On-line surface plasmon resonance biosensing of vascular endothelial growth factor signaling in intact-human hepatoma cell lines

Journal:	*Analytical Chemistry*
Manuscript ID:	Draft
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Mauriz, Elba; University of León, Institute of Biomedicine (IBIOMED) Carbajo-Pescador, Sara; University of León, Institute of Biomedicine (IBIOMED) Ordoñez, Raquel; University of León, Institute of Biomedicine (IBIOMED) García-Fernández, Mª; University of León, Institute of Food Science and Technology (ICTAL) Mauriz, José; University of León, Institute of Biomedicine (IBIOMED) Lechuga, Laura; Institut Català de Nanociencia i Nanotecnología (ICN2), CSIC, Nanobiosensors and Bioanalytical Applications Group González-Gallego, Javier; University of León, Institute of Biomedicine (IBIOMED)

On-line surface plasmon resonance biosensing of vascular endothelial growth factor signaling in intact-human hepatoma cell lines

Elba Mauriz[1, 2,], Sara Carbajo-Pescador[1], Raquel Ordoñez[1], Mª Camino García-Fernández[2], José L. Mauriz[1], Laura M. Lechuga[3], and Javier González-Gallego[1]*

[1]Institute of Biomedicine (IBIOMED), University of León, and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Spain

[2]Institute of Food Science and Technology (ICTAL), University of León, 24007 León Spain

[3]Nanobiosensors and Bioanalytical Applications Group, Institut Català de Nanociencia i Nanotecnología (ICN2), CSIC and Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Barcelona, Spain

Author to whom correspondence should be addressed: Instituto de Biomedicina (IBIOMED), University of León, 24071 León, Spain. Tel: +34 987 291258 Fax: +34 987 291788. E-mail address:elba.mauriz@unileon.es

ABSTRACT:

Surface plasmon resonance (SPR) monitoring of biorecognition events at intracellular levels is a valuable tool for studying the angiogenic response of carcinoma living cells during tumor growth and proliferation. We report here a comparative study of two different strategies to detect human hepatoma cell interactions between transmembrane vascular endothelial growth factor receptor (VEGFR2) and vascular endothelial growth factor (VEGF). To monitor VEGFR2 activation after VEGF stimulation, intact hepatocellular carcinoma HepG2 or Huh7 cells (2×10^5 cells mL^{-1}) were directly immobilized on the sensor chip. Distinguishable SPR sensorgrams were obtained for each cell line depending on the time required for VEGFR2 activation. SPR signals for VEGF/VEGFR2 binding were inhibited by VEGFR inhibitor, CBO-P11. The SPR response after VEGF stimulation/inhibition was in good agreement with the results observed by immunoblotting analysis. In a second approach we used intact cell lines as analytes. SPR analysis was done by injecting HepG2 and HuH7 cell suspensions ($2\text{-}4 \times 10^4$ cells mL^{-1}) onto a sensor surface previously immobilized with VEGF via thiol self-assembled monolayer (SAM). Specificity and reproducibility were evaluated reusing the same chip surface over more than 60 complete regeneration cycles. Comparison between both methods yielded differences in terms of reliability, making the last strategy more effective for the analysis of real samples. The investigation of VEGF signaling in intact human hepatoma living cells by SPR monitoring comprises a novel and promising design for the study of tumor angiogenesis via downregulation of VEGF and VEGFR2 pathways. Further investigation on VEGFR activation and vascular function could contribute to establish a robust and meaningful tool for early cancer diagnostics.

Introduction

Angiogenesis plays a significant role in the development of hepatocellular carcinoma (HCC). The neovascularization process in HCC, in solid tumors and in other angiogenesis-dependent diseases relies on the signaling cascade induced by angiogenic growth factors to generate new blood vessels from pre-existing ones.[1, 2, 3, 4] The signaling triggers the uncontrolled release of pro and anti-angiogenic factors that may lead to tumor growth and metastasis through the proliferation and migration of endothelial cells. At this point, tumor progression depends on the activation of the so-called "angiogenic switch" by shifting the balance from natural angiogenic inhibitors to angiogenic stimulatory growth factors.[5] This process is primarily regulated by the secretion of pro-angiogenic factors like vascular endothelial growth factor (VEGF) and other factors such as angiopoietins, fibroblast growth factor (FGF), transforming growth factor (TGF)-b, platelet derived growth factor (PDGF) and placental growth factor (PLGF)[1]. VEGF is one of the most investigated stimulatory angiogenic factors, because it appears frequently upregulated in most human tumors, and its overexpression has been associated with increased neovascularization, tumor growth and poor prognosis in HCC patients.[6, 7]

In order to induce blood vessel formation, direct binding between VEGF and their tyrosine kinase receptors (VEGFR1, VEGFR2 and/or VEGFR3) is required[8]. These receptors are primarily expressed in the vascular system, while VEGFR2 is mainly expressed on endothelial cells from HCC vessels.[9] Coupling between VEGF and one of its main receptor, VEGFR2, drives its phosphorylation at specific tyrosine residues and its subsequent dimerization and activation. After VEGF binding to its receptor, several intracellular cascades are modulated to induce endothelial cell proliferation. Among them, extracellular regulated kinase (ERK) and PI3K/Akt are the main downstream pathways of VEGFR2, which after been phosphorylated,

lead to expression of several genes implicated in cell survival, angiogenesis, differentiation and motility in cancer cells.[10, 11, 12, 13]

The regulation of VEGFR2 activity throughout the signaling cascade has been used to design different therapeutic strategies in order to block VEGFR2 signal transduction during tumor angiogenesis. The tyrosine kinase inhibitor sorafenib has been already tested and approved for the systemic treatment of advanced HCC by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2007. Other antiangiogenic drugs such as sunitinib, linifanib, brivanib and erlotinib have been also investigated although with worse outcomes.[14] The search for targeted anticancer therapies has required the employment of reliable methods for the evaluation of VEGFR signaling pathways. In this regard, surface plasmon resonance (SPR) biosensor technology provides a promising approach for the label-free characterization of VEGF/VEGFR bindings in real-time. SPR technology has been applied to monitor interactions mediated by tyrosine kinase receptors and their corresponding ligands, including VEGF and other co-receptors, such as extracellular proteoglycans (heparin sulphate proteoglycans HSPGs), neurophilins, integrins, platelet endothelial-cell adhesion molecule-1 (PECAM1), vascular endothelial (VE)-cadherin and integrins.[15, 16, 17, 18, 19, 20, 21] SPR analysis has been carried out by using either the VEGF or the VEGFR immobilized on the sensor chip.[22, 23, 24, 25] Recent SPR studies have also focused on the use of living cells in order to investigate the effect of epidermal growth factor (EGF) stimulation on living cells of several carcinoma cell lines by comparing the variation of patterns in SPR responses,[26, 27, 28] the use of suspension of carcinoma cell lines as analytes,[29] and VEGF expression of carcinoma cells after exposure to stimulatory agents.[30]

We present here two novel and different methods for investigating the phosphorylation of the cell surface signaling receptor VEGFR2 in intact cells after VEGF stimulation. Our approach can

strongly help to fill the lack of functional analyses in the diagnosis of cancer by the characterization of patterns in SPR responses from different carcinoma cell lines. Likewise, SPR direct monitoring of biomolecular interactions occurring *in vivo* in cancer cells will aid to understand the angiogenic process while providing a simple and reproducible method to help in the searching of new antiangiogenic agents. To fulfill this purpose, living cells were directly cultured on the sensor chip or VEGF molecules were covalently attached on the sensor surface. Both immobilization strategies were examined to obtain the most reliable method for monitoring VEGFR/VEGF interactions in real-time. SPR evaluation was validated by immunoblotting under stimulatory and inhibitory agents. The reusability of the VEGF-immobilized surface was evaluated to ensure the reproducibility of the assay whilst the selectivity of results was tested by flowing non-specific analytes onto the activated sensor surface. Results showed that on-line monitoring of intracellular events by SPR technology can be further exploited for target-based drug discovery and early diagnosis of cancer malignancies.

EXPERIMENTAL SECTION

Chemicals and reagents.

Chemicals used for the preparation of the sensor surface: mercaptoundecanoic acid (purity 99 %); N-hydroxysuccinimide (NHS, purity 98 %), and N-(3 dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), the blocking agent ethanolamine hydrochloride, Bovine serum albumin (BSA),the surfactant Tween 20 and the organic solvent trichloroethylene (purity 99.5 %) were obtained from Sigma–Aldrich (Steinheim, Germany). Ethanol (absolute) and Acetone (purissimum) and common chemicals for the preparation of phosphate buffered saline 1× PBS 10 mmol L^{-1}, pH 7.35 (potassium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate) were supplied by Panreac (Barcelona, Spain). Sodium chloride

and sulfuric acid (purity 95–97 %) were purchased from Merck (Darmstadt, Germany), and hydrogen peroxide (approx. 30 % aqueous solution) was provided by Prolabo (Fontenay sous Bois, France).HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was obtained from Sigma-Aldrich (Steinheim, Germany). EDTA (ethylenediaminetetraacetic acid) was from Merk (DarmatadtKGaA, Germany) and SDS (sodium dodecyl sulfate) was obtained from Bio-Rad Laboratories (CA, USA). Gold-coated substrates 10 mm^2 used as sensor chips were supplied bySsens (Hengelo, Netherlands). The matching oil for the sensor chip coupling to the SPR system was from Panreac (Barcelona, Spain). Milli-Q water supplied by a Millipore gradient A apparatus was used for preparing buffer and other working solutions.

