Biosensors and Bioelectronics 39 (2013) 21–25

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

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

The label free picomolar detection of insulin in blood serum

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ARTICLE INFO

Article history:
Received 16 April 2012
Received in revised form
11 June 2012
Accepted 12 June 2012
Available online 6 July 2012

Keywords:
Insulin
Electrochemical impedance spectroscopy
Biosensor
Diabetes
Blood serum

ABSTRACT

Insulin, a polypeptide hormone secreted by pancreatic cells, is a key regulator in glucose homeostasis. Its deficiency leads to insulin-dependent (type I) diabetes whereas resistance to insulin is common in type II diabetes, obesity and a range of endocrine disorders. Its determination is of considerable value, particularly in the clinical diagnosis of diabetes mellitus and the doping control of athletes. It has, additionally, been noted as a potential breast cancer marker (serum insulin levels being found to be raised in comparison to control patients). Electrochemical assays are potentially very cheap, highly sensitive, and very readily transposed to a point of care. Though there exist numerous examples of label free impedimetric or capacitative assaying of biomolecules, these are rarely demonstrated to be effective in complex biological mixtures or to be applicable to low molecular weight targets (since they operate through the interfacial displacement of water/ions and/or the steric blocking of a redox probe). We report herein an ultrasensitive electrochemical and label-free biosensor for insulin in blood serum with a clinically relevant linear range and detection limit of 1.2 pM. The transducing surfaces, based on readily prepared, antibody modified, polyethylene glycol monolayer modified polycrystalline gold surfaces, respond in a highly specific and re-useable manner to the target in up to 50% blood serum.

1. Introduction

Insulin is a polypeptide hormone produced by the pancreas responsible for regulating the metabolism of carbohydrate and blood glucose levels. The human form is a peptide composed of 51 amino acids, a 21-residue A-chain and a 30-residue B-chain linked by two disulfide bonds (Nicol and Smith, 1960). A determination of circulating insulin in serum or plasma is of intrinsic value in the clinical diagnosis/classification of various types of diabetes and related diseases (Carneiro et al., 2002), and doping control in athletes (Graham et al., 2008; Holt and Sonksen, 2008). During the past decade, a variety of detection methods, including those based on radioimmunoassays (Deberg et al., 1998; Lutz and Rand, 1993), mass spectrometry (Ho et al., 2008; Liu and Yan, 2011), fluorescence spectrometry (Mercolini et al., 2008; Pu et al., 2011), and surface plasmon resonance (Frasconi et al., 2010; Gobi et al., 2007) have been developed for the determination of insulin. These methods are, though, either practically laborious or insensitive and are not translatable to a point of care format.

With an increase in the incidence of diabetes (Whiting et al., 2011), the development of an ultrasensitive, cheap, simple and automated diagnostic test would be of considerable value. In potentially meeting these requirements, electrochemical analyses have received increasing attention (Salimi et al., 2007; Salimi et al., 2008; Snider et al., 2008; Wang and Li, 2009; Zhang et al., 2005). Though amperometric sensors based on the oxidation of insulin (Zhang et al., 2005) have been reported (Arvinte et al., 2010; Cox and Gray, 1989; Jaafariasl et al., 2011; Salimi et al., 2007; Salimi et al., 2008; Salimi et al., 2009; Wang and Musameh, 2004; Wang et al., 2002), these operate at high potentials, and accordingly suffer from ascorbic acid and uric acid interference (Salimi et al., 2009). They are also comparatively insensitive; the blood content of insulin is normally in the range of 57–79 pM between meals (Iwase et al., 2001), much lower than the detection limits of most of electrochemical sensors reported to date (Jaafariasl et al., 2011).

Electrochemical impedance spectroscopy (EIS) can sensitively monitor the changes in capacitance or charge-transfer resistance associated with the specific binding of materials to a suitably modified electrode surface. It is a non-destructive, label free and highly multiplexable technique that been applied to the sensitive detection of a number of disease markers (Daniels and Pourmand, 2007; Davis, 2009; Prodromidis, 2010). For example, Ramakrishna and co-workers (Lin et al., 2010) have reported the non-Faradaic EIS measurement of two cardiovascular biomarkers using a sensor based on the immobilization of antibody in a biogenic nanoporous silica substrate on gold, with detection limits at the low fM level. The Suni group (Huang et al., 2008) have reported an assay

