Talanta 109 (2013) 209–215

Contents lists available at SciVerse ScienceDirect

Talanta

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

Real-time detection of the chemokine CXCL12 in urine samples by surface plasmon resonance

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ARTICLE INFO

Article history:
Received 19 October 2012
Received in revised form
30 January 2013
Accepted 5 February 2013
Available online 13 February 2013

Keywords:
CXCR4
lentiviral particle
rheumatoid arthritis
GPCR

ABSTRACT

Surface plasmon resonance (SPR)-based biosensors are established tools for measuring biomolecular interactions between unlabeled analytes in real time, and are thus an ideal method to evaluate G protein-coupled receptor (GPCR) binding interactions. Using as a vehicle lentiviral particles bearing the chemokine receptor CXCR4 in its native plasma membrane context, SPR analysis can be performed using the particles as specific receptors to monitor the CXCR4 interaction with its ligand, CXCL12. The method shows linear correlation in the 5–40 nM range, with low intra- and inter-assay variation, a relative standard deviation $<10\%$, chip-to-chip variation $<12\%$, with stability of the sensor response for more than 150 measurements in the same chip over a four-week period. Our objective was to develop a method for rapid detection and quantification of analytes such as CXCL12 in biological samples, with no need for pretreatment. As a proof of concept, we tested for CXCL12 in urine samples from rheumatoid arthritis patients, who have elevated levels of this chemokine in plasma and synovial fluid. The biosensor method allowed sensitive, reproducible CXCL12 detection in the physiological range, suggesting its value for the diagnosis of autoimmune disorders.

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1. Introduction

Biomarker detection is critical for the diagnosis of many diseases, to precisely define pathological states and to predict patient response to specific treatments for cancer, inflammatory and autoimmune disorders. Efforts have been made in recent years to develop sensitive, reproducible diagnostic assays for these conditions. Among these, label-free nanosensors show considerable potential for the detection of small molecules of clinical interest; these biosensors are ultrasensitive, work in real time, and allow parallel detection of several molecular species [1,2]. Sensors based on surface plasmon resonance (SPR) register biomolecular interactions as increases in refractive index due to mass accumulation at the sensor surface [3]; they have been widely used as detection systems for medical diagnostics and environmental monitoring [4,5].

Although the application of biosensors to analyte detection is not difficult in itself, the complex nature of clinical samples complicates discrimination between specific and non-specific interactions. The use of specific, high affinity antibodies facilitates detection and increases assay sensitivity [6]. Adequate antibodies are not always available, however, and in some cases, specificity is incompatible with the recognition of multiple forms of a protein of interest. This is relevant when evaluating clinical samples, as proteins can be modified by interaction with other proteins, by proteolytic cleavage [7], and/or by other processes including sulphation [8] and citrullination [9,10]. As an alternative to antibodies, the native receptor for a protein can be used. Specific detection is facilitated when the receptor is purified or when, after detergent solubilization, it retains its ability to bind ligands. Some receptors are nonetheless sensitive to solubilization processes, which render them non-functional [11]; this is especially true for the G protein-coupled receptor (GPCR) family. The GPCR are characterized by seven membrane-spanning α-helices (Dixon et al. 1986) that render the receptor extremely hydrophobic; it thus requires a lipid environment to maintain its native conformation [12]. Some attempts have been reported to use biosensors to study membrane-associated GPCR. In all cases,

Abbreviations: GPCR, G protein-coupled receptor; VSVG, vesicular stomatitis virus gene; RA, rheumatoid arthritis; GAG, glycosaminoglycan

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http://dx.doi.org/10.1016/j.talanta.2013.02.018

these experiments were based on capture of detergent-solubilized GPCR prior to reconstitution in a lipid environment [13], introducing detergent-induced variability [14].

GPCR are the largest family of integrated membrane proteins, and include receptors for hormones, neurotransmitters, calcium ions and chemokines, among other molecules; they have become the principal target of pharmaceutical drugs. As key mediators in many diseases, the chemokines and their receptors have attracted considerable attention from researchers. The chemokines are a family of small proteins, originally described as proinflammatory cytokines that regulate leukocyte trafficking in homeostatic and inflammatory processes [15]. Here we focused on the chemokine CXCL12, the only ligand for the CXCR4 receptor; it is expressed in a wide variety of tissues and has a central role in development and hematopoiesis. Mice lacking CXCR4 show defective vascular development, hematopoiesis and cardiogenesis, and die perinatally [16]; CXCL12-deficient mice are characterized by defective B cell lymphopoiesis and myelopoiesis, as well as abnormal neuronal and cardiovascular development [17]. In humans, the CXCR4/CXCL12 axis is implicated in cancer [18], pulmonary fibrosis [19], HIV-1 infection [20] and rheumatoid arthritis (RA) [21,22]. CXCL12 levels are increased in synovial fluid and plasma of RA patients [22]; in this disease, CXCL12 activates fibroblast-like synoviocytes, stimulates angiogenesis [21], and degrades the cartilage matrix by triggering chondrocyte release of matrix metalloprotease 3 [7]. Studies by Grassi et al. also show that elevated CXCL12 levels in RA patient synovial and bone tissue promotes pathological bone loss by recruiting and activating osteoclasts [23].