Other reagents used for the analysis of intracellular events, VEGF and VEGFR were purchased from Sigma–Aldrich (Steinheim, Germany). The polyclonal antibody against VEGF was obtained from Abcam (Cambridge, UK), whereas the polyclonal antibodies against VEGFR, phospho-VEGFR2 Tyr 1175 Anti-CVEGFR2 was from Cell Signaling Technology (Danvers, MA, USA). Phospho-ERK, ERK, phospho-Akt and Akt protein expression were analyzed with polyclonal antibodies obtained from Santa Cruz (CA, USA), anti-p110 was from ABCAM while anti-p85 was purchase from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti β-actin polyclonal antibody was purchased from Sigma-Aldrich. Secondary anti-rabbit/anti-mouse HRP-conjugated antibody from Dako (Glostrup, Denmark) was used to detect primary antibodies. ECL detection kit employed was from Santa Cruz (CA, USA).The VEGF inhibitor, CBO-P11 was from Calbiochem (Merck KGaA, Darmstadt, Germany).

Human carcinoma cell lines.

Human HepG2 and HuH7 hepatocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Stock cells were routinely grown as monolayer cultures in

Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), penicillin (100 UmL^{-1}), streptomycin (100 $\mu g\ mL^{-1}$), glutamine (4 $mmol\ L^{-1}$) and pyruvate (100 $\mu g\ mL^{-1}$) in a humidified 5% CO_2 atmosphere at 37ºC and the medium was changed every other day. Cell culture reagents were from Gibco (Life Technologies, Madrid, Spain).

Cell culture and treatments.

Confluent HepG2 and HuH7 cells growing in complete media were replated in 6 well plates 9.6 cm^2 culture dishes, at a density of 2 x 10^5 cells mL^{-1}, in 2 mL of complete medium. After 24 hours, the plating medium was replaced with 1% FBS fresh medium containing VEGF dissolved in PBS to reach a final concentration of 10 ng mL^{-1}. Cells were treated for 5, 10, 15, 20 or 30 minutes. Moreover, cells were treated with 1 $\mu mol\ L^{-1}$ CBO-P11, a specific inhibitor of VEGFR, 1 hour prior to stimulation with VEGF.

Western blot analysis.

After treatments, cultured cells were washed twice with ice cold PBS and lysed by adding ice cold RIPA buffer containing 50 $mmol\ L^{-1}$ Tris-HCl pH 7,4, 150 $mmol\ L^{-1}$ NaCl, 2 $mmol\ L^{-1}$ EDTA, 0,1% Triton 100X, 10% sodium deoxycholate, 10% SDS, 1 $mmol\ L^{-1}$ NaF and protease cocktail inhibitor (Roche, Basel, Switzerland) and scraped off the plate. The extracts were transferred to a microfuge tube and centrifuged for 10 min at 15,000 g. After the evaluation of the protein concentration in extracts by the Bradford method, equal amounts of the supernatant protein (20µg) were separately subjected to SDS-PAGE and transferred to a PVDF membrane, (Bio-Rad, Hercules, CA). Primary antibodies (Ab) were diluted in blocking solution and incubated overnight at 4°C with polyclonal Ab to phospho-Akt, Akt (1:300 dilution), phospho-ERK, ERK (1:100 dilution), p110, p85 (1:1000 dilution), phospho-VEGFR2 and VEGFR2

(1:700). Equal loading of protein was demonstrated by probing the membranes with a rabbit anti β-actin polyclonal antibody (1:2000 dilution). After washing with PBS-T, the membranes were incubated for 1 hour at room temperature with secondary HRP-conjugated antibody (1:4000) and visualized using ECL detection kit. The density of the specific bands was quantified employing the software ImageJ (National institute of Mental Health, Bethesda, MA, USA) with an imaging densitometer (Scion Image, Maryland, USA).

SPR instrumentation.

Analyses of intracellular events occurring in living cells were done by a custom-designed laboratory SPR sensor platform.[31] The platform is a two-channel SPR instrument working in the Kretschmann configuration. The sensor chip is a thin gold coated slide ($10\times10\times1$ mm^3, thickness 50 nm) which is coupled to the prism by a matching oil with the same refractive index (n = 1.52). The light source is a 3 mW laser diode emitting p-polarized light at 670 nm. The laser beam is divided into two equal beams to enable paralleled and simultaneous detection of two independent analytes. SPR analysis was carried out at a fixed angle of incidence to monitor the binding events happening separately in each sensing channel in real-time. The running of a reference analysis was also possible by using one of the channels as control. Changes of the refractive index over the sensor surface responding to shifts in mass were detected as variations in the reflected light intensity by a multielement photodiode and were subsequently amplified and converted to a digital signal.

The liquid handling system is fully automated and integrated into the SPR sensor platform. The flow delivery system includes two flow cells (300 nL each) which come into contact with the gold coated sensor chip, a peristaltic pump to maintain a constant flow onto the sensing surface, and two diaphragm pumps with their corresponding injection valves for loading the

samples. A flow of assay buffer is pumped continuously onto the sensor chip at a constant speed of 20 μL min^{-1}. The flow-rate for the injection of specific samples and regeneration of the sensing surface may vary from 20 to 70 μL min^{-1} depending on the sensitivity and time of response expected for the biomolecular interaction. A whole assay cycle comprising sample injection and regeneration is complete in 25 min.

Preparation of the sensor surface.

Prior to the coupling onto the SPR platform, the gold-coated sensor chips were comprehensively cleaned with organic solvents. The preparation of the sensor chips included sequential immersion in trichloroethylene, acetone, and ethanol and rinsing in an ultrasonic bath for 15 min. Sensor chips were dipped in a freshly prepared piranha solution (H_2SO_4/H_2O_2, 3:1) afterwards and sonicated again in distilled water for 15 min. At this stage, gold-coated chips were ready to be coupled to the sensor platform.

Functionalization of the sensor surface: Immobilization procedure.

The functionalization of the sensor chips varied depending on the immobilization approach employed to detect VEGFR/VEGF interactions from living cancer cells. For the SPR monitoring after VEGF stimulation, cells were seeded directly on the gold-coated chip (2 x 10^5 cells mL^{-1}). Sensors were cultured overnight and placed immediately afterwards to the SPR platform. Figure 1 shows the aspect of the surface of the cultured sensor chips. To optimize the recognition event, the sensor surface was activated and preconditioned before VEGF injection by the continuous flow of HEPES buffer at 20 μL min^{-1}.

The second approach comprised the immobilization of VEGF via the formation of a standard self-assembled monolayer (SAM) of alkanethiolates. The attachment of VEGF biomolecules through SAMs was expected to provide stable and ordered structures that will reduce non-

specific adsorptions.[32] The thiolated SAM couples the VEGF-A by its amine groups to the NHS chemically activated surface through a carbodiimide linkage. The employment of the SAM chemistry also gives the possibility of the repeatable use of the sensor chips under optimal regeneration conditions, without interfering in the recognition properties of the VEGF-A receptor layer, throughout a large number of assay cycles.

The complete immobilization process was carried out at a constant flow-rate of 20 μL min^{-1} by maintaining a continuous flow of 1 × PBS 0.05 % Tween 20 (PBST). The use of Tween 20 at the concentration normally used in ELISA formats (0.05 %) was considered to overcome potential non-specific interactions. To form the SAM monolayer, alkyl thiolate chains of several lengths were tested to assure the optimal orientation of VEGF-A active binding sites. After characterization of the immobilization conditions, finally a carboxylic terminated SAMs of mercaptoundecanoic acid (MUA) at 0.05 mmol L^{-1} in ethanol was selected for the functionalization of the sensor chip. The activation of MUA carboxylic groups was achieved by using a mixed solution of EDC/NHS (0.2 /0.05 mol L^{-1} in distilled water). The stable intermediate N-hydroxysuccinimide ester acts as a crosslinking reagent allowing the easy coupling to the VEGF-A primary amine groups. Finally, the covalent immobilization was achieved by injecting a solution of VEGFA at 4 μg mL^{-1} in PBST. To avoid non-specific binding and the interference of non-covalently bound VEGFA multilayers the blocking agent ethanolamine 1 mol L^{-1}, pH 8.5, was pumped onto the sensing surface. This final step assures that all remaining unreacted NHS esters are deactivated.

