

2. Materials & methods

2.1. Reagents and materials

Polyamide substrates of 200nm pore size were purchased from GE healthcare Life Sciences (NJ, USA). The cross-linker molecule Dithiobis (succinimidyl Propionate) (DSP) and Dimethyl Sulfoxide (DMSO) were procured from Thermo Fisher Scientific Inc. (MA, USA). The two antibodies, α-interleukin-6 and α-cortisol, and cortisol hormone (hydrocortisone) were purchased from Abcam (MA, USA). Interleukin-6 full length recombinant protein was ordered from Thermo Fisher Scientific Inc. BMIM[BF_4] was purchased from Sigma-Aldrich (MO, USA). Choline[DHP] was purchased as an ionic salt from Ionic-Liquid Technolgies-USA (AL, USA). It was reconstituted into an ionic liquid by mixing in 97.5% (W/V) DI water. Single donor human sweat of pH 5.98 was procured from Lee Biosolutions Inc. (MO, USA) and was stored in −20 °C. Synthetic sweat for zeta potential measurements was prepared in the laboratory according to the protocol from Mathew et al. [21]. The synthetic sweat prepared at pH 2, 4, 6 and 8 comprised of uric acid, lactic acid, ammonia, Na^+, K^+ and Cl^- ions. All dose concentrations of IL-6 and cortisol were performed in human sweat.

2.2. Sensor fabrication

The sensor shown in Fig. 1A was fabricated on a flexible nanoporous polyamide membrane. The fabrication of the sensor was a two-step process. Detailed description of the fabrication process has been provided elsewhere [22]. Briefly, 125 nm gold electrodes were deposited using e-beam deposition. The active region at the center of the electrode for immunoassay functionalization was sputtered using magnetron sputter with 100 nm thick ZnO.

2.3. ATR-IR spectroscopy setup

The infrared spectra (IR) was collected using Nicolet iS-50 FTIR (Thermo Scientific Inc.) in Attenuated Total Reflectance mode. The tool comprised of deuterated triglycine sulfate (DTGS) detector and KBr window. The spectra were collected using Germanium crystal for 256 scans at a resolution of 4 cm^{-1} in the wavelength range 4000 cm^{-1} to 600 cm^{-1}. Samples were prepared on glass substrates deposited with Gold/ZnO (Au/ZnO) similar to the deposition parameters of the sensor substrate. Similar to the sensor immunoassay (described in detail in 2.5), the Au/ZnO surface was immobilized with DSP. α IL-6 and α cortisol antibodies in 100% PBS, 90% BMIM[BF_4] and 90% Choline [DHP] were incubated separately on the DSP functionalized ZnO surface.

2.4. Zeta potential measurements

Zeta Potential measurements provide the charge state of protein by measuring the electrophoretic mobility in a buffer solution. In this study, zeta potential (ZP) (ζ) was measured for the two antibodies, IL-6 and cortisol in various concentration dilutions of two RTILs in synthetic sweat using Malvern Zetasizer NanoZS (Malvern Instruments, UK). ZP measurements for the two antibodies were performed in 25% and 60% BMIM[BF_4] in varying synthetic sweat of pH 2, 4, 6 and 8 and in 25%, 60% and 97.5% Choline[DHP] in synthetic sweat at pH 2, 4, 6 and 8. Also, ZP measurements were performed in only synthetic sweat of pH 2, 4, 6 and 8. Viscosity of the various volumetric ratios of ionic liquids in synthetic sweat was performed using μVisc viscometer (Rheosense, San Ramon, USA). Smoluschowski approximation was used to compute the zeta potentials from the measured electrophoretic mobility [23].

2.5. Electrochemical impedance spectroscopy (EIS) measurements on ZnO sensor

The immunoassay of the biosensor was developed by functionalization of DSP thiol-cross linker on ZnO deposited PA substrate using 3μL of sample volume. 10 μg/mL of α-IL-6 and 100 μg/mL α-Cortisol antibody in aqueous PBS buffer and in both RTILs, BMIM[BF_4] and Choline[DHP] was then incubated for 30 min on DSP functionalized biosensor. 3 μL of human sweat was dispensed to compute the baseline response of the sensor. This was followed by dispensing various dilutions 0.2–200 pg mL^{-1} of IL-6 and 0.1–200 ng mL^{-1} of cortisol. In order to determine the specificity of the biosensor, cortisol doses in the range 0.2–200 pg mL^{-1} was dispensed on sensor functionalized with IL-6 antibody and IL-6 doses in the dose concentration range 0.1–200 ng mL^{-1} was dispensed on sensor functionalized with cortisol antibody.

EIS measurements were performed over the frequency range of 1Hz- 1 MHz with an AC input voltage of 10 mV using a potentiostat (Gamry Instruments, USA). The sensor response was calculated for n = 3 replicates. The ratio impedance change with respect to the baseline impedance was computed for developing calibration dose response curve of the sensor at 100 Hz. Data is represented as mean ± standard error of mean.

3. Results & discussion

The present work is aimed at understanding the hybrid electrical double layer interface that is formed when an ionic liquid interacts with an aqueous polar fluid at one interface and the semiconducting electrode on the opposite interface towards the detection of protein biomolecules. In this paper, we are investigating the EDL modulation when integrated with ionic liquids for detection of IL-6 and cortisol from aqueous ionic buffer with varying pH namely human sweat.

Interleukin-6 (IL-6) and cortisol are the two biomarkers detected and quantified using affinity based assays with the use of a monoclonal antibody mechanism. This work focuses on (i)