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http://dx.doi.org/10.1016/j.bios.2012.06.014

of peanut protein in phosphate buffer with Faradaic EIS, at levels down to less than 0.3 nM. More recently, Mount and co-workers (Ciani et al., 2012) have reported the pM to nM sensitive detection of three wound infection biomarkers using Faradaic EIS. It is particularly noteworthy that all prior immunoassays of this type are associated with the detection of relatively large protein molecules (MW > 20 kD), the interfacial binding of which is expected to cause significant impedance change. There exists, to the best of our knowledge, no prior report on the EIS assaying of a small polypeptide, (insulin molecular weight < 6 kDa) in either spiked electrolyte or in complex biological fluid. We report herein, then, the first, EIS based, electrochemical sensor for insulin in blood serum using electrodes which are readily fabricated, potentially re-useable, highly target specific, and report sensitively and linearly on log insulin across the entire clinically relevant range.

2. Materials and methods

2.1. Chemicals and reagents

Human insulin, human blood serum, strepavidin and bovine serum albumin (BSA) were purchased from Sigma Aldrich. Monoclonal anti-insulin antibody (mouse IgG1 isotype) was purchased from Santa Cruz Biotechnology, Inc. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimde (NHS) and dimethyl sulphoxide (DMSO) were purchased from Sigma Aldrich. Polyethylene glycol (PEG) containing thiol HS–$(CH_2)_{11}$–$(EG)_3$–OCH_2–COOH was purchased from Prochimia Surfaces, Poland. Phosphate buffered saline (PBS) with Tween-20 (PBST, 10 mM, pH 7.4) was prepared by dissolving PBS tablets (Sigma Aldrich) in ultrapure water with 0.2% v/v Tween-20 added, and filtered using a 0.22 μm membrane filter. All other chemicals were of analytical grade. Ultrapure water (18.2 M Ω/cm) was obtained from a Milli-Q system and used throughout.

2.2. Apparatus

Electrochemical experiments were performed on an Autolab Potentiostat 12 equipped with an FRA2 module (Metrohm Autolab B.V.). A conventional three-electrode system with gold disk working electrodes (1.6 mm diameter, BASi), a platinum wire counter, and a silver/silver chloride (Ag/AgCl, filled with 1.0 M KCl) reference (CH Instruments) was used. All potentials reported are relative to the Ag/AgCl reference.

2.3. Sensor Surface Preparation

Gold electrodes were first polished sequentially with 3.0, 1.0 and 0.1 μm diamond spray (Kemet International Ltd.) then ultrasonically washed in water (ca. 5 min) prior to immersion in hot piranha solution (concentrated H_2SO_4: 30% H_2O_2, v/v 3:1. Caution: please treat with extreme care!) for 15 min. Finally, the electrodes were electrochemically pre-treated according to a previous report (Xiao et al., 2007) with some alteration. Briefly, cyclic voltammetry (CV) scans were conducted in 0.5 M KOH over the potential range from −0.35 V to −1.35 V at a scan rate of 2 V/s, until curves were stable. Following this treatment, scans were carried out in 0.5 M H_2SO_4 over the potential range from −0.35 to 1.5 V (4 V/s) until a stable sharp cathodic peak is attained. The effective surface area of the gold electrode can be calculated during this procedure (Hoogvliet et al., 2000).

The pre-treated gold electrodes were dried in a flow of nitrogen gas and immediately immersed in a solution of 50 μM HS–$(CH_2)_{11}$–$(EG)_3$–OCH_2–COOH in ethanol for 12 h at room temperature. Such PEG films have well-established biocompatibility (Cho et al., 2011; Klapshina et al., 2010) and antifouling characteristics (Harder et al., 1998; Schilp et al., 2009). After monolayer formation electrodes were rinsed with ethanol, then water and dried in a flow of nitrogen gas, prior to incubation in a solution containing 0.4 M EDC and 0.1 M NHS to activate the terminal carboxyl groups (~40 min). Insulin antibodies were subsequently immobilized by dipping the gold electrode in a 1.0 μM antibody solution (PBST, pH 7.4) for 10 h at 4 °C. In order to block the active sites on the electrode surface, the modified electrode was finally soaked in 100 μM BSA for 6 h at 4 °C, then thoroughly rinsed with PBST prior to analysis.

2.4. Electrochemical impedance spectroscopy

EIS measurements were conducted with an Autolab Potentiostat 12 equipped with an FRA2 module using a three-electrode system and a frequency range of 0.01–10 kHz. The amplitude of the applied sine wave was 10 mV with the direct current potential set at 0.22 V (The E_0 of the used redox probe). All analyses were carried out in 10 mM PBST solution containing 1.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl, and plotted in the form of complex plane diagrams (Nyquist plots) subsequently fitted using a standard Randles equivalent circuit.