SPR detection formats.

The immobilization strategies determined the detection format of the binding events occurring at the sensing surface. Initially, cells cultured on sensor chips and placed onto the SPR platform

were stimulated by the injection of 10 ng mL^{-1} of VEGF-A in PBS at 20 µL min^{-1}. The effect of the biomolecular interaction on the SPR response was monitored for up to 30 min. The inclusion of Tween 20 in the PBS solution used as running buffer was prevented in order to avoid cell lysis.

For the recognition of living cells interactions over VEGF-immobilized sensor chips, cell suspensions (2-4 x 10^4 cells mL^{-1}) were dispersed in ice-cold PBS containing 3mM EDTA and pumped over the sensor surface at 70 µL min^{-1}. The same solution of PBS containing 3 mmol L^{-1} EDTA was used as running buffer for the SPR monitoring. The use of both ice cold cells and precooled conditions at the SPR system was recommended to prevent receptor internalization before temperature increases.[29, 33, 34] Likewise, EDTA anticoagulatory properties and its ability to stabilize solutions were exploited to control non-specific associations and to avoid cell aggregation. After cell injection, flow-rates were adjusted to 20 µL min^{-1} to prevent cell diffusion at the moment of reaching the sensor surface. A solution of sodium dodecyl sulfate (SDS) 0.5% at 70 µL min^{-1} was used as regeneration agent to disrupt cell association with the VEGF interactant. The duration of a complete regeneration cycle was 6 min.

RESULTS AND DISCUSSION

In spite of the unmet need of having a real-time diagnostics tool for tumor cell monitoring and early cancer detection, the study of biological processes in living cells by SPR biosensing has been poorly addressed. In particular, SPR investigation on the expression and activation of angiogenic growth factors in carcinoma cell lines is limited to a low number of publications. [26, 27, 28, 29, 30]

In this work, we suggest a novel approach to recognize changes in hepatoma cell lines in response to VEGF stimulation by evaluating the changes in the intensity of the reflected light of the interacting plasmon wave over the activated sensor surface. Monitoring of intra-cellular events was carried out by using two carcinoma cell lines (HepG2 and HuH7) that highly express VEGF. The effect of VEGF activation over sensor chips previously cultured with the hepatoma cell lines was examined by comparing SPR sensorgrams.

Cells immobilized on sensor chips.

For gold chips cultured overnight with human HepG2 cells and prepared to be coupled to the SPR system, a significant signal increase was observed after VEGF injection from minutes 5 to 10 of the analysis. Signal reached a maximum at minute 12 and decreased slowly afterwards (see Figure 2(A)). For HuH7 cultured sensor chips, SPR sensorgrams showed a moderate increase at the beginning of the analysis that kept constant up to 20 minutes. The SPR signal reached the top value at this stage and did not vary for the last 10 minutes of monitoring (Figure 2 (B)). SPR response in both HepG2 and HuH7 cells indicated the activation of tyrosine kinase cell receptors after VEGF stimulation as control did not give any signal response. The difference between SPR sensorgrams for both cell types correlates well with the evaluation done by immunoblot analysis (see Figure 2). The study of the optimal VEGF concentration which assures VEGFR activation showed no relevant differences in the range tested (10-100 ng mL^{-1}). The SPR response did not depend on VEGF concentration to generate cell activation as it has been already suggested by Hiragun et al.[26] Therefore, the minimal VEGF concentration (10 ng mL^{-1}) was selected for VEGF injection.

Western blot analyses showed that VEGFR2 phosphorylation was increased between 5-15 minutes after VEGF stimulation in HepG2 cells, while in HuH7 cells receptor activation

increased after 10 minutes and was maintained for 30 minutes (Figure 2 (A) and (B)). Moreover, the main molecules of intracellular signaling pathways related to VEGFR2 activation were also analyzed. It was observed that p110 and p85, the subunits that conform PI3K structure, and phospho-Akt, as well as phospho-ERK, increased between 5-10 minutes after the post-stimulation in HepG2 cell line, correlating with the VEGFR2 activation observed. In Huh7, their expressions increased at 5 minutes after VEGF addition and were maintained for 20 minutes, which is in accordance to VEGFR2 activation. Total Akt and ERK protein levels were no modified under our study conditions, indicating that VEGF stimulation only affects their phosphorylation status (Figure 2).

The effect of VEGF inhibitor, CBO-P11, was also tested in both hepatoma cell lines. CBO-P11 is a 17-amino acid peptide that blocks VEGF binding to VEGFR-2 as well as angiogenesis and other cellular functions mediated by VEGF receptors. In our study, CBO-P11 was pumped over the cell-cultured sensor chips for 20 minutes before VEGF injection. The SPR values obtained in response to VEGF stimulation was monitored for up to 30 minutes (Figure 2). SPR sensorgrams for HepG2- and Huh7-cultured substrates showed plain SPR signals that decrease slightly from the initial baseline. The pattern of the SPR response differed significantly from those obtained without previous CBO-P11 incubation, indicating the inhibition of the tyrosine phosphorylation and the blocking of the VEGFR signaling cascade. CBO-P11 prevented VEGFR activation by inhibiting VEGF binding to their tyrosine kinase cell receptors. Immunoblotting analyses demonstrated that VEGFR2 phosphorylation in cells previously treated with CBO-P11 was drastically diminished in both cell lines, showing a progressive reduction from 10 to 30 minutes after VEGF administration (Figure 2).

The selectivity of the analysis was investigated by monitoring the extend of non-specific interactions. Bovine serum albumin (BSA) was used as control in the reference channel to study possible interferences of non-related compounds. Simultaneous injections of BSA and VEGF were performed independently in each sensing channel at the same flow-rate and under the same conditions. The SPR response exhibited for the BSA-channel was maintained constant from injection to the end of the analysis while the sensing channel monitoring VEGF stimulation showed the pattern described above. Therefore, it was confirmed that the SPR signal complies with VEGFR2 activation and that the recognition properties of cell-cultured sensor chips were not affected by the presence of compounds of similar molecular weight.

The variability of the SPR signals was studied by comparing single injections of VEGF samples over distinct gold sensor chips cultured with both types of hepatoma cell lines. The SPR signal was represented by the mean of 7 measurements. Variations in SPR signal intensity were not significant, while differences in the pattern between analyses were minimal (data not shown).

Cells as analytes.

The use of cells as analytes implies a novel route in order to investigate the angiogenic process in hepatocellular carcinoma cell lines. This strategy includes the immobilization of VEGF on the sensor chip and the monitoring of a cell solution passing over the sensing surface. Several SPR-based methods have exploited VEGF immobilization for the detection of interactions with either receptors or inhibitors[22, 25] (using immobilization techniques as dextran-based hydrogels, self-assembly layers, biotynilated surfaces, electrostatical binding, etc).

The most common immobilization format to monitor biomolecular interactions is the covalent attachment of the biomolecule used as ligand to a chemically modified substrate. The ligand is covalently cross-linked to a previously functionalized gold surface by means of a thiolate

bonding. In our study we use the well-known thiol SAM chemistry to immobilize VEGF covalently in a reliable and reproducible way. The covalent immobilization conditions were optimized in order to obtain the most sensitive surface to monitor VEGF interaction with its tyrosine kinase cell receptor. The assay conditions (assay buffer concentration, pH, and flow rates) were maintained constant throughout the immobilization procedure.

Since alkyl thiol chains of different lengths are generally used to improve the orientation and prevent the steric hindrance of the immobilized biomolecules, a SAM formed by a mixed layer of a carboxylic terminated-thiol (mercaptoundecanoic acid) and a non-functionalized alkyl chain of mercaptohexanol (1:9) was compared with the immobilization of a mercaptoundecanoic single monolayer at the same concentration (0.05 mmol L^{-1}). The SPR sensorgrams were recorded using the two channels of the same sensor chip. After SAM functionalization, the SPR response against an anti-VEGF specific antibody was significantly higher for the flow channel immobilized with the single alkyl thiol compound. Therefore, the SAM layer of mercaptoundecanoic acid was selected for VEGF immobilization.

The influence of VEGF concentration was also evaluated by comparison of the SPR response throughout the immobilization process and after the interaction with the anti-VEGF antibody. The SPR response was examined for several concentrations of VEGF (0.5, 1, 2 and 4 μg mL^{-1}). Similar SPR immobilization signals were obtained at 2 and 4 μg mL^{-1} concentrations. However, the best binding rates for the immunoreaction with the anti-VEGF antibody were observed for the highest VEGF concentration and consequently this was selected as the optimal ligand concentration.

Monitoring of binding events was carried out using one SPR sensing channel as reference. Both flow channels were chemically functionalized, but only one of them was utilized for VEGF

immobilization, the other channel was blocked with ethanolamine after NHS/EDC activation (Figure 3 (A)).