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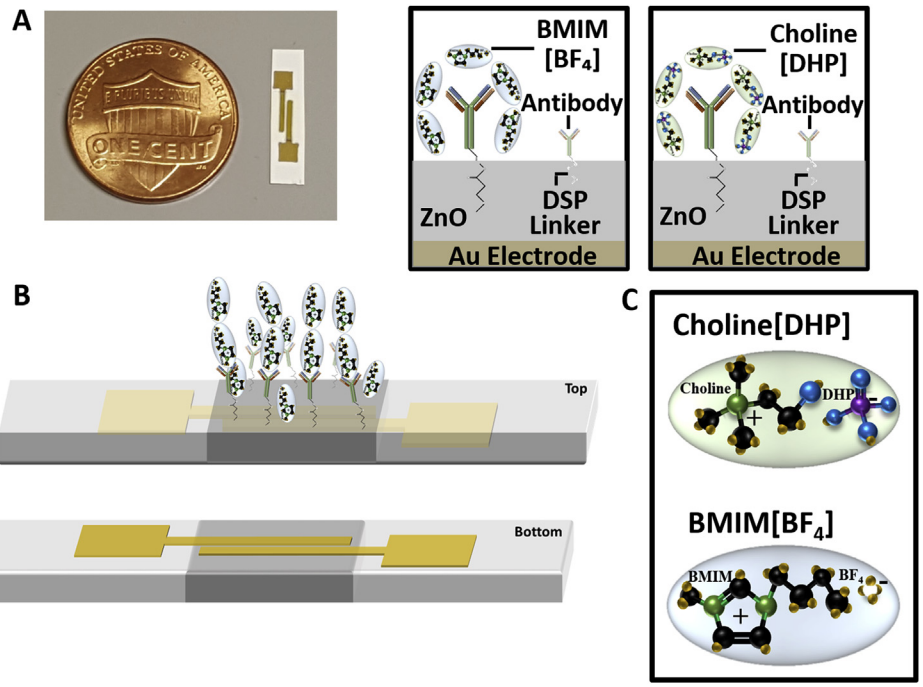

Fig. 1. A. Picture of the sensor, B. Schematic representation of functionalized sensor with Ab in RTIL introduced from front side. The inset shows the schematic representation of RTIL molecules surrounding the protein C. Molecular structures of RTILs, BMIM[BF4] and Choline[DHP].

investigating the role of the ionic moieties of the RTIL buffer on the electrical double layer (EDL) formed in confined volume nanoporous systems (ii) method of interaction of the biomolecules present in aqueous sweat buffer onto semiconductor electrode surfaces mediated through the ionic liquid and (iii) the biosensing performance of affinity based antibody capture probes This section has been systematically categorized into the various sections of the hybrid EDL interface: 1. Characterization of the gold(Au)/ZnO electrode surface for formation of the hybrid RTIL-aqueous sweat buffer EDL towards the development of immunosensor 2. ATR-IR analysis for biomolecule interactions towards the formation of the immunoassay on the RTIL/aqueous buffer interface within the electrolyte 3. Zeta potential measurements of capture probe antibodies in room temperature ionic liquids across varying synthetic sweat pH for investigating stability of the protein biomolecules in the RTIL buffer 4. Investigation of modulation of the hybrid EDL resulting in the measurement of the human sweat biosensor's analytical performance metrics using electrochemical impedance spectroscopy studies.

3.1. Characterization of the gold (Au)/ZnO electrode surface for formation of the hybrid RTIL-aqueous sweat buffer EDL

The substrate used for characterizing the biosensing performance comprised of a metal/semiconductor (Au/ZnO) electrode deposited on nanoporous polyamide (PA) membrane substrate, which was used for transduction of biomolecular binding events into electrochemical signal. Fig. 1A shows the sensor design. A schematic of surface functionalization of the electrode with capture probe antibodies in RTILs is represented in Fig. 1B. Sweat (aqueous buffer) was introduced on the opposite side of the Au/ZnO deposited polyamide surface, to allow for the diffusion of the analyte through the pores of the membrane, thus, resulting in a size based matching and selective binding to the antibody [1]. The capture probe antibodies in both the RTILs, BMIM[BF_4] and Choline[DHP] are represented in Fig. 1D. In order to achieve a robust EDL with reliable electrochemical performance as characterized through the signal to noise ratio at the hybrid interface, it is necessary to have a uniform deposition of ZnO. Fig. 2A shows the SEM image of ZnO deposited uniformly on the PA membrane. The EDX spectra shown as an inset, confirms the presence of ZnO with a peak corresponding to L-shell of Zn at 1KeV.

3.2. ATR-IR analysis for biomolecule interactions towards the formation of the immunoassay on the RTIL/aqueous buffer interface within the electrolyte

In order to establish that the mechanism of interaction of the biomolecules under study through the immunoassay mechanism remains persevered within the RTIL buffer, ATR-IR spectroscopy was performed. It is necessary to have a stable immunochemistry on the sensor surface for developing a robust biosensor. The ATR-IR spectra for DSP functionalized ZnO surface along with α IL-6 antibody and α cortisol antibody in aqueous PBS buffer bound to DSP cross-linker is represented in Fig. 2B. The symmetric carbonyl stretch of NHS ester bond of DSP is indicated by peaks at wavenumbers between 1777 and 1780 cm^{-1}. The peak at 1742 cm^{-1} indicates the presence of free carboxylic acid. The methylene scissors of DSP is indicated by the peaks at 1438 cm^{-1} and 1417 cm^{-1}. A peak at 1316 cm^{-1} indicates symmetric C-N-C stretch of DSP [24,25]. Thus, the spectra of DSP bound ZnO confirms the binding of DSP to ZnO. Further, the conjugation of the antibodies, IL-6 and cortisol, was verified using IR spectra. The binding of antibody to DSP linker results in the breaking of C-O bond of NHS ester in DSP. The primary amine group of antibody binds to that position through aminolysis. This phenomenon was evaluated from IR spectra, with decrease in peak height at 1780 cm^{-1} and changes in peak at 1665 cm^{-1} at DSP-antibody step, as compared to DSP step. The IR spectra of IL-6 antibody in PBS bound to DSP functionalized ZnO shows no peaks in the 1780 cm^{-1} region. A peak shift with an increased peak height at 1652 cm^{-1} in the IL-6 antibody bound to DSP is observed. These confirm that IL-6 antibody was bound to the DSP-ZnO surface.

