Multimodal electrochemical and nanoplasmonic biosensors using ferrocene crowned nanoparticles for kinase drug discovery applications

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Abstract

The use of on-chip multimodal sensing approaches is very promising towards integrated biosensing systems, which measure different parameters involved in biomolecular interactions and provide automated validation of true positives. In this report we investigate a proof of concept that enables multiple detection technologies for screening inhibitors of kinase activity, which is a crucial process in drug discovery applications. We demonstrate the integration of electrochemical techniques on the same chip, namely differential pulse voltammetry, impedance spectroscopy and direct open circuit potential measurements. Gold nanoparticles that attach to the thio-phosphorylated proteins facilitate localized surface plasmon resonance detection. The addition of thiolated ferrocene, which attaches to the nanoparticles like a crown, enables sensitive electrochemical amperometric detection of

kinase activity. This novel multimodal biosensor provides a more rigorous measurement of biomolecules, with wide significance in biomedical, environmental and pharmaceutical applications.

Keywords: multimodal biosensor; open circuit potential; electrochemical impedance; amperometric sensing; localized surface plasmon resonance; protein phosphorylation

1. Introduction

Complementary characterisation techniques are often required in the study of biomolecular interactions and on the validation of biomolecular detection. Different electrochemical techniques can be used for fast, miniaturised biosensing systems, with each technique providing different information, which can then be correlated to a certain concentration of analyte. Often more than one technique is used to validate the biosensor or provide complementary information on the biological recognition system (e.g. kinetics and concentration). A few reports have integrated different techniques on the same measurement system in order to look at different properties of an interaction or validate the detection of an analyte [1,2]. Such integrated systems open the doors towards generic biosensors that can be used for a range of applications, where the user selects which techniques to use according to the properties of the biomolecule to be measured or the information required.

We here describe the combination of several electrochemical (open circuit potential, electrochemical impedance spectroscopy, differential pulse voltammetry) and nanoplasmonic (localized surface plasmon resonance) techniques on a linear array of electrodes in a flow cell. These have been applied for the detection of protein phosphorylation driven by kinases. Abnormal phosphorylation of proteins, interceded by kinases, is an important cellular regulation in several aspects of neoplasia [3]. Furthermore, there are over 400 diseases now linked either directly or indirectly to the activity of protein kinases [4-7]. This makes protein kinases an abundant source of potential drug targets to intervene in several diseases. However, the current kinase inhibitor drug market is very premature with less than 30 protein kinase inhibitors approved by the United States Food and Drug Administration until 2014 [8]. This is primarily due to the time consuming, cost inefficient and laborious detection techniques, like mass spectroscopy and western blot, used in pharmaceutics to understand the

kinase activity. In addition, some of these assays require the usage of toxic chemical reagents, such as radioactive tags/label, and are unsuitable for high-throughput applications [9]. The need to develop high-throughput biosensors that are capable of screening large libraries of kinase inhibitors is therefore very high. In this work, we present a novel 3-step assay that enables parallel detection of protein phosphorylation by multiple electrochemical and nanoplasmonic techniques. In the first step, phosphorylation of myelin basic protein by PKC-α kinase is achieved by using 5'-[γ-thio] triphosphate (ATP-S) as a source of phosphate in the presence and absence of known inhibitor PKC-α, GF 109203X. The phosphorylation process is monitored in real time using an in-house built open circuit potential (OCP) measurement system. In the second step, gold nanoparticles are added to the reaction, which attach spontaneously to the thio-phosphoryl groups. This facilitates detection of phosphorylated proteins using a localized surface plasmon resonance technique as reported in our previous work [2,10]. Finally, in the last step thiolated ferrocene (Fc) is added to the system, which is grafted onto the gold nanoparticles allowing detection of phosphorylation using differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS).

These techniques act as complementary biosensors to each other, providing on-chip self-validation of the results for effective and reliable measurements. The application of this multimodal approach towards the detection of protein phosphorylation, serves as an example on how the engineering of the biorecognition interaction enables the use of signal probes, such as nanoparticles and redox molecules, that are only bound to the surface in the case of a true positive signal.