The selectivity of the SAM monolayer was evaluated measuring the ability to recognize interactions with their specific interactants: anti-VEGF and VEGFR2. SPR responses displayed by paralleled biosensing showed no variation of the SPR signal for non-specific antibodies on the covalently immobilized VEGF channel whereas no distinctive changes from baseline were observed after the injection of anti-VEGF and VEGFR2 on the reference channel (Figure 3 (B)). These results ensured the robustness of the VEGF-coated surface to specifically recognize only complementary compounds and the capability of the reference channel to be a useful control for cell interactions.

At this stage, the sensing surface was ready for the determination of cell interactions using one of the channels as reference. SPR evaluations were done in triplicate and PBS EDTA 3 mmol L^{-1} was used as running buffer. Different concentrations of hepatoma cell lines were tested in order to optimize the assay detection. Initially, cell suspensions ranging from 1 x 10^7 to 2 x 10^4 cells mL^{-1} were injected onto the activated sensor chip. As cell concentration increased, larger non-specific responses were obtained at the reference channel. The injection of cells over the reference channel produced a low remaining response which corresponded to the non-specific aggregation of cells onto the surface (25-30% of the SPR signal). Therefore, the optimal cell suspension for analysis was the lowest concentration giving the highest signal required to ensure the performance of the analysis. SPR response can maintain a minimum reliable signal that permits the clear differentiation between specific and non-specific interactions. Following these criteria, HepG2 cells were flowed over the VEGF-coated surface at 4 x 10^4 cells mL^{-1} while 2 x 10^4 cells mL^{-1} was selected as the best option to monitor HuH7 cells interactions. The SPR

responses for several cell concentrations represented as a calibration curve is shown in Figure 4 (A).

SPR evaluations were done in triplicate and VEGF/VEGFR binding was monitored in real-time. The paralleled biosensing allowed the simultaneous injection of the same samples in both the VEGF-coated and the reference channel. Comparison of responses showed the same behavior for cell-inhibitor mixed solutions and inhibitor free samples on the channel used as control. No significant decline of the SPR baseline was obtained for samples containing either CBO-P11 inhibitor or VEGF as competitor on the reference channel (Figure 4 (B)), indicating the reliability of SPR analysis to selectively detect specific interactions. For the quantification and comparison of intracellular events, SPR responses obtained on the activated channel were normalized by expressing the SPR signal (SPR_{signal}) of cell blank solutions as the percentage of the maximum response [100 x ($SPR_{signal}/SPR_{signal,max}$].

After defining assay conditions, we evaluated the reproducibility of the assay under the repeated use of the same sensor chip. SPR biosensors take advantage of their capacity to reproduce measurements by monitoring interactions in a reusable surface. Non-covalently bound interactants may be removed without loss of the physicochemical properties of the immobilized molecule by a regeneration agent. In our study, the stability of the VEGF-coated surface under regeneration conditions was estimated using solutions of HCl 0.01 mol L^{-1} or SDS. The complete removal of non-covalently bound cells was solely attained by flowing SDS 0.5 % at 70 μL min^{-1}. The binding capacity of the VEGF-coated surface was evaluated by monitoring the response against anti-VEGF specific antibody. It was proved that the same sensing surface could withstand 68 regeneration cycles without significant loss of the SPR signal operating along 40

days of continuous analysis. Previous studies based on VEGF immobilization formats have reported a maximum of five detection-regeneration cycles.[24]

To study the repeatability and accuracy of the SPR analysis, we compared the intra and inter-assay variability of cell measurements. For the estimation of intra-assay variation, the difference in SPR signal resulting from cell interactions at the same concentration was calculated. The variation of SPR signal from the initial value to the end of the analysis was minimum (14.3 %). Inter-assay variability was considered as the chip-to-chip reproducibility. The difference between SPR responses for specific anti-VEGF interactions from sensor chips immobilized on 4 different days was 20.8 % (RSD). The feasibility of the VEGF-coated surface was demonstrated by the low coefficients of variation obtained after repeated operation.

Finally, the VEGF inhibitor CBO-P11 was co-injected with hepatoma cells to confirm the specificity of the SPR response. The detection format involved the use of mixture solutions of cell suspensions at the optimized concentration and the VEGF inhibitor at different dilutions. After a previous incubation step, only free VEGF receptors could interact with covalently immobilized VEGF at the sensor chip. Consequently, cell binding to the VEGF-coated surface was inhibited by increasing concentrations of CBO-P11 and the SPR signal corresponding to VEGF-VEFGR-2 interaction diminished as CBO-P11 concentration increased (Figure 5 (A)).

Particularly, HepG2 and HuH7 hepatoma cell lines at 2 and 4 x 10^4 cells mL^{-1}, respectively, were incubated during 30 minutes with CBO-P11 at 3 concentrations (from 5 to 20 μmol L^{-1}) in ice-cold PBS EDTA 3 mmol L^{-1} from a stock solution of 1 mmol L^{-1} in PBS. HepG2 samples containing 5, 10 and 20 μmol L^{-1} of CBO-P11 showed a drop in SPR response of 32.3, 39.2 and 56.8 % respectively with regard to free cell samples, whereas HuH7 mixed solutions exhibited a decrease of the SPR signal of 33, 42.8 and 53.8 % for the same CBO-P11 concentrations (Figure

5 (B)). The differences in values between both cell lines were not significant. Similarly, the competitive effect of VEGF was evaluated in HuH7 cell samples showing a signal decrease of 25.3 and 39.6 % at 10 and 20 ng mL^{-1} concentrations, respectively.

The investigation of tumor angiogenesis via downregulation of vascular endothelial growth factors with their tyrosine kinase cell receptors by our SPR platform confirms previous biological studies on the detection of autophosphorilation of VEGFR2 after stimulation with VEGFA in intact cells.[8, 13] To our knowledge this is the first SPR-based study which demonstrates VEGF/VEGFR2 interactions with hepatoma intact cells. Although SPR detection of intracellular events in intact cells has been already reported,[26, 27, 28, 29, 30] our major advance is the enhancement of the assay performance by measuring analytical relevant parameters. In comparison with previous studies this work provides additional and relevant results on the study of specificity, inter- and intra-assay variability, reusability and reproducibility, as critical issues for the development of SPR-based analytical assays.

CONCLUSIONS

The aim of this study was to evaluate the application of surface plasmon resonance technology to the investigation of the angiogenic process in intact carcinoma living cells. VEGF/VEGFR interactions in HepG2 and HuH7 hepatoma cell lines were studied by two different assay detection formats involving either the immobilization of intact cells or the VEGF functionalization of sensor chips. Comparison between both methods suggests that the direct VEGF immobilization approach provides higher stability and better reproducibility for the monitoring of interactions. The capacity of the sensor surface to recover the initial assay conditions without alteration of VEGF active binding sites affords significant benefits over

conventional analytical methods. Other substantial advantages are the real- time monitoring of label-free biomolecular interaction with a low-response time.

To further enhance the feasibility of SPR detection future work will deal with the improvement of the assay sensitivity, minimizing non-specificity and increasing affinity parameters. Possible applications of our twofold-format SPR method may provide functional knowledge on signaling transduction events of angiogenic growth factors and can significantly contribute to the development of beneficial angiogenic therapies based on membrane-receptor targeted agents.

ACKNOWLEDGEMENTS

CIBERehd and CIBER-BBN are funded by Instituto de Salud Carlos III (Spain). Sara Carbajo-Pescador is supported by the Consejería de Educación (Junta de Castilla y León, Spain) and Fondo Social Europeo. Raquel Ordoñez is supported by the program Formación del Profesorado Universitario from the Ministry of Education (Spain). This work has been partially supported by Fundación Investigación Sanitaria en León.

REFERENCES

(1) Semela, D.; Dufour, J. F. *J.Hepatol.* **2004**, *41*, 864-880.

(2) Hanahan, D.; Weinberg, R. A. *Cell.* **2011**, *144*, 646-674.

(3) Wu, X. Z.; Xie, G. R.; Chen, D. *J.Gastroenterol.Hepatol.* **2007**, *2*, 1178-1182.

(4) Sun, H. C.; Tang, Z. Y. *J.Cancer Res.Clin.Oncol.* **2004**, *30*, 307-319.

(5) Hanahan, D.; Weinberg, R. A. *Cell.* **2011**, *144*, 646-674.

(6) Chekhonin, V. P.; Shein, S. A.; Korchagina, A. A.; Gurina, O. I. *Curr.Cancer.Drug Targets.* **2012**, *13,* 423-443.

(7) Tischer, E.; Mitchell, R.; Hartman, T.; Silva, M.; Gospodarowicz, D.; Fiddes, J. C.; Abraham, J. A. *J.Biol.Chem.* **1991**, *266*, 11947-11954.