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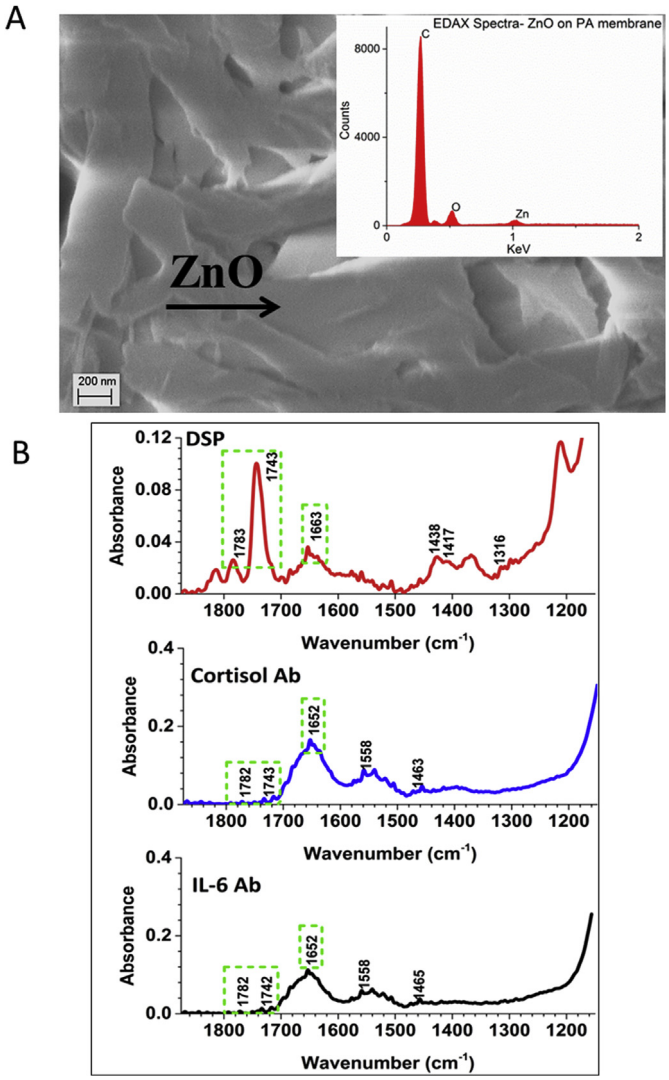

Fig. 2. A. SEM image of uniform ZnO deposition in intercalated pores of Polyamide substrate. Inset shows EDX spectra with Zn peak at 1KeV, B. ATR-IR spectra (i) DSP functionalized on ZnO surface (ii) Cortisol antibody in PBS bound to DSP (iii) IL-6 antibody in PBS bound to DSP functionalized ZnO surface.

Further, cortisol antibody in PBS bound to DSP exhibits similar changes as IL-6 antibody conjugated to DSP (Fig. 2B). No peaks were observed in the 1780 cm^{-1} region. A peak shift with increased peak height at 1652 cm^{-1} indicates aminolysis of DSP and binding of cortisol antibody to DSP, thus confirming antibody conjugation to DSP functionalized ZnO surface.

Once the immunochemistry on the Au/ZnO surface with antibodies in aqueous PBS buffer was confirmed, the stability of the immunoassay for α IL-6 and α cortisol antibodies in RTILs was evaluated using ATR-IR spectra. Conjugation of antibodies in RTILs to DSP functionalized ZnO surface is represented in Fig. 3A. The IR spectra of conjugation of α IL-6 antibody in BMIM[BF_4] shows no peak at 1780 cm^{-1} and 1740 cm^{-1} indicating the breaking of C-O bond of NHS ester of DSP. A peak shift from 1663 cm^{-1} of the DSP only spectra to a peak at 1647 cm^{-1} of α IL-6 antibody in BMIM[BF_4] bound DSP spectra indicates aminolysis and conjugation of antibody to DSP. Similarly, the IR spectra of cortisol antibody in BMIM [BF_4] bound DSP indicates the breaking of C-O bond of DSP at 1774 cm^{-1} and 1740 cm^{-1}, and aminolysis peak at 1631 cm^{-1} confirming a stable conjugation of antibody in BMIM[BF_4] to DSP. Further, similar results for binding of α IL-6 and α cortisol in Choline [DHP] bound to DSP were observed. No peaks were observed at 1780 cm^{-1} and 1740 cm^{-1} for both the antibodies in Choline[DHP] bound to DSP. Aminolysis peak at 1652 cm^{-1} for α IL-6 in Choline [DHP] bound DSP and 1650 cm^{-1} for α cortisol in Choline[DHP] bound DSP were observed confirming the binding of both the antibodies in Choline[DHP] to DSP. The peak positions have been described in Supplementary Table S1.

3.3. Zeta potential measurements of capture probe antibodies in RTILs across varying synthetic sweat pH for investigating stability of the protein biomolecules in the RTIL buffer

Although FTIR provides information on the stability and binding of proteins, it does not provide information on the charge states and electrochemical behavior of proteins in various buffer solutions. Zeta potential measurements provide information pertaining to the surface charge and stability of proteins in buffers such as human sweat with varying pH, ionic composition and water content under applied electric field. Electrophoretic mobility of protein is used to measure the charge state/zeta potential of protein i.e. under an applied electric field, the protein is surrounded by counter-ions and co-ions to acquire a charge a neutral state. This protein and counter-ion complex, held by strong electrostatic forces, acquire a charge neutral state and travel to the opposite electrode [23]. The mobility with which they travel towards the electrode is used to determine the zeta potential, thus providing the charge state of the protein in that buffer. Further, if the zeta potential is around 0 mV, the protein is at its isoelectric point (IEP) and tends to form aggregates and becomes unstable. In this paper, we have assessed the electrochemical surface charge modulation reflective of the modulation to the EDL when the protein is dispersed into hybrid ionic liquid/aqueous buffer at various volumetric dilutions. The primary objective of this study was to understand stability of protein in RTIL upon interaction with sweat buffer.

Fig. 3B represents the zeta potential (ZP) measurements of α-IL-6 antibody and α-cortisol antibody in volumetric ratios of 25% and 60% BMIM[BF_4] with synthetic sweat at pH 2, 4, 6, 8 and 25%, 60% and 97.5% Choline[DHP] with synthetic sweat at pH 2, 4, 6, 8. Zeta potential of antibody in only aqueous buffer (100% synthetic sweat and 0% RTIL) was also performed to compare the electrophoretic stability of capture probe antibodies in aqueous and RTIL buffers. The zeta potential of α-IL-6 antibody in 0% RTIL varied between 8.6 and −4.2 mV across pH 2−8 with a crossover from positive to negative zeta potential occurring at pH 5. Similarly, the zeta potential of α IL-6 in 25% BMIM[BF_4] is between ~2.3 and 0.7 mV and does not vary much with varying pH. However, when the ratio volumetric concentration of BMIM[BF_4] is increased to 60%, the zeta potential varies from ~90 to 60 mV and does not meet the IEP. The ZP varied between ~4 and -4mV for α-cortisol antibody in 0%RTIL with varying pH (2−8) of synthetic sweat. In 25% BMIM[BF_4], the ZP varies between 3.8 and 0.5 mV. However, a higher magnitude of ZP change between ~80 and 30 mV was observed for α-cortisol antibody in 60% BMIM[BF_4] with varying pH between 2 and 8, indicating stability of antibody under applied electric field.

