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Short communication

# Receptor specific adhesion assay for the quantification of integrin–ligand interactions in intact cells using a microplate based, label-free optical biosensor

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ABSTRACT

In this proof of principle study, a sensitive, label-free, whole cell approach for determination of the affinity of ligands to cell adhesion receptors is presented. The employed optical biosensor is capable of recording, in real time, the kinetics of cell adhesion with high resolution in a microplate based format. The assay was made receptor specific by measuring cell adhesion on integrin ligand displaying surfaces while tuning the concentration of the integrin ligand under study in the employed cell suspensions. As a case study, Arg-Gly-Asp (RGD) displaying polymer surfaces were used and cell adhesion was quantified by measuring the antagonistic action of echistatin, a disintegrin also containing the RGD motif, on the adhesion of cancer cells. Using this novel methodology the half maximal inhibitory concentration ($IC_{50}$) for echistatin in living cancer cells was determined to be in the range of 20–40 nM. The introduced methodology is fast, sensitive, and does not require isolated receptors because biological effects are measured in intact, living cells. The utility of this method for study of other type of adhesion receptors and ligands is discussed.


## 1. Introduction

Integrins are transmembrane heterodimers, a group of plasma membrane receptors that mediate adhesion of cells to the extracellular matrix (ECM). Integrins bind to ECM proteins mainly via the Arg-Gly-Asp (RGD) sequence motif and are crucially involved in many physiological events, including immune responses, cell growth and differentiation, and tissue morphogenesis. In addition, integrins are associated with pathophysiological conditions (e.g., thrombosis, some genetic and autoimmune diseases, and metastatic development), and thus, represent important therapeutic target structures [1].

Disruption of binding to integrin receptors is an important topic in drug discovery, comprising the design, synthesis and biomedical applications of new integrin-targeting drugs [2]. Snake venom disintegrins, a family of low molecular weight proteins, also typically contain the RGD sequence, and are known to effectively block integrin activities with high affinity [3]. However, the binding of such small molecules to integrin is technically difficult to track in real time by conventional *in vitro* ligand binding assays, and often requires isolation and labeling of the receptor of interest. High throughput assays using biosensors offer potential solutions to this problem.

Several studies have described biosensor techniques based on excitation of surface plasmons to provide information on integrin–ligand interactions. For example, binding of small mono- and oligomeric ligands to membrane-embedded integrins has been monitored by surface plasmon-enhanced fluorescence spectroscopy [4]. In addition, molecular mechanisms of binding of $\alpha_{IIb}\beta_3$ integrin to echistatin have been examined by surface plasmon resonance spectroscopy (SPR) [5]. Similarly, binding between the I domain from integrin $\alpha_X\beta_2$ and its ligand iC3b has been studied by SPR [6]. In these studies, integrins were isolated and attached to the sensor surface or were reconstituted in tethered phospholipid bilayers. Note, these are rather time-intensive preparations.

The resonant optical waveguide grating based Epic BenchTop (BT) biosensor technique is a powerful tool for high-throughput, label-free detection of stimulation-mediated cell responses [7]. Previous studies have measured the dependence of cell spreading kinetics on the average surface density of integrin ligand RGD

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motifs [8], evaluated differences between the adherence of primary human monocytes, dendritic cells, and macrophages [9], and studied complex signaling events during B cell activation [10]. This method enables real-time monitoring of a cell–surface interaction within a 100–200 nm layer above the sensor surface by analyzing the alterations in refractive index exactly where focal adhesions (i.e., sites where integrins and intracellular protein scaffolds link the actin cytoskeleton to the ECM) occur. During cellular adhesion, proteins, lipids, and ions enter this sensing zone above the biosensor surface, and consequently the local refractive index and the biosensor signal increases, allowing the adhesion process to be followed in real time with extremely high resolution [8,9].

In the present study, we examine the feasibility of using the resonant waveguide grating technology for analysis of integrin–ligand interactions by measuring the kinetics of cell adhesion. As a model, the smallest known natural disintegrin – echistatin from *Echis carinatus* – was chosen for inhibition of integrin-mediated cell adhesion via selective recognition by $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [11]. By coating the sensor surfaces with an RGD displaying polymer film, and using different concentrations of echistatin to block the cell adhesion molecules in the cell suspensions transferred onto the sensing surfaces, inhibition of binding was determined using a biosensor assay that uses intact cells, does not require ligand labeling, or isolation of receptors.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals and reagents were obtained from Sigma-Aldrich, unless stated otherwise. The stock solution of echistatin from *Echis carinatus* was prepared in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) Hank's balanced salt solution (HBSS), pH 7.0.