(8) Olsson, A. K.; Dimberg, A.; Kreuger, J.; Claesson-Welsh, L. *Nat.Rev.Mol.Cell Biol.* **2006**, *7*, 359-371.

(9) Yamaguchi, R.; Yano, H.; Nakashima, Y.; Ogasawara, S.; Higaki, K.; Akiba, J.; Hicklin, D. J.; Kojiro, M. *Oncol.Rep.* **2000**, *7*, 725-729.

(10) Pitt, S. C.; Chen, H. *Surgery.* **2008**, *144*, 721-724.

(11) Hoeben, A.; Landuyt, B.; Highley, M. S.; Wildiers, H.; Van Oosterom, A. T.; De Bruijn, E. A. *Pharmacol.Rev.* **2004**, *56*, 549-580.

(12) Kohno, M.; Pouyssegur, J. *Ann.Med.* **2006**, *38*, 200-211.

(13) Takahashi, T.; Ueno, H.; Shibuya, M. *Oncogene.* **1999**, *18*, 2221-2230.

(14) Dekervel, J.; van Pelt, J.; Verslype, C. *Curr.Opin.Oncol.* **2013**, *25*, 409-416.

(15) Shinkai, A.; Ito, M.; Anazawa, H.; Yamaguchi, S.; Shitara, K.; Shibuya, M. *J.Biol.Chem.* **1998**, *273*, 31283-31288.

(16) Cunningham, S. A.; Tran, T. M.; Arrate, M. P.; Brock, T. A. *J.Biol.Chem.* **1999**, *274*, 18421-18427.

(17) von Tiedemann, B.; Bilitewski, U. *Biosens.Bioelectron.* **2002**, *17*, 983-991.

(18) Jeltsch, M.; Karpanen, T.; Strandin, T.; Aho, K.; Lankinen, H.; Alitalo, K. *J.Biol.Chem.* **2006**, *281*, 12187-12195.

(19) Herve, M. A.; Buteau-Lozano, H.; Vassy, R.; Bieche, I.; Velasco, G.; Pla, M.; Perret, G.; Mourah, S.; Perrot-Applanat, M. *Am.J.Pathol.* **2008**, *172*, 167-178.

(20) Delcombel, R.; Janssen, L.; Vassy, R.; Gammons, M.; Haddad, O.; Richard, B.; Letourneur, D.; Bates, D.; Hendricks, C.; Waltenberger, J.; Starzec, A.; Sounni, N. E.; Noel, A.; Deroanne, C.; Lambert, C.; Colige, A. *Angiogenesis.* **2013**, *16*, 353-371.

(21) Iwata, H.; Imamura, S.; Hori, A.; Hixon, M. S.; Kimura, H.; Miki, H. *Bioorg.Med.Chem.* **2011**, *19*, 5342-5351.

(22) Rusnati, M.; Bugatti, A.; Mitola, S.; Leali, D.; Bergese, P.; Depero, L. E.; Presta, M. *Sensors (Basel).* **2009**, *9*, 6471-6503.

(23) Nandini, C. D.; Mikami, T.; Ohta, M.; Itoh, N.; Akiyama-Nambu, F.; Sugahara, K. *J.Biol.Chem.* **2004**, *279*, 50799-50809.

(24) Pimkova, K.; Bockova, M.; Hegnerova, K.; Suttnar, J.; Cermak, J.; Homola, J.; Dyr, J. E. *Anal.Bioanal Chem.* **2012**, *402*, 381-387.

(25) Anderson, S. M.; Chen, T. T.; Iruela-Arispe, M. L.; Segura, T. *Biomaterials.* **2009**, *30*, 4618-4628.

(26) Hiragun, T.; Yanase, Y.; Kose, K.; Kawaguchi, T.; Uchida, K.; Tanaka, S.; Hide, M. *Biosens.Bioelectron.* **2012**, *32*, 202-207.

(27) Yanase, Y.; Hiragun, T.; Kaneko, S.; Gould, H. J.; Greaves, M. W.; Hide, M. *Biosens.Bioelectron.* **2010**, *26*, 674-681.

(28) Hide, M.; Tsutsui, T.; Sato, H.; Nishimura, T.; Morimoto, K.; Yamamoto, S.; Yoshizato, K. *Anal.Biochem.* **2002**, *302*, 28-37.

(29) Mizuguchi, T.; Uchimura, H.; Kataoka, H.; Akaji, K.; Kiso, Y.; Saito, K. *Anal.Biochem.* **2012**, *420*, 185-187.

(30) Liu, C.; Lei, T.; Ino, K.; Matsue, T.; Tao, N.; Li, C. Z. *Chem.Commun.(Camb).* **2012**, *48*, 10389-10391.

(31) Mauriz, E.; Garcia-Fernandez, C.; Mercader, J. V.; Abad-Fuentes, A.; Escuela, A. M.; Lechuga, L. M. *Anal.Bioanal Chem.* **2012**, *404*, 2877-2886.

(32) Shankaran, D.; Miura, N. *Journal of Physics D.* **2007**, *40*, 7187–7200.

(33) Lang, K.; Hatt, H.; Niggemann, B.; Zaenker, K. S.; Entschladen, F. *Scand.J.Immunol.* **2003**, *57*, 350-361.

(34) Haigler, H.; Ash, J. F.; Singer, S. J.; Cohen, S. *Proc.Natl.Acad.Sci.U.S.A.* **1978**, *75*, 3317-3321.

Figure 1. Photographs of HepG2 and HuH7 cells inmobilized on SPR sensor chips after being cultured overnight and immediately before being placed on the SPR platform.

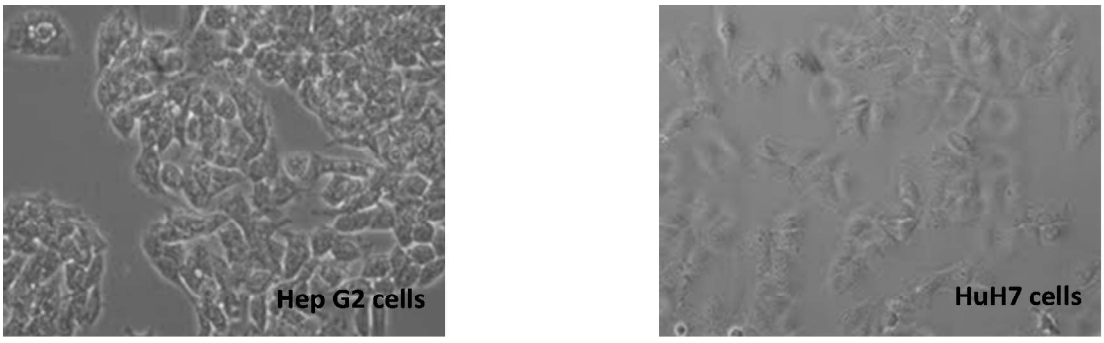
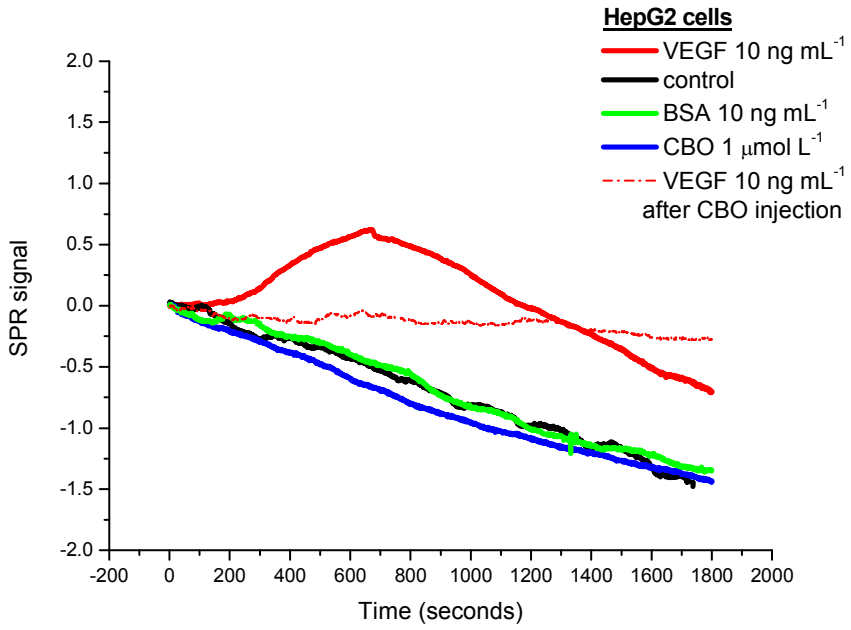
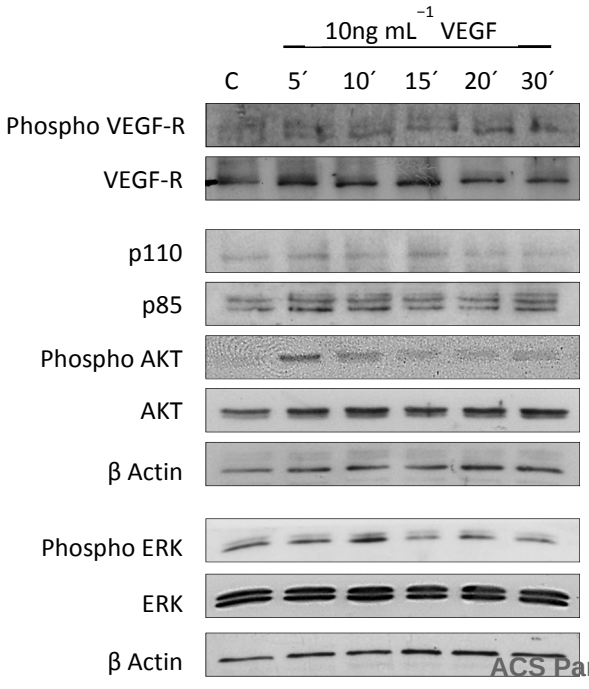
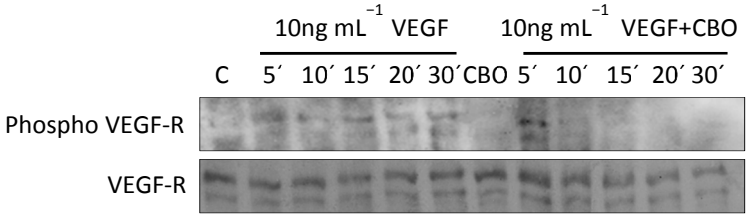