In choline [DHP], both α-IL-6 and α-cortisol have negative ZP at volumetric ratios greater than 60% but the ZP remains closer to the IEP when the volumetric ratio of choline[DHP] is 25% in synthetic sweat. The ZP varied between 4 and −3.7 mV with a cross over at pH 5 for α-IL-6 in 25% Choline[DHP]. However, in 60% Choline[DHP] and 97.5% Choline[DHP], the ZP varied between −3.7 and −19.5 mV and −8.7 to −16.5 mV respectively, and did not meet the IEP. The ZP for α-cortisol in 25% Choline[DHP] almost remained constant between ~3 and −1.8 mV across pH 2−8. However, for 60% Choline [DHP], ZP changed between −2.4 and −19.6mV across pH 2−6 and

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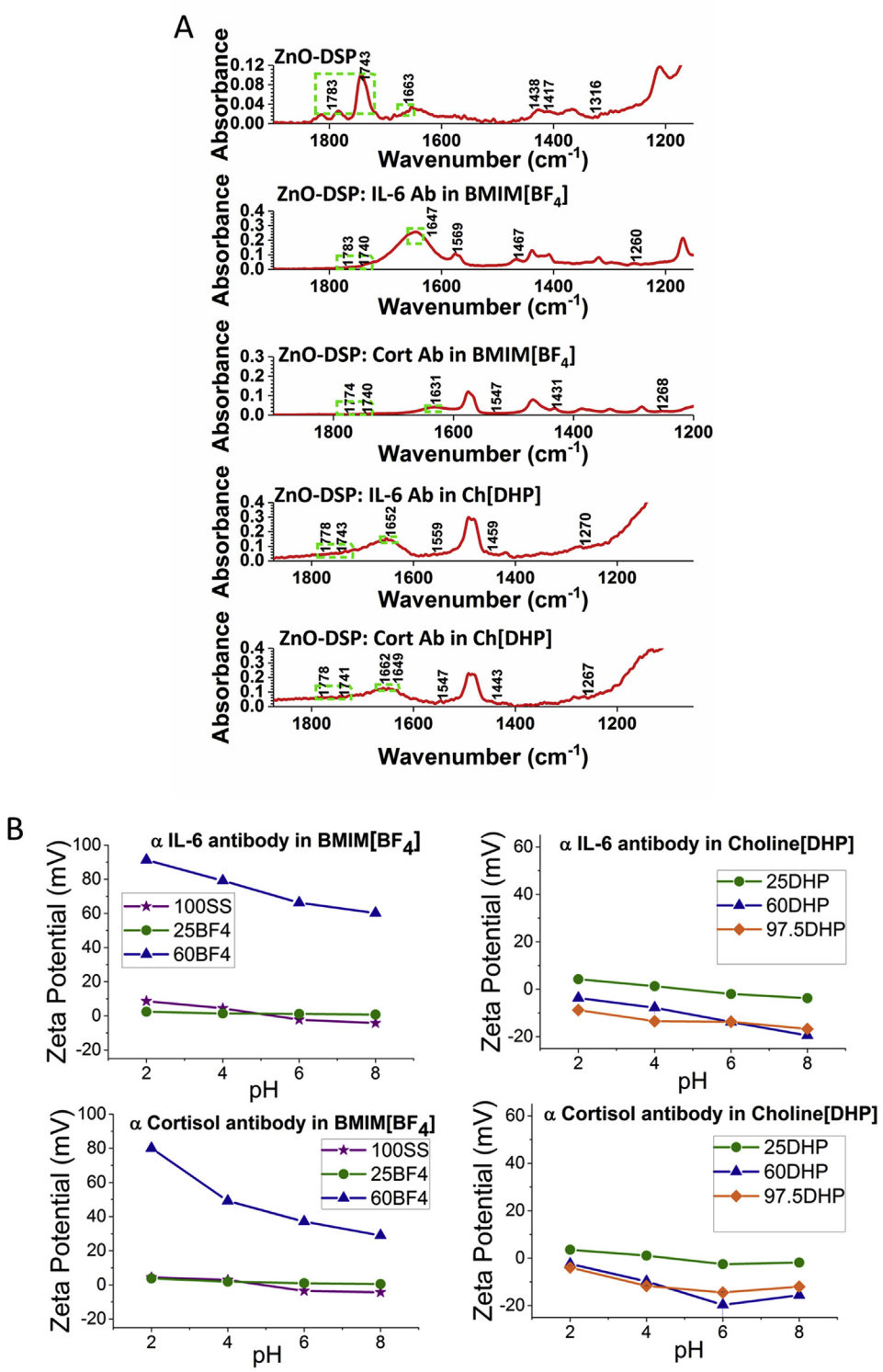

Fig. 3. A. FTIR spectra of (i) DSP functionalized on ZnO surface (ii) Conjugation of IL-6 Ab in BMIM[BF_4] to DSP (iii) Conjugation of Cortisol Ab in BMIM[BF4] to DSP (iV) IL-6 Ab in Choline[DHP] bound to DSP and (V) Cortisol Ab in Choline[DHP] bound to DSP. B. Zeta Potential of IL-6 and cortisol antibody in 100% synthetic sweat and various volumetric dilution ratios of 25%, 60% BMIM[BF_4] and 25%, 60%, 97.5% Choline[DHP] in synthetic sweat at varied pH of 2, 4, 6 and 8.

the ZP dropped slightly to −15.6 mV at pH 8. Similarly, the ZP of α-cortisol in 97.5% Choline[DHP] varied between ~ −4 to −14 mV across pH 2–6 and slightly dropped to −12 mV at pH 8. This indicates that the antibody was stable in Choline[DHP] concentrations greater than 60%. The low ZP in aqueous buffer (no RTIL) and RTIL buffers with volumetric ratios <25% indicates the formation of aggregates of the antibody, thus leading to denaturation. Since the amount of water content in the buffer solution is in a larger content at <25% RTIL concentration (in volume), the water molecules may heavily interact with the protein, thus causing aggregates. While at concentrations ≥60% RTIL, the RTIL forms a shield around the protein, preventing interaction with water that causes denaturation

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of protein. Furthermore, higher magnitude of ZP indicates the stability of protein in the buffer solution. At higher concentrations of RTIL, more protein molecules are surrounded by RTIL molecules. This results in repulsion of protein molecules possessing similar charge and hence higher ZP.