2.5. Sensor operation

For the detection of insulin, the prepared receptive interfaces were incubated in 10 mM pH 7.4 PBST with 1.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl containing specific concentrations of insulin at room temperature for 30 min, and EIS responses were recorded in the same solution. To evaluate sensor selectivity, strepavidin was initially used. For "in situ" assays in serum (10% v/v with 10 mM PBST, 1.0 mM $[Fe(CN)_6]^{3-/4-}$, 0.1 M KCl), electrodes were incubated in different concentrations of insulin spiked PBST for 30 min prior to EIS analysis in the same solution. "Ex situ" assays were also run in spiked serum (1–80% v/v with 10 mM PBST, 1.0 mM $[Fe(CN)_6]^{3-/4-}$, 0.1 M KCl) by electrode incubation for 30 min prior to PBST rinsing and EIS analysis in 10 mM pH 7.4 PBST with 1.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl. Used electrode interfaces were regenerated by incubation in 0.2 M Gly–HCl buffer at pH 2.0 containing 1% DMSO for 5 min to disassociate the attached insulin, prior to PBST rinsing and re-use (Kandimalla et al., 2004).

3. Results and discussion

3.1. Biosensor fabrication

Nyquist impedance plots include a semicircle portion at high frequencies and a linear portion at lower frequencies corresponding to charge-transfer and diffusion limited processes, respectively. The former can be quantified, through the semicircle diameter, as the charge-transfer resistance (R_{ct}) of the modified electrode, when fitted using the standard Randles equivalent circuit (inset in Fig. 1 Guo et al., 2012; Vyas et al., 2010). Faradaic EIS analyses were initially used to characterize the construction of the receptive surfaces, noting, predictably, sharp increases in R_{ct} on PEG SAM formation (< 50 Ω–~120 kΩ) and antibody immobilization (~200 kΩ, Fig. 1).

3.2. Detection of insulin in buffer

The label free assaying of low molecular weight targets is particularly demanding since their association with a suitably prepared receptive interface will typically yield only small

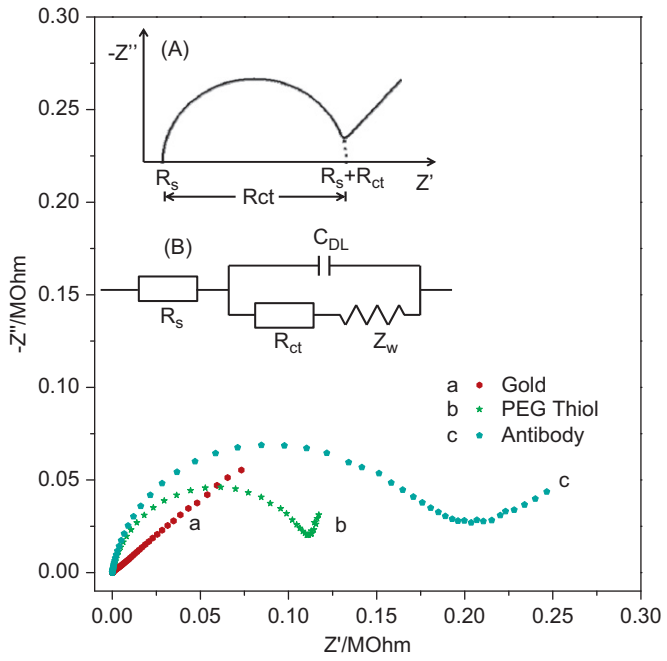

Fig. 1. Nyquist plots recorded in PBST (10 mM, pH 7.4) solution containing 1.0 mM $Fe(CN)_6^{3-/4-}$ and 0.1 M KCl. (a) Bare gold electrode; (b) PEG SAM interfaces and (c) subsequently prepared antibody modified interfaces. Inset: (A) an ideal typical Nyquist plot of Faradaic EIS and (B) the Randles equivalent circuit used for data fitting.