Figure 2. SPR monitoring of VEGFR activation via injection of VEGF 10 ng mL^{-1} at 20 µL min^{1} compared to SPR signals of control, BSA and VEGF injection after on-line flowing of CBO-P11 VEGF inhibitor over intact cell immobilized-sensor chips and immunoblotting results for: (A) HepG2 and (B) HuH7 hepatoma cancer cell lines. (C) Scheme of VEGFR phosphorylation pathway.

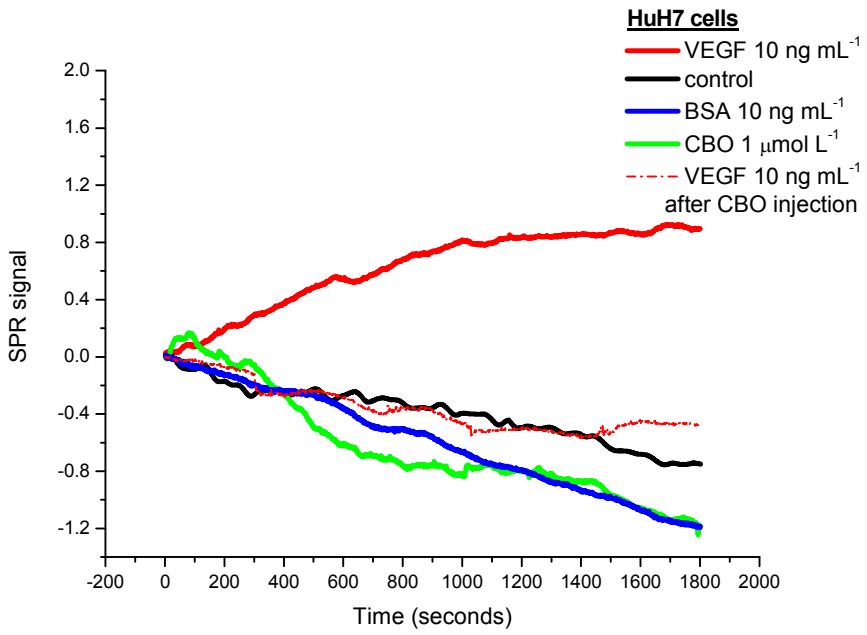
(A)

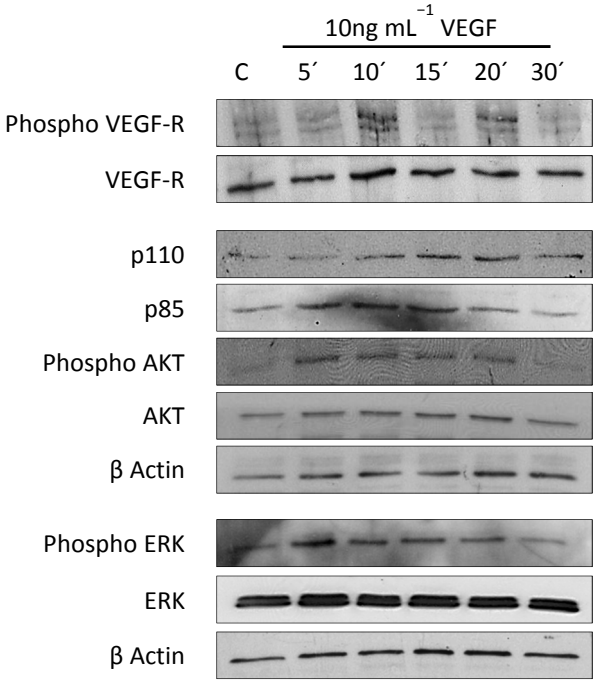


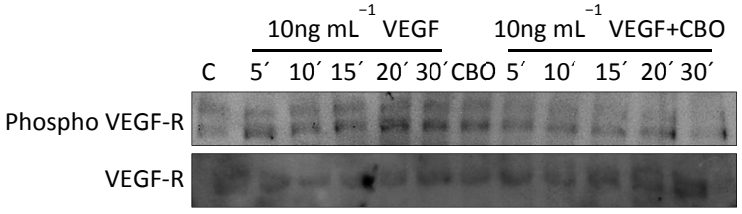




(B)


HuH7 cells
VEGF 10 ng mL-1
control
BSA 10 ng mL-1
CBO 1 μmol L-1
VEGF 10 ng mL-1
after CBO injection
SPR signal
Time (seconds)
2.0
1.6
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
-200
0
200
400
600
800
1000
1200
1400
1600
1800
2000


10ng mL-1 VEGF
C
5´
10´
15´
20´
30´
Phospho VEGF-R
VEGF-R
p110
p85
Phospho AKT
AKT
β Actin
Phospho ERK
ERK
β Actin


10ng mL-1 VEGF
10ng mL-1 VEGF+CBO
C
5´
10´
15´
20´
30´
CBO
5´
10´
15´
20´
30´
Phospho VEGF-R
VEGF-R

(C)

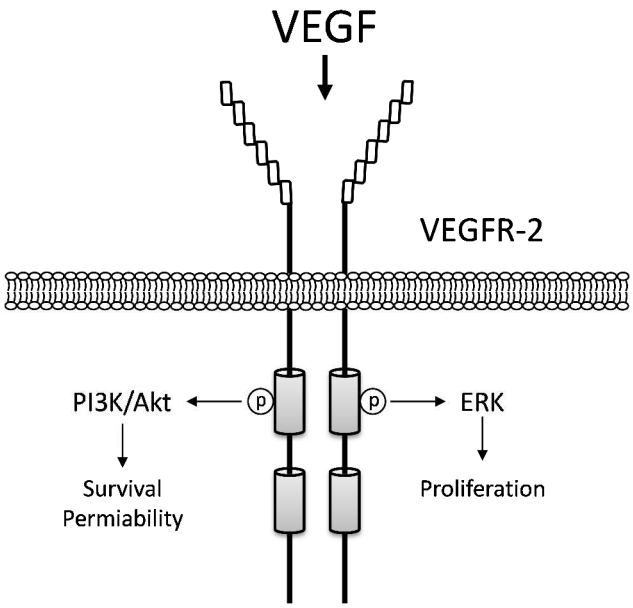
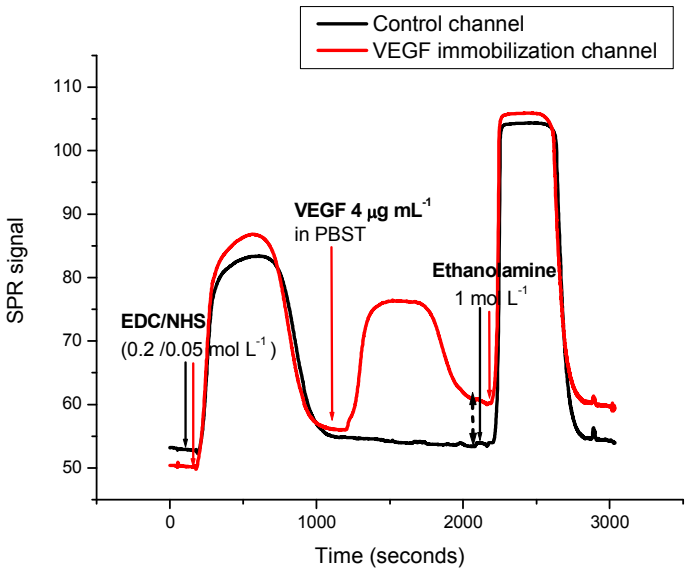

VEGF
VEGFR-2
PI3K/Akt
p
p
ERK
Survival
Permiability
Proliferation

Figure 3. SPR sensorgrams obtained for: (A) VEGF immobilization on mercaptoundecanoic activated sensor chips; (B) monitoring of specific and non-specific interactions in both functionalized and reference channel.