ZP is a characteristic of the electrical double layer formed between the protein-counter ion complexes. The higher magnitude of ZP also indicates the formation of compact EDL around the protein. The ZP measurements indicate that at RTIL concentrations greater than 60%, compact and stable EDLs are formed around the protein. These results can be easily translated to techniques such as Electrochemical Impedance Spectroscopy (EIS) that leverage the formation of EDL for biosensing.

3.4. Investigating the modulation of the hybrid EDL resulting in measurement of the sweat biosensor's analytical performance metrics using electrochemical impedance spectroscopy studies

The immunosensor performance for the detection of IL-6 and cortisol with the antibodies in aqueous PBS, BMIM[BF_4] and Choline [DHP] was evaluated using non-faradaic Electrochemical Impedance Spectroscopy technique. This technique is highly effective to capture the biomolecular binding events at the electrode-solution interface with small voltage perturbations. The biomolecular binding events modulate either the dielectric properties or Debye length of the EDL. This phenomenon can be captured in the low to mid frequency regime typically between 10 and 1000 Hz [11,26]. In this work, the analytical biosensing performance metrics have been characterized at 100 Hz primarily due to high signal-to-noise ratio and ease of integration into portable electronics for translation of this biosensing system to point-of-care diagnostics.

In order to assess the biosensing performance using non-faradaic EIS, it is necessary to understand binding mechanism at the electrode-solution interface that can be modeled as an equivalent circuit. The electrode-solution interface primarily consists of electrical double layer (EDL) and bulk diffusion. EDL comprises of 1. Compact stern layer formed by the ions of opposite charge attracted to the electrode surface and 2. Diffuse layer [27]. The biomolecular binding events occurring within the EDL can be captured using non-faradaic EIS by measuring the impedance change at the electrode-solution interface. The impedance change due to the binding of molecules, when modeled as an equivalent circuit, can be attributed to either a change in charge-transfer resistance or double layer capacitance depending on the buffer solution used.

RTILs have excellent electrochemical, capacitive properties and offer more stability to biomolecules [28–30]. Here, we compare the biosensing performance for two biomolecules, interleukin-6 (IL-6) and cortisol in human sweat with their capture probe antibodies in aqueous PBS buffer and ionic liquids. Further, a comparison on the choice of ionic liquid specific to a biomolecule for better biosensing performance was evaluated.

Fig. 4 shows the calibration dose response (CDR) curve for the detection of IL-6 and cortisol in human sweat. Specific antibody to capture these biomolecules, dissolved in three buffer solutions PBS, BMIM[BF_4] and Choline[DHP] was used. The CDR curves are plotted at 100 Hz as ratio change in impedance (Z_{mod}) from the baseline step without any target biomolecule. As represented in Fig. 4A, maximum change in Z_{mod} ratio was observed to be ~0.45, ~0.5 for IL-6 with its antibody in PBS and BMIM[BF_4] respectively and Z_{imag} ratio of ~0.32 in choline[DHP]. The limit of detection was interpreted as the smallest concentration of detection molecule that can be reliably detected over the signal to noise threshold of 3 [31]. The specific signal threshold (SST) was calculated as three times the standard deviation of multiple human sweat buffer washes with no detection molecule over baseline impedance signal. It is observed that the Limit of detection (LOD) with IL-6 antibody in PBS is 20 pg mL^{-1}. However, the LOD is much lower at 0.2 pg mL^{-1} when the antibody in ionic liquids (BMIM[BF_4], Choline[DHP]) is used as the capture probe for detection. This low LOD in both the ionic liquids is achieved by the selective binding of IL-6 to the IL-6 antibody confined by the RTIL, thereby, negating any changes in impedance contributed by the buffer. In other words, the confinement of biomolecules within the RTIL allows for the detection of capacitive changes at the electrode-solution interface. This electrode-solution interface was modeled using a modified Randle's circuit to understand the metric that contributes for change in impedance due to biomolecular binding. The circuit fit parameters along with their equivalent circuits are represented in Fig. 4A. It is observed that, broadly, the circuit for aqueous buffer, and both the ionic liquids have similar circuit elements. The R_s represents the bulk solution resistance in series with a parallel R_{ZnO} and C_{ZnO}. This combination is in series with another parallel RC circuit at lower frequencies, charge-transfer resistance (R_{ct}) and double layer capacitance (C_{dl}). The biomolecular binding is usually captured at frequencies <1000Hz and is represented by changes in R_{ct}- C_{dl} complex. The circuit-fit parameters highlighted in green in Fig. 4A show that the binding of IL-6 to IL-6 antibody in PBS is primarily driven by changes in R_{ct}. The changes in R_{ct} could also be due to the adsorption of ions from the buffer at the electrode interface. This results in higher noise signal contributed by the buffer and therefore, the LOD is higher with aqueous buffer as compared to both the RTILs. On the contrary, the binding of IL-6 antigen to IL-6 antibody in RTILs causes a modulation of C_{dl} (as highlighted in green in the circuit-fit parameters of Fig. 4A), that occurs due to the specific binding of IL-6 to IL-6 antibody enclosed with in the RTIL. The C_{dl} signal is enhanced by the charge storing capacitive property of RTILs.

When the antigen molecule binds to the antibody, it results in the modulation of the permittivity of the double layer and also the Debye-length gets shifted depending on the size of the biomolecule. The modulation of 'ε_r' and 'd' results in the change in double layer capacitance i.e. when an antigen molecule docks onto the antibody, the RTIL surrounding the antibody modulates the height of the double layer (Debye length) slightly. Furthermore, the docking of the antigen molecule causes a change in permittivity within the EDL. The modulation of C_{dl} is hence used to quantify the binding of antibody-antigen complex. The CDR curves for IL-6 in both the RTILs have similar LOD of 0.2 pg mL^{-1} indicating that IL-6 can be reliably detected even at low concentrations in these RTILs. The increasing dose concentrations of IL-6 in BMIM[BF_4] shows higher changes in Z_{mod} ratio of up to 0.5 as compared to change in Z_{imag} ratio of Choline[DHP] of ~0.39. However, Choline[DHP] has better R^2 value of 0.94 as compared to BMIM[BF_4] of 0.85.