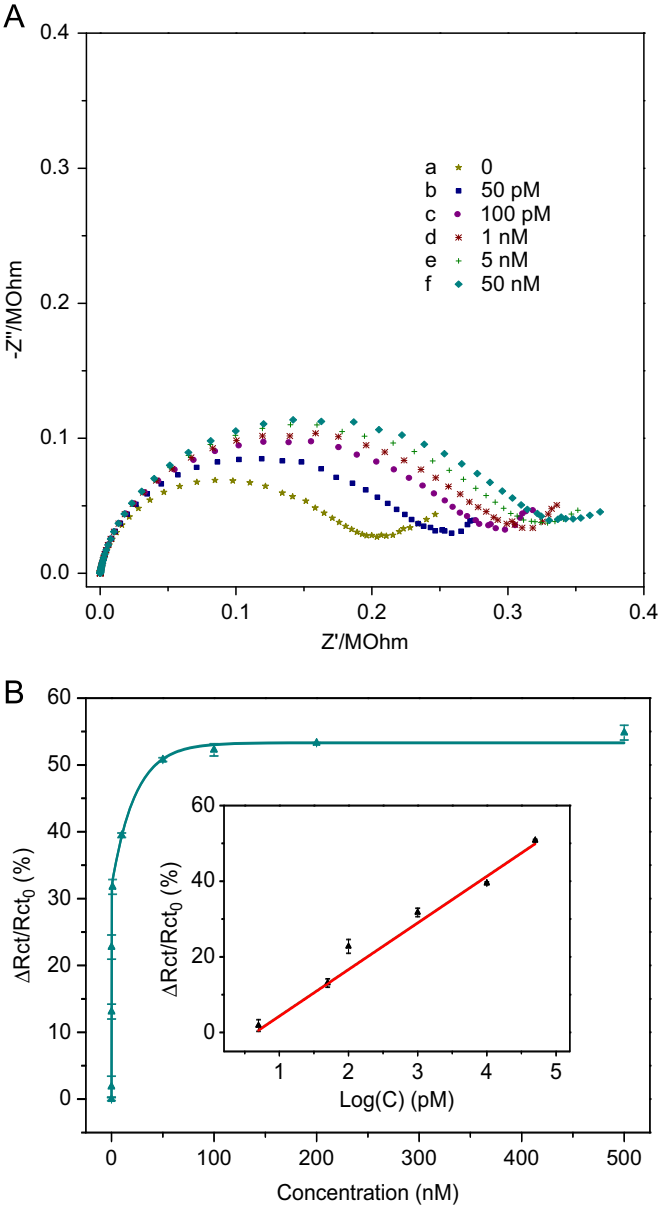

Fig. 2. (A) Typical Faradaic impedance spectra corresponding to the biosensor after incubation in PBST solutions of different insulin concentration: curves from inner to outer represent 0 pM, 50 pM, 100 pM, 1 nM, 10 nM, and 50 nM insulin, respectively. (B) Normalized charge-transfer resistance (R_{ct}) changes of the biosensor as a function of insulin analyte concentration. The inset shows the corresponding calibration curve for the insulin biosensors. Error bars represent the standard deviations across three repeats.

perturbations in adsorbed mass (QCM), dielectric (SPR) or interfacial resistance (EIS) compared to that observed with larger targets. The binding efficacy and interfacial sensitivity resolved herein, however, is sufficient for prepared surfaces to be detectably responsive to insulin in PBST even in the low picomolar range (Fig. 2). It is likely that this sensitivity is, at least partially, aided by the insulin negative charge (the isoelectric point of human insulin is about 5.4 (Thomsen et al., 2008)) at this pH and the resulting electrostatic repulsion of the redox probe. More detailed analyses reveal a good linear correlation (R^2=0.994) between R_{ct} and the logarithmic value of insulin concentration, a linear range of 5 pM–50 nM, and a detection limit of 1.24 ± 0.01 pM (Fig. 2). This lies much lower than that of prior amperometric assays of insulin (Jaafariasl et al., 2011; Salimi et al., 2009), and equivalent to an antibody displacement surface plasmon resonance assay in relatively noncompetitive media (Frasconi et al., 2010). The interfacial disassociation constant K_D, was calculated to be 0.11 ± 0.01 nM, a value in very good agreement with a previous laser-induced fluorescence determination (Tao and Kennedy, 1997). The prepared interfaces exhibit negligible response (< 5% change in baseline signal) to streptavidin or BSA concentrations of up to 100 nM, indicating excellent selectivity.