(A)



(B)

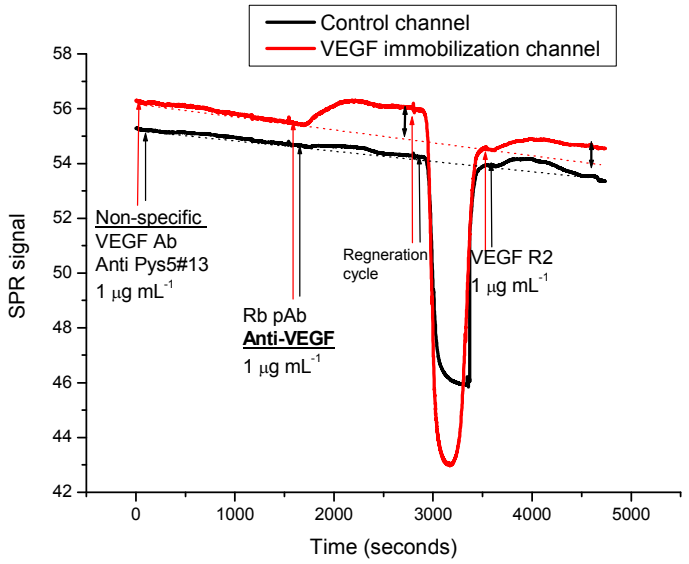
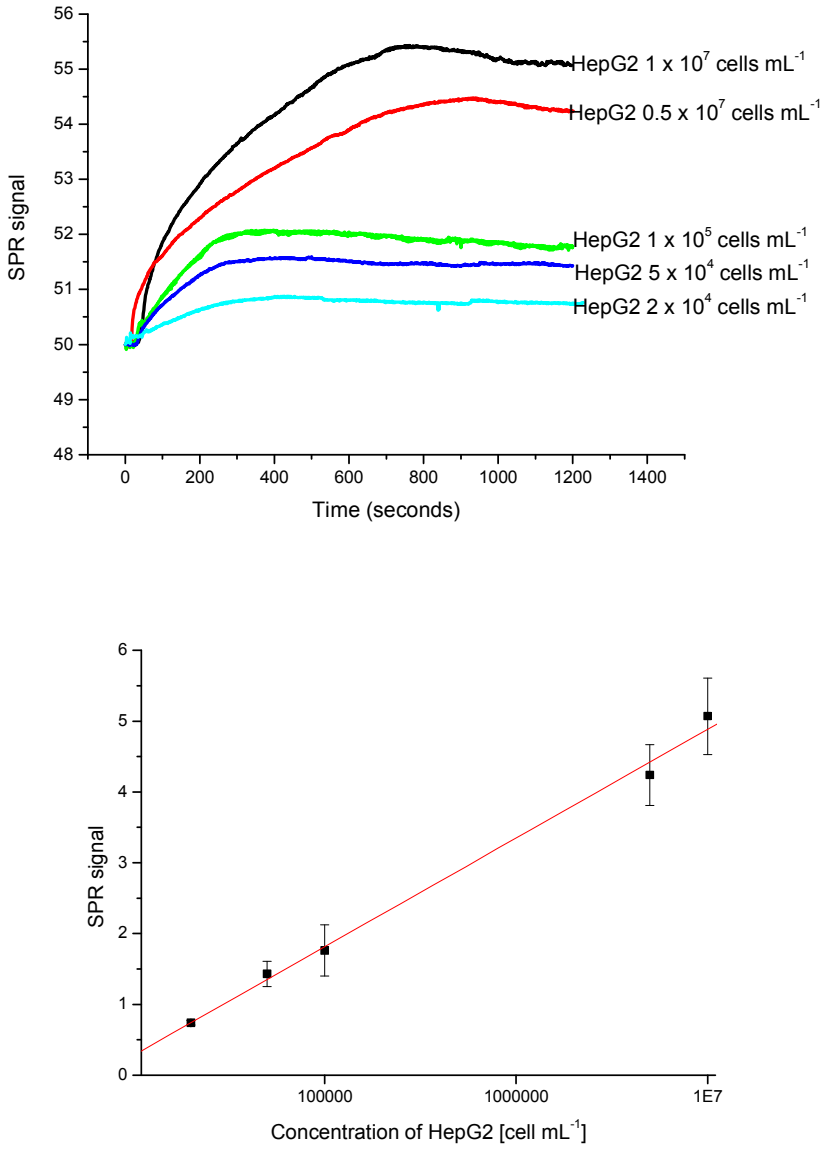
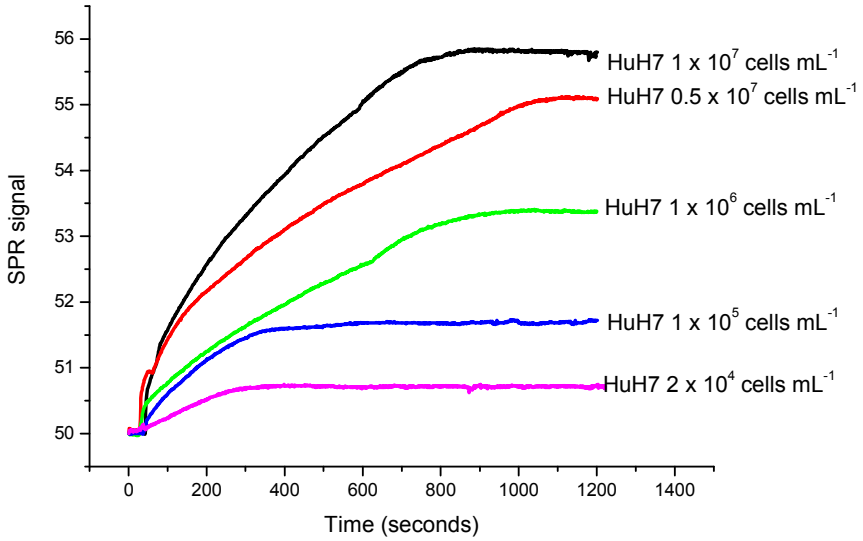
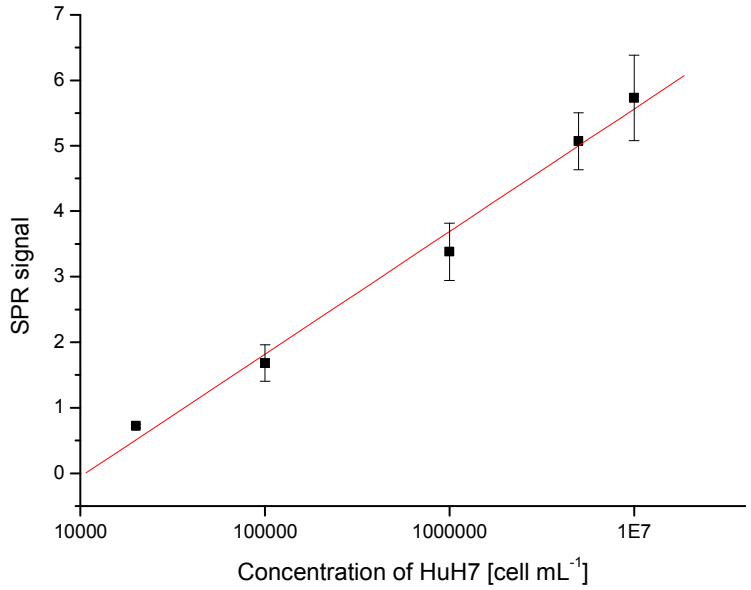


Figure 4. SPR monitoring of HepG2 and HuH7 cell interactions on VEGF-immobilized sensor surfaces at different concentrations and their respective calibration curves (A) and SPR sensorgram for sequential injections of HepG2 cells in the presence of CBO inhibitor at 5 and 10 μmol L^{-1} (B).