2. Experimental

2.1 Reagents

All chemicals were of analytical grade and were used as received, unless otherwise specified. Aqueous solutions were made with double de-ionised water, 18.2 MΩ cm, with a Pyrogard filter (Millipore, USA). Tris base, magnesium chloride ($MgCl_2$), sodium chloride (NaCl), acetone, mercaptoundecanoic acid (MUA), 6-mercapto-1-hexanol (MCH), 6-(ferrocenyl)hexanethiol, ethanolamine, ethanol, N-hydroxysuccinimide (NHS), ethyl-dimethylaminopropyl carbodiimide (EDC), 3-(N-morpholino)propanesulfonic buffer (MOPS), PKC-α kinase inhibitor GF 109203X, 5'-[γ-thio] triphosphate (ATP-S) and gold nanoparticles (20 nm average spherical diameter, in 0.1 mM PBS) were purchased from

Sigma-Aldrich (UK). Dephosphorylated myelin basic protein (MBP) and PKC lipid activator were purchased from Millipore. PKC-α human recombinant kinase produced in Sf9, was procured from ProSpec-Tany TechnoGene Ltd (Israel).

2.2 Protein phosphorylation assay

The biosensor principle is described in Figure 1. A mixed self-assembled monolayer (SAM) made of 1 mM MUA and MCH in a ratio of 1:9 MUA:MCH was immobilized overnight onto gold electrodes. In order to cover possible pinholes on the gold surface and to reduce non-specific binding, a 1 h backfilling step was performed with 1 mM MCH. All further modification steps were carried out under a flow rate of 100 µl/min using a peristaltic pump (Watson Marlow 323-Du) and an in-house built polytetrafluoroethylene (PTFE) flow cell with an inner chamber of approximate dimensions 25×8×5 mm^3 (length × width × height) and an internal volume of 1 ml accommodating the array of electrodes. The SAM was activated by injecting 1 ml of an aqueous mixture of 40 mg/ml EDC and 10 mg/ml NHS. For protein attachment, 200 µl of 156 µM MBP protein (in 10 mM MOPS pH 7.0 containing 128 mM $MgCl_2$, 641 mM EDTA, 1.134 µg inactive lambda phosphatase and 0.05% sodium azide) were flown into the cell; unreacted carboxyl groups were blocked using 500 µl of 10 mM ethanolamine, pH 8.5, in aqueous solution. Phosphorylation of MBP was carried out in 10 mM Tris base, pH 7.4 containing 6 mM NaCl and 0.4 mM $MgCl_2$. (hereafter referred to as *reaction buffer*). The whole reaction volume was fixed to 100 µl for all replicates and their controls. 1 µM ATP-S and 4 units of PKC-α (1 unit per 25 µl) were subsequently added. To initiate the phosphorylation reaction, 5 µl of PKC lipid activator (1:20 of reaction volume) containing 0.5 mg/ml phosphatidylserine and 0.05 mg/ml diacyglycerol in 20 mM MOPS (pH 7.2), 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithiothretiol and 1 mM $CaCl_2$, was added. Control reactions were performed by adding 0.1 µM PKC kinase inhibitor (GF 109203X) to the phosphorylation reaction.

2.3 Electrochemical measurements

A CompactStat potentiostat (Ivium Technologies, The Netherlands) was used to perform impedance measurements and DPV in a three-electrode cell with an Ag/AgCl reference electrode (BASi, USA) and a platinum wire counter electrode (ALS, Japan). A linear array of 4 gold electrodes with 2 mm diameter was prepared in-house by thermal evaporation (BOC Edwards, USA) of 20 nm Cr and 100 nm Au on glass slides. The electrode array was

sonicated for 3 min in acetone followed by rinsing with ethanol and then ultrapure water. Impedance measurements were recorded in the above mentioned *reaction buffer*, scanning the frequency between 100 KHz and 0.1 Hz applying a 10 mV a.c. voltage superimposed to the OCP. Since an ideal impedimetric sensor will measure the response at a single frequency rather than for a wide spectrum, localized values of resistance (R_l) and capacitance (C_l) were used for comparing the EIS signals. R_l and C_l were measured at the frequency that minimizes the imaginary response of the complex capacitance (defined as $1/j\omega Z$) – in our system this was observed to be at 1 Hz throughout the different steps. Moreover, recording an EIS signal for a single frequency allowed to shorten the time of the measurement from ~3.5 min (sweeping the whole frequency range) to ~20 s. DPV was performed to monitor the Fc peak scanning the potentials *vs* Ag/AgCl and each measurement could be performed in ~10 s. All the measurements were carried out at least in triplicates.