### 2.2. Preparation of the biosensor surface coating for cell adhesion studies

The biomimetic poly(L-lysine)-*graft*-poly(ethylene glycol) copolymer with poly(ethylene glycol) and RGD (RGD = –Gly-Tyr-Gly-**Arg-Gly-Asp**-Ser-Pro-Gly-$NH_2$) (PLL-*g*-PEG-RGD [PLL(20)-g(3.5)-PEG(2.3)/PEG(3.4)-RGD]) was obtained as a powder from SuSoS AG (Dübendorf, Switzerland) and was stored at −20 °C until use. A stock solution of 1.0 mg/ml PLL-*g*-PEG-RGD was prepared in 10 mM HEPES (pH 7.4) and sterilized by filtration. Surface coating was created by incubation with 250 μg/ml PLL-*g*-PEG-RGD for 30 min at room temperature, and reagent excess was removed by rinsing the surface three times with 20 mM HEPES HBSS, pH 7.0.

### 2.3. Cell culture

HeLa cells (93021013 Sigma-Aldrich) were maintained in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum (Biowest SAS, France), 4 mM L-glutamine, 100 U/mL penicillin and 100 μg/ml streptomycin solution, and 0.25 μg/ml amphotericin B. Cells were cultured in a humidified atmosphere containing 5% $CO_2$ at 37 °C. On reaching 80% confluence, cells were detached every 3–4 days using 0.05% (w/v) trypsin, 0.02% (w/v) EDTA solution. HeLa cells were kept with low passage number (below 20), since higher passage numbers might influence the state of the cells.

Before the cell adhesion experiments, HeLa cells were removed from tissue culture dishes using a standard procedure (0.05% (w/v) trypsin, 0.02% (w/v) EDTA). Trypsin digestion was terminated by the addition of completed medium and the harvested cells were centrifuged at 380*g* for 3 min. The cell pellet was resuspended in assay buffer (20 mM HEPES HBSS, pH 7.0).

### 2.4. The epic BT resonant waveguide grating (RWG) imager biosensor

All measurements were performed in 384-well Epic sensor microplates (Corning, USA). The bottom of each well of the microplate contained a 2 × 2 mm resonant waveguide grating (RWG) sensor. The grating areas couple the incident light into a $Nb_2O_5$ wave-guiding layer and thus an evanescent optical field decaying exponentially with the distance from the sensor surface (with a penetration depth of 150 nm) is created [8]. The characteristic wavelength, at which the resonant evanescent field is created (i.e., the resonant wavelength) is affected by any changes in the refractive index inside the penetration depth. During cellular adhesion proteins, lipids, ions are entering into this sensing zone above the biosensor surface, and consequently the local refractive index and the biosensor signal is increasing. The adhesion process can be followed in real time with extremely high resolution. The employed microplate format allows 384 parallel determinations by reading the resonant wavelength between 825 and 840 nm in every 3 s, and the distribution of the resonant wavelength is imaged in each well by high speed complementary metal-oxide semiconductor camera.

### 2.5. The biosensor based label-free cell adhesion assay

Prior to addition of cell suspensions, a baseline was recorded in the sensor wells of the Corning Epic sensor microplate for 1 h with 30 μL 20 mM HEPES HBSS, pH 7.0. After the stable baselines had been established for all wells, the HeLa cells were harvested and the inhibitor solutions at varying concentrations were added to the cell suspensions, resulting in a final cell density of 8000 cells/well. Then, 30 μL of cell suspensions were seeded into the wells and the biosensor responses were recorded for 2 h. Untreated HeLa cells were used as negative control in the experiment. All measurements were replicated at least three times at room temperature.

## 3. Results and discussion

In a previous study, the biosensor signal in the Epic BT method was used to follow the progression of the cell adhesion and spreading process on a given surface. In addition, effects of direct interaction with the adhesion receptors and density of focal contacts of the cells on this process were determined [8,9]. In this study, concentration dependent antagonism by echistatin of cell attachment to PLL-*g*-PEG-RGD on the sensor surface was evaluated in *in vitro* cell adhesion tests. Echistatin has been chosen for the current study for its known activity to bind to $\alpha_{IIb}\beta_3$ (also called *GPIIb/IIIa*), $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins expressed in HeLa cells [12,13] with very high affinity and it is commonly used positive control for many integrin binding assays.

In the label-free biosensor measurement, a change in the wavelength of the guided light ($\Delta\lambda$) occurs when HeLa cells adhere and spread on the sensor surface until an adhered cell monolayer is formed. A signal is detected as a shift in the resonant wavelength ($\Delta\lambda$), the magnitude of which is proportional to the area of the sensor covered by spread cells. Cell adhesion molecules and complexes are located in the evanescent field ~150 nm above the sensor surface. Therefore, non-adhering cells, which are located in the assay medium, are not detected by the biosensor and are excluded from the measurement [8], as indicated e.g. by the flat kinetic curves, when the spreading of HeLa cell was completely blocked by preincubation with echistatin at high concentration (Figs. 1 and 2 **A** ).

Fig. 1 illustrates schematically the working principle of the introduced label-free cell adhesion assay. First, surface coating of the

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