3.3. Detection of insulin in blood serum

The direct detection of a comparatively small target such as insulin in complex biological fluids is highly desirable, more practical from a point of care perspective, but very demanding. Though previous insulin assays in blood serum have been reported, (Frasconi et al., 2010; Gobi et al., 2007). For example, using surface plasmon resonance, thus far these operate only at high levels of dilution (5–10 fold), and with low sensitivity and a narrow linear range. We have evaluated herein insulin serum assays in two ways. In the first instance, "in situ" assays were carried out with spiked 10% serum in PBST (Fig. 3). Under such conditions reliable linear assessments were possible across the clinically relevant range with a detection limit of 4.70 ± 0.64 pM. By extrapolation of data acquired through such analyses, the levels of insulin present in native (non spiked) serum could be determined at 60.1 ± 3.9 pM, within the reported normal range (Iwase et al., 2001).

Subsequent "ex situ" analyses were carried out with insulin spiked blood serum at controllable dilution in PBST (Fig. 4). Biosensors were incubated in these solutions and then measured after rinsing with PBST. As is evident, the assays are effective and remain robust at 50% blood serum, with an interference response of less than 3% of the assay response in pure buffer solution. Fig. 5 shows the "ex situ" assay calibration curve of insulin detection in 50% blood serum. A similar linear range to that of the assays in pure buffer solution was obtained, with a detection limit of 4.77 ± 0.99 pM.

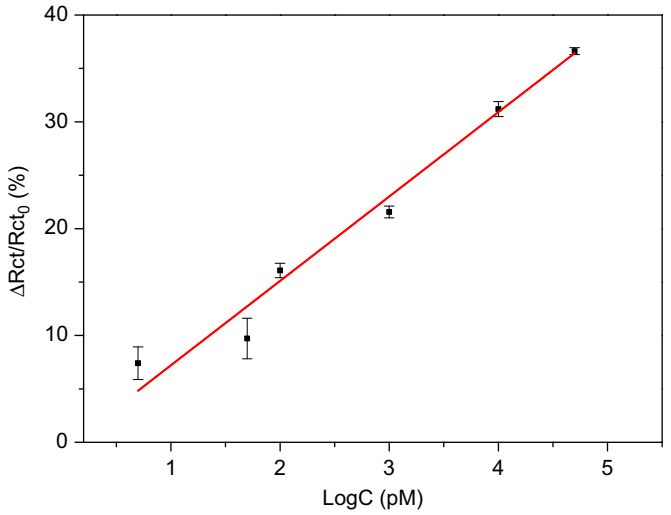

Fig. 3. Calibration curve for "in situ" assays of insulin in 10% blood serum. The biosensor surfaces were incubated in PBST (10 mM, pH 7.4) solution containing 1.0 mM $Fe(CN)_6^{3-/4-}$, 0.1 M KCl and 10% blood serum, spiked with different concentrations of insulin. EIS measurements were subsequently carried out in the same solution. Error bars represent the standard deviations across three repeats.

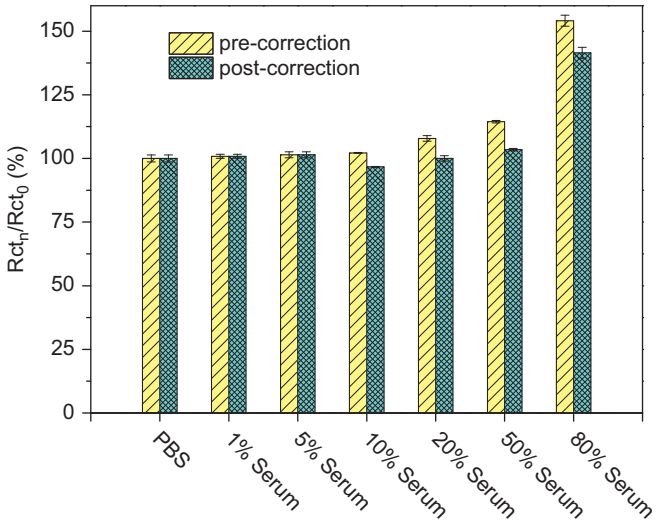

Fig. 4. The effect of blood serum concentration on the sensor response to insulin before (left column) and after (right column) correction. Electrode surfaces were incubated in PBST (10 mM, pH 7.4) solution containing 100 pM insulin and different volume percents of blood serum for 30 min, and then rinsed with PBST before "ex situ" EIS measurements in PBST containing 1.0 mM $Fe(CN)_6^{3-/4-}$ and 0.1 M KCl. The measured data were then corrected by subtraction of EIS contributions from the insulin in the serum itself as calculated from the calibration curve. Error bars represent the standard deviations across three repeats.