Fig. 4B represents the CDR curve at 100 Hz for cortisol in human sweat with cortisol antibody in all 3 buffers i.e. PBS, BMIM[BF_4] and Choline[DHP]. The circuit and fit parameters are represented below the CDR curves respectively. Similar to the detection of IL-6, it is observed that the LOD for antibody in PBS buffer is higher (10 ng mL^{-1}) as compared to both the RTILs. The changes in R_{ct} dominate for the PBS buffer. However, in RTILs the binding mechanism remains similar to that of IL-6, where the modulation of C_{dl} contributes to the changes in impedance. The LOD and the change in Z_{mod} ratio for BMIM[BF_4] is 0.1 ng mL^{-1} and 0.52 respectively. Since the binding mechanism is largely attributed to the changes in C_{dl}, and the equivalent circuit for Choline[DHP] shows only C_{dl}, a ratio change in impedance of Z_{imag} over $Z_{imag\ baseline}$ is used to represent the CDR curve. The LOD and change in impedance ratio for cortisol detection using Choline[DHP] is similar to BMIM[BF_4] of 0.1 ng mL^{-1} and 0.52 respectively. The R^2 value for both the ionic liquids is ~0.94.

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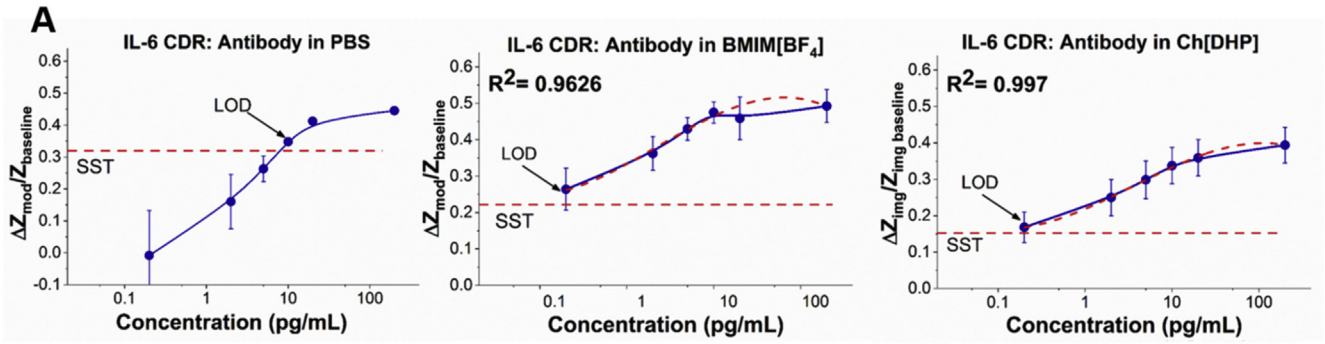
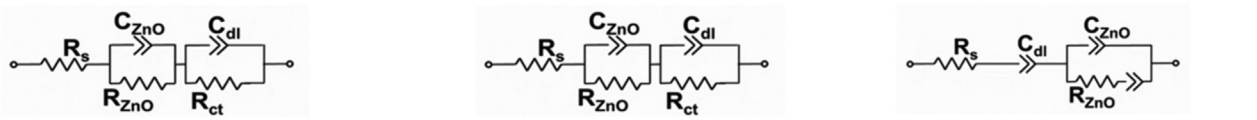
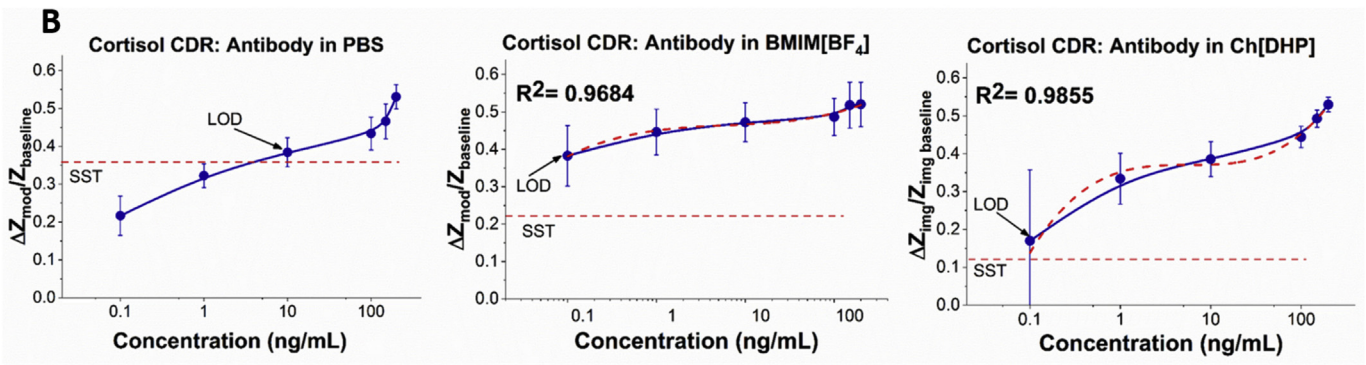




Dose (pg/mL)	R_{ct} (kΩ)	C_{dl} (F)	R_{ZnO} (kΩ)	C_{ZnO} (F)	R_s (Ω)
HS	99	2.15E-06	5.96	6.36E-07	4076
0.2	59	3.93E-06	23	6.36E-07	8412
2	30.2	3.68E-06	10.3	3.68E-06	5476
5	21	2.47E-06	11.4	6.28E-06	3995
10	18.7	3.35E-06	10	7.15E-06	3233
20	15.4	5.15E-06	10.2	5.50E-06	2789
200	11.1	5.05E-06	10.0	5.91E-06	2473

Dose (pg/mL)	R_{ct} (kΩ)	C_{dl} (F)	R_{ZnO} (kΩ)	C_{ZnO} (F)	R_s (Ω)
HS	73	2.95E-06	7.5	1.18E-06	1744
0.2	114	2.30E-06	4.8	3.83E-06	1845
2	106.2	2.06E-06	3.9	3.31E-06	1609
5	132.6	1.64E-06	3	2.50E-06	1433
10	156.3	1.36E-06	2.3	2.03E-06	1260
20	155	1.31E-06	2.1	1.90E-06	1153
200	153.6	1.32E-06	2.0	1.88E-06	1077