2.4 Open Circuit Potential

The open circuit potential was measured in real time using a complete monolithic FET-input ultra low input bias current instrumentation amplifier INA116 (Texas Instruments, USA) [11]. Difet® inputs, a very low input bias current of 3 fA at 25°C, with special guarding techniques were used to achieve the very low input bias current performance and provide an accurate differential measurement. The circuit was designed in house with 4 amplifiers operated at ±15 V on a single PCB circuit board. A power supply circuit was made to provide ±15 V to minimise the mismatch of the power rails. The outputs of the amplifiers are connected to a 4 channel-input analog-to-digital converter with input resolution of 24bits measuring a range of ±1 V (NI 9219 DAQ, National Instruments, UK). The array of 4 functionalized gold electrodes and the Ag/AgCl reference electrode were connected directly to the differential inputs of the amplifiers, with a platinum wire immersed in the cell connecting the electrolyte to ground to prevent common-mode rejection. LabVIEW (National Instruments, UK) was used to monitor and capture the open circuit potential measurement in real time simultaneously from the 4 digital outputs of the DAQ from the amplifiers array.

2.5 Localized surface plasmon resonance measurements

Once the electrochemical recordings were terminated, LSPR was measured using an in-house built system with discrete components, all bought from Ocean Optics (USA). The system had 3 parts: reflection probe (R400-7-UV-VIS) with both detector and light source, connected to a Tungsten Halogen source (LS-1-LL) and a spectroscope (USB4000-UV-VIS-ES). The data

was obtained through absorption mode on a cross platform spectroscopy operation software (SPECTRASUITE) provided by Ocean Optics; each measurement lasted only a few seconds.

3. Results and Discussion

Prior to monitor thio-phosphorylation, the coverage of pinholes on the electrode surface by MCH backfilling was confirmed by means of EIS measurements: a decrease in capacitance signal of 11.3 ± 0.6% was observed, indicating the effectiveness of the backfilling process. OCP was monitored in real time upon thio-phosphorylation of MBP immobilised onto the gold electrodes in a flow cell (Fig. 2A). OCP showed a change of -30 mV upon protein thio-phosphorylation in comparison to -1.7 mV in the presence of PKC-α kinase inhibitor. The negative shift indicates the addition of the negatively charged phosphate groups to MBP.

Thio-phosphorylation events enabled an easy and rapid attachment of gold nanoparticles by means of sulphur bonds. The modification with gold nanoparticles and following washing step required only 10 min in flow conditions. Upon AuNP addition, a voltage shift between 5.4-6.8 mV is observed in the thio-phosphorylation and inhibition reactions. This shift is mostly attributed to the changes in buffer conditions upon introducing gold nanoparticles (in 0.1 mM PBS) in the flow cell. Upon addition of thiolated Fc to the system, a further OCP shift of 5.3 mV was recorded in the case of the thio-phosphorylated samples. However, for the control samples and those with inhibitors the shift was lower than 1.5 mV, which confirms that only a minimum amount of AuNPs is present for those samples. A further confirmation that the OCP signals (upon gold nanoparticles attachment) were due to the buffer effect was supported by the LSPR detection of nanoparticles in reflection [10]. The presence of gold nanoparticles was observed only for thio-phosphorylated samples (Fig. 2D): a consistent red shift of 5.8 ± 0.4 nm for samples where thio-phosporylation took place was recorded, compared to 0.9 ± 0.2 nm shift for reactions in presence of phosphorylation inhibitor. The gold nanoparticles attachment provided a wide surface accessible for thiolated ferrocene grafting. This allowed both amperometric and impedimetric detection of phosphorylation of the proteins. A 10 min step was required for thio-ferrocene modification by adding 500 µl of 1 mM 6-(Ferrocenyl)hexanethiol dissolved in ethanol and finally diluted 1:100 in the *reaction buffer*. Prominent signal changes due to the electrochemical double layer modification were induced in electrodes where thio-phosporylation occurred by means of the Fc-crown-like-structure. Not only real time OCP measurements but also successive

impedimetric and voltammetric recordings could be performed in the same cell without the need of changing measurement solution, simply by flowing suitable reagents to the array.