We developed a rapid SPR method to determine whether CXCL12 is present in urine, and tested it in samples from RA patients to assess whether it can be used as a diagnostic marker. The technique uses CXCR4 in the context of the cell membrane, which is incorporated into lentiviral particles. The virions are prepared from transfected cells bearing the receptor of interest; they are easily purified and attached covalently to the biosensor surface. Lentiviral particles bearing CXCR4 are generated in mild conditions; in a previous study, we used ligand binding assays and a specific antibody that recognizes a conformational CXCR4 epitope to demonstrate that this complex protein remains unaltered [24].

The method enables multi-analyte detection using parallel flow cells, requires no sample pretreatment, and is sufficiently sensitive and reproducible to detect variations in protein concentration at physiological levels. This procedure could have general applications, as it would allow identification of a wide variety of physiologically active analytes in biological samples; nonetheless, other receptors must be studied to establish this technique as a reference for receptor-based interaction studies. Using lentiviral particles attached to the surface of the SPR biosensor, we show that SPR is a valid technique for detection of CXCL12 binding to its native receptor, and that it can be used to detect this chemokine in urine samples from RA patients.

2. Material and methods

2.1. Cell and reagents

Human embryonic kidney 293 T cells (HEK293T) were from the American Type Culture Collection (CRL-11268; Manassas, VA). Antibodies used were CXCR4-01 [25], IgM-SPRD (Bioscience, San Diego, *CA*) and anti-VSVG mAb (Abcam, Cambridge, UK). AMD3100 was from Sigma-Aldrich (St. Louis, MO), CXCL12 and CCL3 chemokines from Peprotech (Rocky Hill, NJ), and LVTHM, PAX2 and VSVG plasmids from Tronolab (Lausanne, Switzerland).

Mercaptoundecanoic acid, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were from Sigma-Aldrich. Trichloroethylene, acetone, ethanol, sulfuric acid and hydrogen peroxide were from Merck (Darmstadt, Germany). PBS (10 mM phosphate pH 7.4, 137 mM NaCl, 2.7 mM KCl) and Tween 20 were from Panreac Quimica (Barcelona, Spain). Ethanolamine hydrochloride was from Acros Organics (Geel, Belgium). The Quantikine Human CXCL12/SDF-1α immunoassay kit was from R&D Systems (Abingdon, UK).

2.2. Lentiviral particle production, purification and characterization

Lentiviral particles were produced by JetPei (Polyplus, Illkirch, France) cotransfection of HEK293T cells with LVTHM, PAX2 and VSVG plasmids at a 1:1:1 ratio. At 72 h post-transfection, the supernatant was collected, and cell debris removed by low-speed centrifugation and 0.22 μm filtration. The supernatant was centrifuged through a 20% sucrose cushion in a Beckman SW55 rotor (247,000xg, 2 h, 4 °C) and the pellet resuspended in PBS. Lentiviral particles were aliquoted [10^7 transducing units (TU)/ml; 234 ± 28 CXCR4 molecules/particle] and stored at −80 °C [24].

For CXCR4 siRNA lentiviral particle production, two siRNA duplexes of CXCR4 were designed [24] and transfected into HEK293T cells at a final concentration of 120 nmol/L using JetPei. Lentiviral particles were produced and characterized as above.

2.2.1. Flow cytometry analysis

HEK293T cells were plated in V-bottom 96-well plates (2.5×10^5 cells/well) and incubated (30 min, 4 °C) with 50 μl/well anti-human CXCR4-01 mAb (30 μg/ml), followed by IgM-SPRD. Cell-bound fluorescence was determined in a Profile XL flow cytometer (525 nm; Beckman Coulter, Miami, FL). For characterization by flow cytometry, lentiviral particles were coupled to latex beads (15 min at RT) and stained for CXCR4 as above.

2.2.2. Western blot and enzyme-linked immunosorbent assays

Lentiviral particles were analyzed in Western blot using antibodies specific for vesicular stomatitis virus G (VSVG) protein and for CXCR4 [25]. For urine samples, the Quantikine Human CXCL12/SDF-1α immunoassay kit was used according to manufacturer's instructions. Linearity of detection was between 0.312 pg/ml and 10 ng/ml CXCL12.

2.3. Urine samples

Second morning urine samples were collected from five healthy donors and five patients with active rheumatoid arthritis (DAS28 ≥ 5.1) at the Hospital Puerta de Hierro (Madrid, Spain). pH was measured and samples were aliquoted and stored at -20 °C. Samples were not centrifuged, filtered or pH neutralized. All patients and controls were female, mean age 45 years old (range 35–55 years). All study participants gave informed consent.