Dose (pg/mL)	C_{dl} (F)	R_{ZnO} (kΩ)	C_{ZnO} (F)	R_s (Ω)
HS	1.83E-06	3.2	8.90E-07	390.5
0.2	2.17E-06	2.87	8.79E-07	337.4
2	2.25E-06	3.2	9.37E-07	326.6
5	2.39E-06	3	9.10E-07	307.2
10	2.50E-06	2.68	8.80E-07	289.8
20	2.70E-06	2.3	8.40E-07	279.2
200	2.87E-06	2.1	8.60E-07	267.8



Dose (ng/mL)	R_{ct} (kΩ)	C_{dl} (F)	R_{ZnO} (kΩ)	C_{ZnO} (F)	R_s (Ω)
HS	260.5	3.98E-06	27	2.59E-06	15603
0.1	100.43	4.16E-06	20.6	6.57E-06	12675
1	72.74	4.86E-06	26.9	1.01E-05	9692
10	53.85	6.91E-06	56	1.32E-05	8736
100	51.5	4.62E-06	24.65	1.16E-05	7462
150	46.5	4.66E-06	23.54	1.20E-05	6727
200	39.7	4.66E-06	23.54	1.60E-05	4222

Dose (ng/mL)	R_{ct} (kΩ)	C_{dl} (F)	R_{ZnO} (kΩ)	C_{ZnO} (F)	R_s (Ω)
HS	126	1.13E-06	25.2	4.59E-06	2444
0.1	237.2	1.48E-06	22.3	7.73E-06	1778
1	65.8	1.53E-06	16.8	9.49E-06	1625
10	22.3	1.53E-06	12.1	9.98E-06	1552
100	26.7	1.94E-06	10.8	1.06E-05	1457
150	89.4	2.12E-06	10.7	5.03E-06	1457
200	83.5	2.28E-06	11.2	5.21E-06	1369

Dose (ng/mL)	C_{dl} (F)	R_{ZnO} (kΩ)	C_{ZnO} (F)	R_s (Ω)
HS	1.72E-06	42.2	1.12E-06	5040
0.1	2.51E-06	38.6	1.33E-06	3863
1	2.60E-06	29.8	7.59E-07	4139
10	3.11E-06	27.6	9.03E-07	3668
100	3.98E-06	27.79	1.98E-06	3484
150	5.49E-06	35.26	4.62E-06	2708
200	5.93E-06	21.59	2.85E-06	2727

Fig. 4. A. Calibration dose response curves for detection of IL-6 using IL-6 antibody in PBS, BMIM[BF_4] and Choline[DHP]. The equivalent circuits for the corresponding buffer solution are given below the CDR curves. The table shows the circuit-fit parameter values with the column highlighted in green indicating the parameter being modulated due to the binding of IL-6 to IL-6 antibody. B. Calibration dose response curves for detection of Cortisol using cortisol antibody in PBS, BMIM[BF_4] and Choline[DHP]. The equivalent circuits for the corresponding buffer solution are given below the CDR curves. The table shows the circuit-fit parameter values with the column highlighted in green indicating the parameter being modulated due to the binding of Cortisol to Cortisol antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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3.5. Selectivity to IL-6 and cortisol in RTILs

After establishing a better biosensing performance with capture probe in RTILs as compared to aqueous buffer, the specificity for detecting IL-6 and cortisol was performed in both the RTILs. Further detailed analysis was performed to assess if there exists a specific combination of RTIL-capture probe for biosensing i.e. for each capture probe (IL-6 and cortisol antibody), we evaluated if better biosensing was achieved in BMIM[BF_4] or choline[DHP] using the cross-reactivity studies.

The cross-reactive experiments were performed for IL-6 antibody in BMIM[BF_4] and Choline[DHP] with cortisol as the detection and vice-versa. Fig. 5 shows the comparison of the cross-reactive molecule and the actual CDR. It is observed that in both the RTILs, the biosensor response is specific to the biomolecule of interest and no cross-talk occurs. However, IL-6 antibody in BMIM [BF_4] and cortisol antibody in Choline[DHP] show a negative change in ratio Z_{mod} when the cross-reactive molecule cortisol and IL-6 are added respectively. This negative change implies that upon addition of the cross-reactive molecule, the impedance increases from the baseline. This change in impedance is opposite to the behavior when a molecule of interest is captured by its capture probe, thus, implying no cross-talk occurs. Since, the binding mechanism within the EDL is dominated by the changes in C_{dl}, we further evaluated to confirm if the negative change in impedance was not as a result of modulation of C_{dl}. Fig. S2 in supplementary represents the circuit fit parameters extracted for the cross-reactive experiments. It is observed that the C_{dl} values do not change with increasing dose concentration of cross-reactive molecules. This indicates that no binding occurs between the capture probes and cross-reactive molecules. The RTILs enclose the antibody capture probe and a modulation of C_{dl} occurs only upon specific antigen docking onto the capture probe. Table 2 represents a comparison of this work to other biosensors that do not use RTILs for the detection of IL-6 and cortisol in various biological fluids. It is observed that the use of RTILs provide an enhanced and specific signal response of the detection biomolecule. The enhanced sensor performance metrics using RTILs demonstrated on a flexible nanoporous platform can be leveraged for translation as wearables. Typically, wearable biosensors require: 1. stable immuno-chemistry/life-time, 2. small form factor, 3. enhanced sensor metrics. The stable immunoassay chemistry upto 1 week on a wearable form-factor with enhanced lifetime of the biosensor was demonstrated in our previous work Munje et al. [12]. In addition, this work demonstrates enhanced sensor metrics for detection of biomolecules in sweat leveraged through optimal choice of ionic liquids with a stable immunochemistry, showing a proof-of-feasibility for translation as wearables.

Table 2
Comparison of biosensing performance in aqueous and RTILs for various body fluids.