For phosphorylation detection, impedimetric data obtained before phosphorylation were compared with the signal acquired upon the Fc-crowned gold nanoparticle attachment. Both R_l (Z' at 1 Hz) and C_l ($-1/2\pi f Z''$ at 1 Hz) changes were correlated to the presence and absence of kinase inhibitor as well as where thio-phosphorylation compounds (i.e. kinase, ATP-S and kinase activator) were not used (Fig. 2B). Percentage shifts of 32 ± 10% and -16 ± 4.8% were recorded for R_l and C_l, respectively, in the case of thio-phosphorylation. Negligible signal changes were observed for both control reactions where thio-phosphorylation compounds were not used ($\Delta R_l = -1.4 \pm 2.9\%$ and $\Delta C_l = 0.6 \pm 1.9\%$) and where the experiment was carried out in presence of thio-phosphorylation compounds with addition of PKC kinase inhibitor ($\Delta R_l = -0.9 \pm 3.6\%$ and $\Delta C_l = 0.4 \pm 3.1\%$). The former can give an indication of the non-specific interactions between Fc and protein. The latter is noticeably the most essential control for drug discovery application as it allows proving the efficacy of new possible drugs in terms of preventing protein phosphorylation.

The final and perhaps most powerful tool to crosscheck the thio-phosphorylation event was achieved by performing DPV measurements on the same platform (Fig. 2C), which clearly showed the Fc peaks. An Fc peak of 1.31 ± 0.26 µA height (using baseline subtraction) was detected at 0.59 V, while no peak was observed for the controls.

4. Conclusions

This study describes a new multimodal biosensor approach, demonstrating the potential in combining multiple sensing techniques on a single platform to achieve high levels of accuracy and reliability in the measurements.

This multimodal biosensor was successfully applied using a small array of electrodes in a flow cell for detecting phosphorylation of proteins. We tested phosphorylation of MBP by PKC-alpha in the presence of a known inhibitor, however this approach can be used to assay activity of any kinase-protein pair in the presence of known/unknown inhibitors. This opens opportunities to develop an integrated device with multiple sensing technologies, as demonstrated, for pharmaceutical industries and academia to discover drugs. Moreover, it also exploits simple and cost-effective consumables to build a biosensor system for screening inhibitors of kinase.

Our multimodal approach can easily be translated to a range of other biosensing applications, such as biomedical and environmental, where different electrochemical techniques can be used to perform on-chip validation of true positives. The engineering of the biorecognition elements/reagents can allow the use of nanoparticles, redox molecules or other types of signal probes for signal validation and amplification.

References

[1] Z. She, K. Topping, M.H. Shamsi, N. Wang, N.W.C. Chan, H.B. Kraatz, Investigation of the utility of complementary electrochemical detection techniques to examine the in vitro affinity of bacterial flagellins for a toll-like receptor 5 biosensor. *Anal. Chem.* 87 (2015) 4218-4224. DOI: 10.1021/ac5042439

[2] N. Bhalla, M. Di Lorenzo, G. Pula, P. Estrela, Protein phosphorylation detection using dual-mode field-effect devices and nanoplasmonic sensors. *Sci. Rep.* 5 (2015) 8687. DOI: 10.1038/srep08687

[3] K. Parang, G. Sun, Protein kinase inhibitors in drug discovery. *Pharm Sci. Enc.* 27 (2010) 1-67. DOI: 10.1002/9780470571224.pse027

[4] P. Cohen, D.R. Alessi, Kinase drug discovery – what's next in the field? *ACS Chem. Biol.* 8 (2013) 96-104. DOI: 10.1021/cb300610s

[5] P. Cohen, Protein kinases – the major drug targets of the twenty-first century? *Nat. Rev. Drug Discov.* 1 (2002) 309-315. DOI: 10.1038/nrd773

[6] H. Weinmann, R. Metternich, Drug discovery process for kinase inhibitors. *ChemBioChem* 6 (2005) 455-459. DOI: 10.1002/cbic.200500034

[7] E. ter Haar, W.P. Walters, S. Pazhanisamy, P. Taslimi, A.C. Pierce, G.W. Bemis, F.G. Salituro, S.L. Harbeson, Kinase chemogenomics: targeting the human kinome for target validation and drug discovery. *Mini Rev. Med. Chem.* 4 (2004) 235-253. DOI: 10.2174/1389557043487367

[8] R. Roskoski Jr., USFDA approved protein kinase inhibitors. http://www.brimr.org/PKI/PKIs.htm. Last accessed 1 May 2015

[9] S. Laufer, S. Thuma, C. Peifer, C. Greim, Y. Herweh, A. Albrecht, F. Dehner, An immunosorbent, nonradioactive p38 MAP kinase assay comparable to standard radioactive liquid-phase assays. *Anal. Biochem.* 344 (2005) 135-137. DOI: 10.1016/j.ab.2005.06.015