3.4. Biosensor regeneration

Biosensor regeneration was achieved by surface immersion in 0.2 M Gly–HCl buffer containing 1% DMSO for 5 min to disassociate the insulin antibody–antigen complex. Subsequent to this, the electrodes can be re-used in assays with minimal detriment to sensitivity ($<4\%$ deviation in sensitivity across 4 repeated generations assessments and regenerations—see Fig. 6).

4. Conclusions

The assaying of insulin has implications in the doping control of horseracing and human athletics, diabetes management and a host of human disease states. Levels of the polypeptide mediate the confinement of glucose levels in human blood to within a narrow concentration range and an assay constitutes a powerful diagnostic predictor of diabetes and trauma. We have reported herein, the generation and utilization of a facile impedimetric biosensor capable of the ultrasensitive and label free detection/calibration of insulin in diluted blood serum (up to 50%). The assays are highly selective, extend linearly across the entire clinically relevant concentration range, and exhibit low pM limits of detection. The interfaces are readily regenerated and reused in a format that is translatable to a point of care.

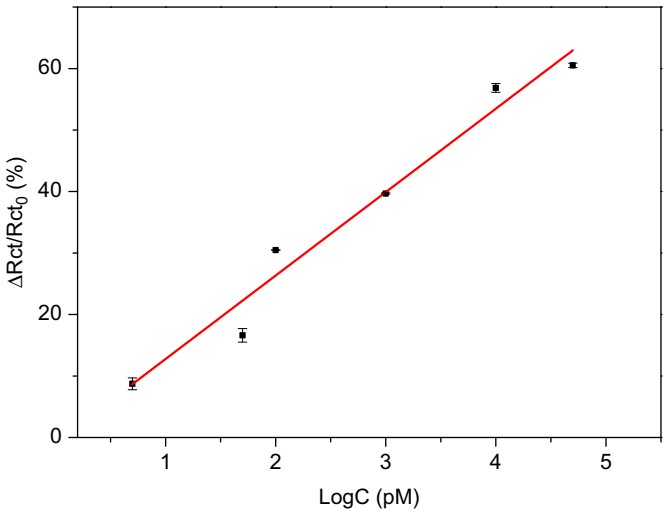

Fig. 5. Calibration curve for the "ex situ" assays of insulin in 50% blood serum. Electrode surfaces were incubated in PBST (10 mM, pH 7.4) solution containing 1.0 mM $Fe(CN)_6^{3-/4-}$, 0.1 M KCl and 50% blood serum, spiked with different concentrations of insulin. EIS measurements were carried out in PBST (10 mM, pH 7.4) solution containing 1.0 mM $Fe(CN)_6^{3-/4-}$ and 0.1 M KCl after rinsing with PBST. Error bars represent the standard deviations across three repeats.

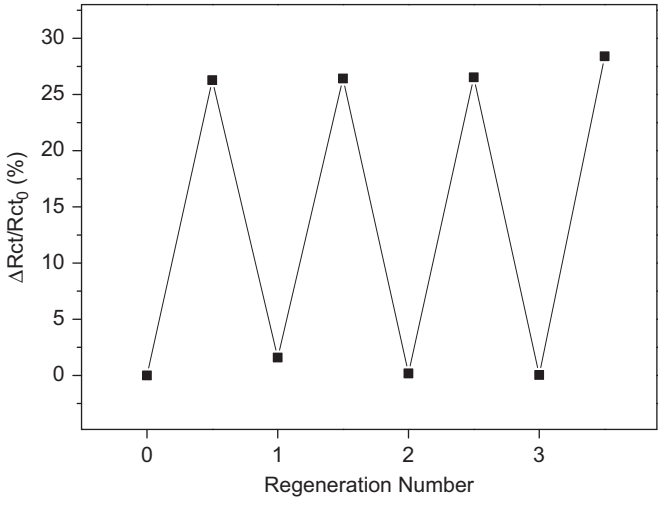

Fig. 6. Sensory surface regeneration by immersion in 0.2 M Gly–HCl buffer containing 1% DMSO for 5 min prior to rinsing with PBST, and impedance analysis in PBST (10 mM, pH 7.4) solution containing 1.0 mM $Fe(CN)_6^{3-/4-}$ and 0.1 M KCl. Shown here are the normalised R_{CT} values in the absence or presence of 100 pM insulin.

Acknowledgments

This research project has been supported by a Marie Curie International Incoming Fellowship of the European Community's seventh Framework Program (contract no. PIIF-GA-2010-271775).

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