Biomolecule	Analyte Buffer	LOD	RTIL	Reference
Interleukin-6	Blood	10 pg mL^{-1}	No	[32]
	Serum	1.37 pg mL^{-1}	No	[33]
	Saliva/urine	0.39 pg mL^{-1}	No	[34]
	Human Sweat	**0.2** pg mL^{-1}	Yes	**This work**
Cortisol	Blood	0.6 ng mL^{-1}	No	[35]
	Human Sweat	1 ng mL^{-1}	No	[1]
	Human Sweat	**0.1** ng mL^{-1}	Yes	**This work**

4. Conclusion

In this work, we have demonstrated the detection of IL-6 and cortisol in human sweat using electrochemical affinity based biosensing with capture probe antibodies in aqueous and ionic liquid buffers. A better biosensing performance with capture probes in ionic liquids was achieved. A comprehensive analysis was performed to understand the effect of type of buffer solution on the signal transduction mechanism. In other words, the impedance

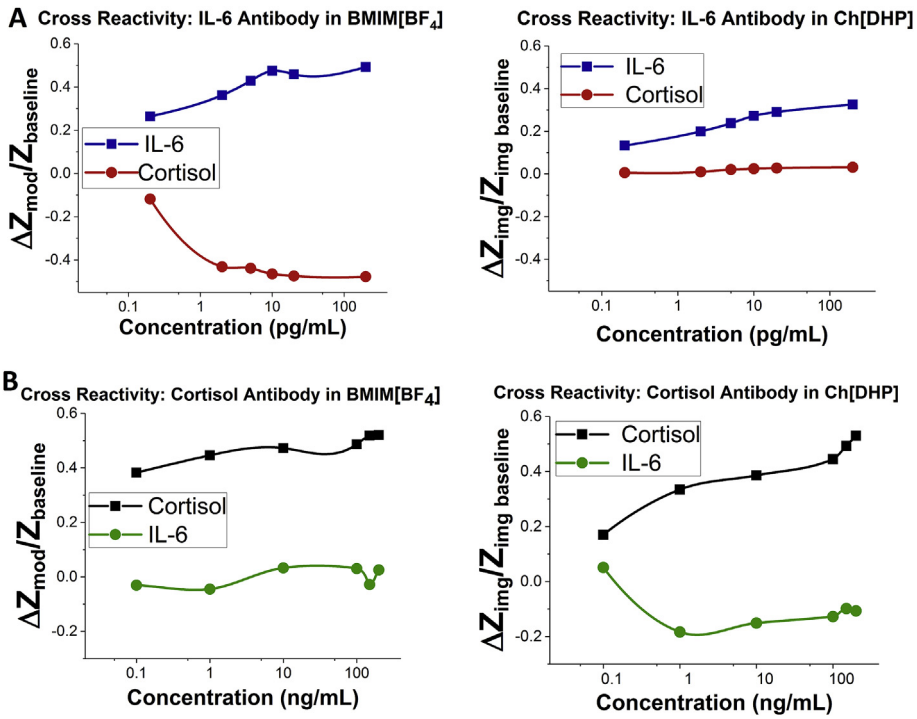

Fig. 5. Sensor selectivity for A) IL-6 antibody in BMIM[BF_4] and Choline[DHP] with Cortisol as cross-reactive molecule and B) Cortisol antibody in BMIM[BF_4] and Choline[DHP] with IL-6 as cross-reactive molecule.

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response due to biomolecular binding when modeled as an equivalent circuit at the electrode-solution interface, exhibited modulation of different circuit elements upon binding of target analyte to capture probe in aqueous PBS buffer and ionic liquids.

The formation of stable immunoassay chemistry on the biosensor to capture the target analyte was established using ATR-IR analysis. Zeta potential measurements showed stable surface charge state of the capture probe antibodies in BMIM[BF_4] and Choline[DHP] upon interaction with aqueous sweat buffer of varying pH, when a bias voltage was applied. This technique confirmed for the translatability of the developed immunoassay for biosensing using electrochemical impedance spectroscopy.

The biosensor showed a LOD of 10 pg mL^{-1} for IL-6 using α IL-6 antibody in PBS. However, a better LOD of 0.2 pg mL^{-1} was achieved using α-IL-6 antibody in both the ionic liquids, BMIM[BF_4] and Choline[DHP]. Similarly, a LOD of 10 ng mL^{-1} was achieved for cortisol with α cortisol antibody in PBS buffer and a LOD of 0.1 ng mL^{-1} was achieved in BMIM[BF_4] and Choline[DHP]. The key reason for better biosensing performance with capture probe antibodies in ionic liquids as compared to aqueous PBS buffer is the difference in the binding mechanism modality and formation of compact EDLs in ionic liquids. The binding using antibody in PBS buffer results in modulation of charge-transfer resistance which could also be due to the noise signal by the ions in the buffer. Thus, the SNR is low in PBS buffer. However, the key mechanism of biomolecular binding in ionic liquids is the modulation of the double layer capacitance (C_{dl}). The electrochemical capacitive properties of ionic liquids further enhance the signal by formation of compact EDLs, surrounding the capture probe antibody there by, negating any interaction from the buffer ions and allowing selective binding to the target analyte.

Although the two ionic liquids have similar LOD for IL-6, a wider dynamic change in sensor response is exhibited by BMIM[BF_4] (~0.45) as compared to Choline[DHP] (~0.32) in ratio change in impedance. However, a better R^2 value of 0.99 was observed in Choline[DHP] as opposed to BMIM[BF_4] of R^2 value of 0.96. Further, high selectivity to IL-6 was established from the cross-reactive experiments through negative interaction to cross-reactive molecule for BMIM[BF_4] and no signal response to cross-reactive molecule for choline[DHP]. Similarly, in cortisol cross-reactive studies, no signal response was observed for BMIM[BF_4] and negative interaction to cross-reactive molecule was observed for Choline[DHP]. A R^2 value of 0.96 and 0.98 for cortisol CDR was obtained using both BMIM[BF_4] and Choline[DHP] respectively. Since highly selective and reliable detection is achieved in both the ionic liquids, based on the specific application, either of the RTILs can be chosen for detection of IL-6 and cortisol in human sweat.

Acknowledgments

We thank Vignesh Ravindranath for helping with schematic representations. We also thank Richard Alexander Willis for helping with data compilation program. This work was partially supported by Cecil H. and Ida Green endowed fellowship at the University of Texas at Dallas.

Conflict of interests statement

Drs. Shalini Prasad and Sriram Muthukumar have a significant interest in Enlisense LLC, a company that may have a commercial interest in the results of this research and technology. The potential individual conflict of interest has been reviewed and managed by The University of Texas at Dallas, and played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report, or in the decision to submit the report for publication.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.02.013.

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