(A)




SPR signal
Time (seconds)
HuH7 1 x 10^7 cells mL^-1
HuH7 0.5 x 10^7 cells mL^-1
HuH7 1 x 10^6 cells mL^-1
HuH7 1 x 10^5 cells mL^-1
HuH7 2 x 10^4 cells mL^-1


SPR signal
Concentration of HuH7 [cell mL^-1]

(B)

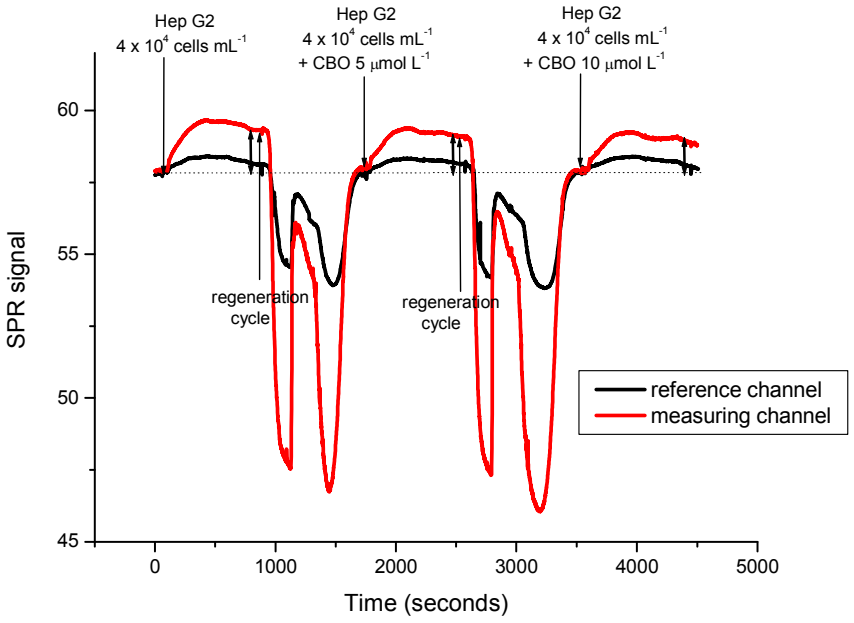
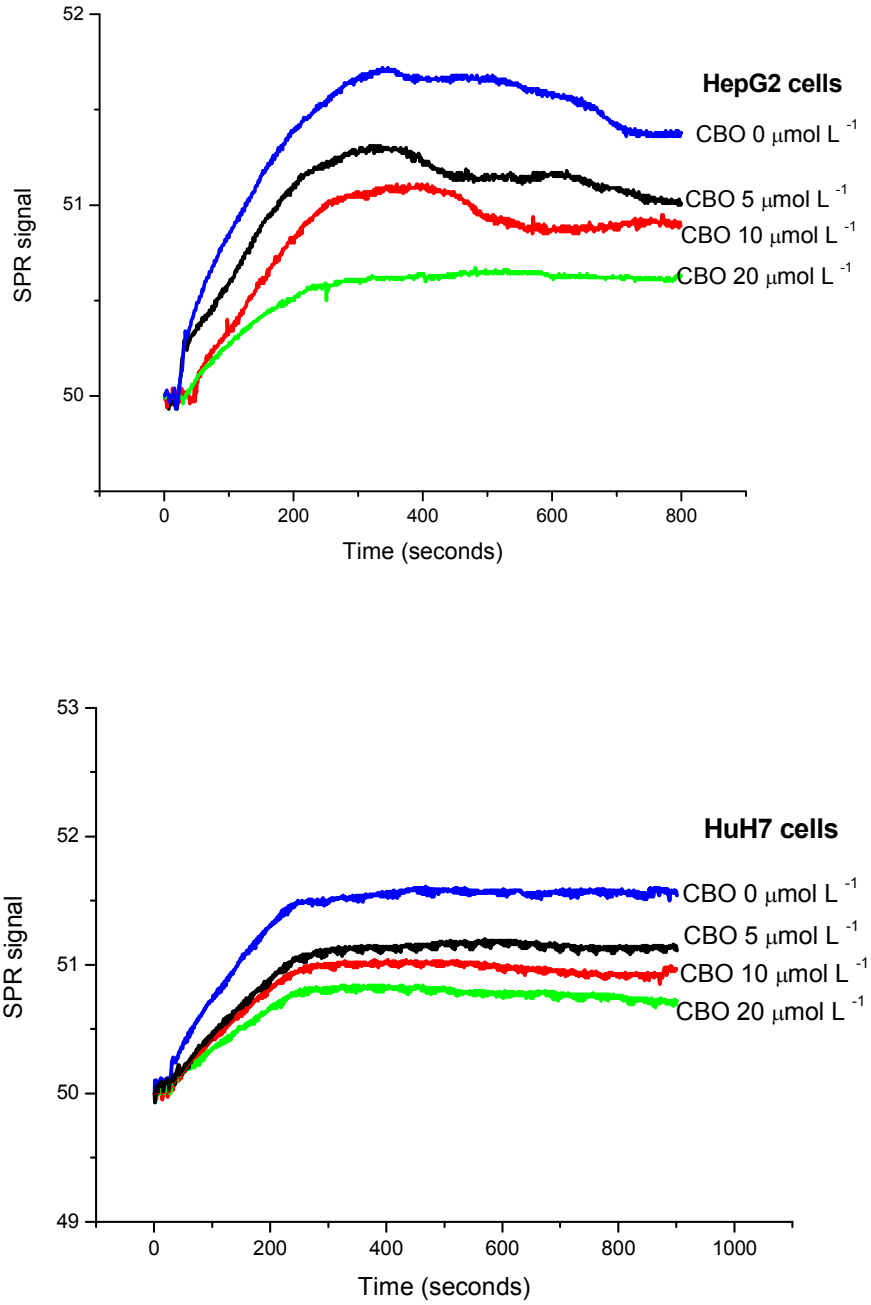
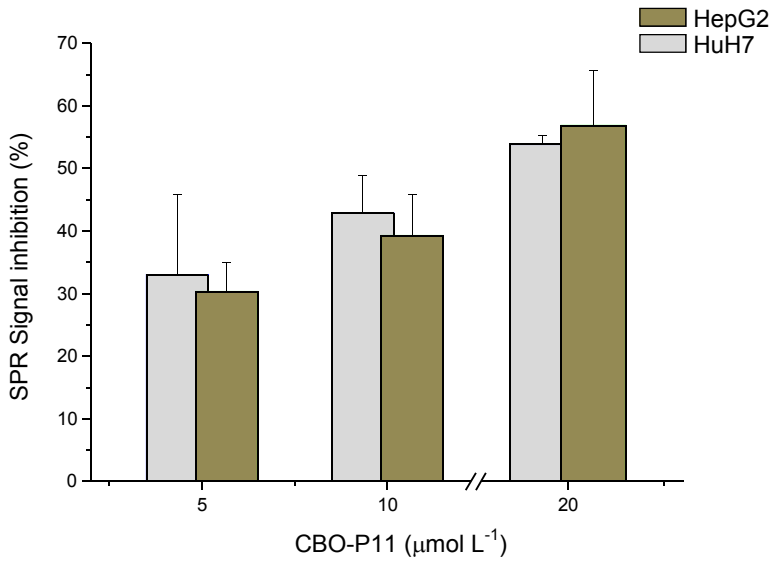

Hep G2
4 x 10^4 cells mL^-1
Hep G2
4 x 10^4 cells mL^-1
+ CBO 5 μmol L^-1
Hep G2
4 x 10^4 cells mL^-1
+ CBO 10 μmol L^-1
regeneration cycle
regeneration cycle
reference channel
measuring channel
SPR signal
Time (seconds)
60
55
50
45
0
1000
2000
3000
4000
5000

Figure 5. SPR sensorgrams showing the reduction of SPR signal due to the presence of CBO-P11 in mixed solutions of HepG2 and HuH7cells (A) and representation of the inhibitory and competitive effects of CBO and VEGF in both cell lines (B).

(A)



(B)


HepG2
HuH7
SPR Signal inhibition (%)
0
10
20
30
40
50
60
70
5
10
20
CBO-P11 (μmol L-1)

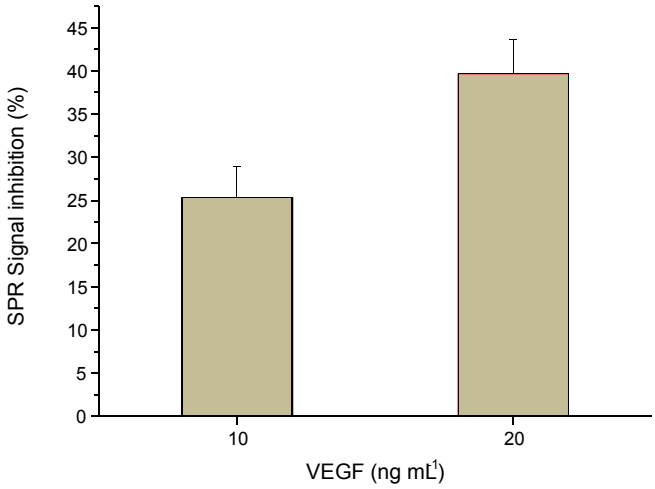

SPR Signal inhibition (%)
0
5
10
15
20
25
30
35
40
45
10
20
VEGF (ng mL-1)

Table of contents graphic: for TOC only