[10] N. Bhalla, N. Formisano, A. Miodek, A. Jain, M. Di Lorenzo, G. Pula, P. Estrela, Plasmonic ruler on field-effect devices for kinase drug discovery applications. *Biosens. Bioelectron.* 71 (2015) 121-128. DOI: 10.1016/j.bios.2015.04.020

[11] P. Estrela, D. Paul, P. Li, S.D. Keighley, P. Migliorato, S. Laurenson, P Ko Ferrigno, Label-free detection of protein interactions with peptide aptamers by open circuit potential measurement. *Electrochim. Acta* 53 (2008) 6489-6496. DOI: 10.1016/j.electacta.2008.04.036

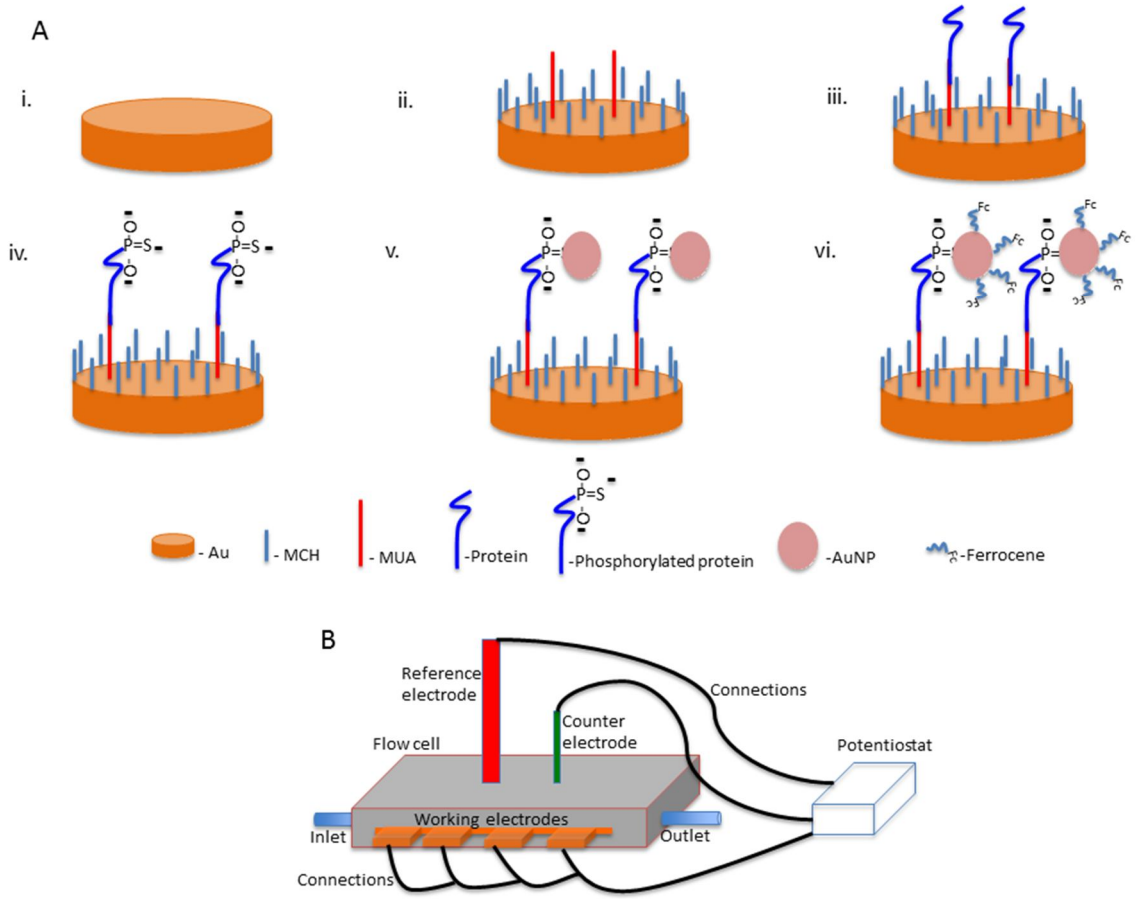

Figure 1. Scheme for analysis of protein phosphorylation: **A)** Kinase assay showing (i) bare gold electrode, (ii) surface functionalization using MUA/MCH, (iii) protein immobilization, (iv) thio-phosphorylation, (v) attachment of gold nanoparticles, (vi) nanoparticles with attached ferrocene. **B)** Dummy schematic representation of the in-house built flow cell and measurement system for the electrochemical detection.

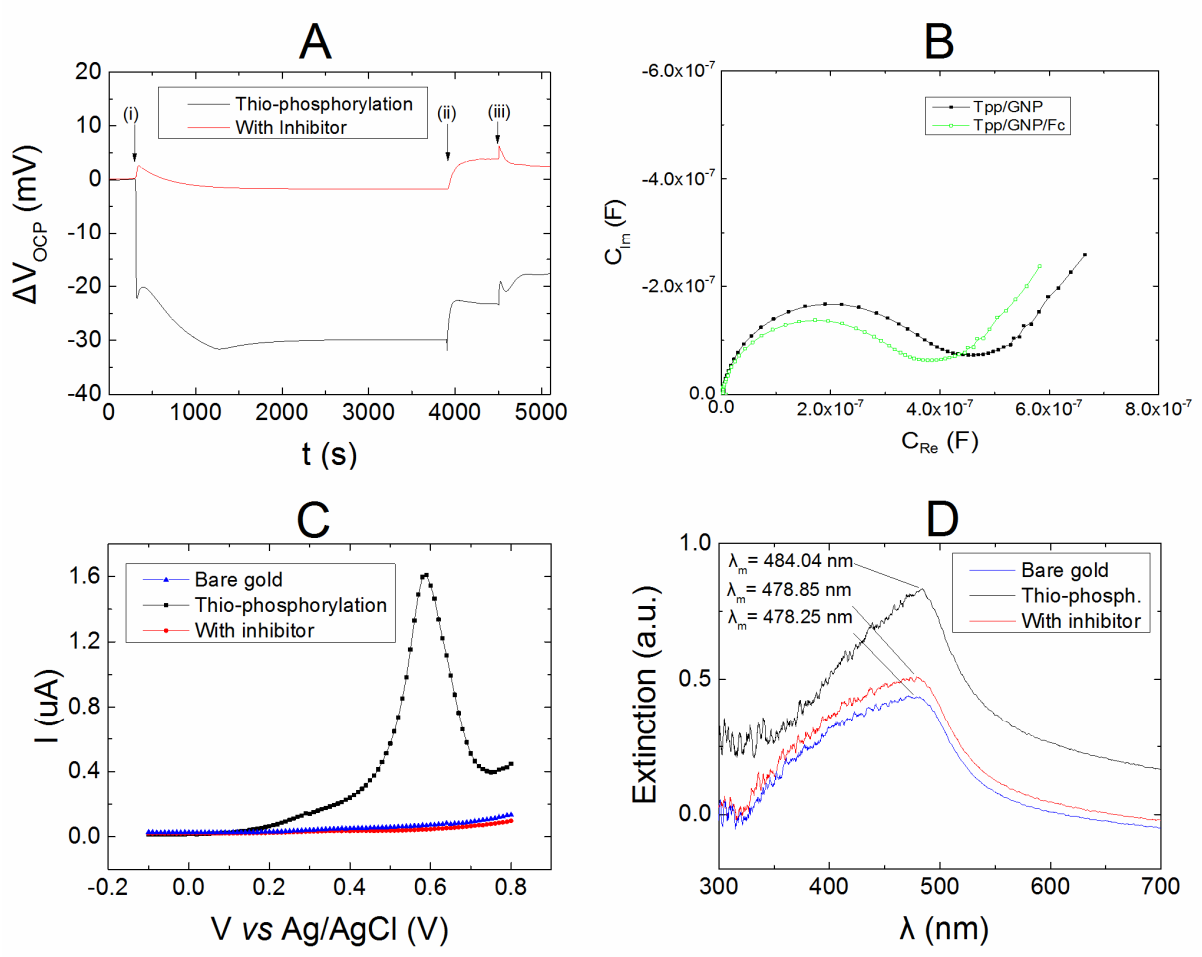

Figure 2. Representative multimodal detection of protein phosphorylation for a sample without inhibitor (black lines) and a sample in the presence of inhibitor (red lines): **A)** Real time OCP measurements upon injection into the flow cell of (i) ATP-S, PKC-α and PKC lipid activator as well as PKC-α kinase inhibitor in one sample, (ii) gold nanoparticles, (iii) Fc. **B)** Cole-Cole plot (imaginary *vs.* real part of the complex capacitance) for thio-phosphorylated samples before (black line) and after Fc injection (green line); the relative minimum for the imaginary part of the capacitance is obtained at the frequency of 1 Hz. **C)** DPV measurements recorded after Fc injection. **D)** Raw LSPR spectra in the window of 300-700 nm obtained at the end of the electrochemical experiments; the spectra for a bare gold sample is shown for comparison.