2.4. Instrumentation

The surface plasmon resonance device is home-made and works in the Kretschmann configuration using a glass prism coupler structure [4]. The sensor has two flow cells with a volume of 0.3 μl each, and permits two independent sample measurements. The prototype incorporates other required optics components and an electronic module, computer-controlled pumps, valves and automated fluid injection. All measurements in this study were performed by sample injection using the flow delivery system, to ensure injection of 350 μl of sample while maintaining

a continuous flow of running buffer (20-60 μl/min). Further description of this SPR system can be found elsewhere [4].

2.5. Immobilization of viral particles

Gold chips were cleaned with trichloroethylene, acetone and ethanol, immersed in piranha solution [H_2SO_4:H_2O_2 (3:1)], rinsed with water, ultrasonicated (5 min) and dried with an N_2 stream. The chip was placed over the flow cells and the prism was optically coupled to the chip using refractive index matching oil. A constant flow speed of 20 μl/min was maintained throughout the immobilization process. A carboxyl-terminated alkanethiol self-assembled monolayer (SAM) was formed on the gold chip by flowing mercaptoundecanoic acid (0.05 mM solution in ethanol; 20 min); alkanethiol excess was rinsed with ethanol, followed by water before the next immobilization step. The carboxylic surface was activated with 0.2 M EDC and 0.05 M NHS to form an N-hydroxysuccinimide ester intermediate, followed immediately by injection of lentiviral particles diluted in acetate buffer (10 mM NaOAc). Lentiviral particles were coupled covalently to the ester via amine groups. The surface was blocked with 1 M ethanolamine, pH 8.5. Once particles were immobilized, PBS-T (0.05% Tween20, pH 7.4) flow was adjusted to 60 μl/min. All procedures were performed in controlled room temperature conditions (20–24 °C).

2.6. Data analysis

Sensorgrams were corrected for signals obtained in a reference flow chamber in which buffer without CXCL12 was used. Statistical analysis was performed using a non-linear regression equation applied to the Mann-Whitney model with a 95% confidence interval and unpaired Student's *t*-test (GraphPad PRISM 5.0).

3. Results and discussion

3.1. Characterization of CXCR4 on the lentiviral particle surface

HEK293T cells were used to generate lentiviral particles. These cells, which express endogenous CXCR4 as analyzed by Western blot (Fig. 1A), were transiently cotransfected with plasmids bearing genes to generate the lentiviral particles (LVPX4). We evaluated CXCR4 on LVPX4 by coupling particles to latex beads, followed by flow cytometry analysis with a CXCR4-specific antibody (Fig. 1B). Results were confirmed by Western blot; the VSVG viral core protein was used as control (Fig. 1A).

3.2. Lentiviral particles on the biosensor surface

The standard technique for attaching biomolecules to the gold surface of a SPR chip is to generate a self-assembling monolayer (SAM) [26]. A SAM generally consists of long-chained (C12 and higher) n-alkylthiols with organic functional groups, which link easily to the gold film via thiol groups [27]. We studied the influence of pH on LVPX4-conjugate covalent coupling to the SAM using buffer solutions over a pH range of 4–8 (Fig. 1C). Optimal lentiviral particle immobilization was found using 10 mM acetate buffer pH 6, which was fixed for the rest of the experiments. After testing several LVPX4 concentrations, we used LVPX4 at saturation (3×10^5 TU/ml; 234 ± 28 CXCR4 molecules/particle). Lentiviral particles were attached simultaneously to the two activated sensor surfaces (Fig. 1D).

3.3. SPR characterization of LVPX4

We tested the ability of CXCR4 present on LVPX4 to bind the specific ligand CXCL12. For this direct detection format (Fig. 1E), the SPR sensor flow cell was loaded with running buffer, the baseline stabilized, and a 360 μl sample was injected over the sensor surface (6 min, 60 μl/min). The sample was replaced by running buffer to stabilize the signal. As an appropriate assay buffer can greatly reduce non-specific binding to matrix components, we analyzed PBS in a pH range from 6.5-8 and ionic strength from 150–500 mM NaCl. For CXCL12, buffer with pH 7–7.5 increased specific interactions (Fig. 2A); in addition, lentiviral particles were damaged at ionic strengths >200 mM (not shown). The running buffer used for experiments was thus PBS-T (137 mM NaCl, pH 7.4, 0.05% Tween-20). The SPR response was linear for CXCL12 concentrations from 5 to 40 nM, with SPR values of 0.1-1.3 arbitrary units (Fig. 2B), and saturation was reached at 80 nM. As a negative control, we tested binding of CCL3, which does not interact specifically with CXCR4; no SPR signal was detected (Fig. 2C). The presence of CXCR4 in the immobilized virions was always confirmed by injecting anti-CXCR4 antibody, which showed an increase in signal ($\Delta \cong 2.1$ a.u. for 10 μg/ml); an isotype-matched control antibody IgM gave no SPR signal (Fig. 2C).

The basis of the biosensor is to establish a stable, specific complex between an analyte and the chip-immobilized ligand. To analyze the specificity of the interaction between CXCR4 and CXCL12, we tested inhibition of CXCL12 binding to its receptor by the CXCR4 antagonist AMD3100 [28,29]. AMD3100 was added to running buffer at a saturating concentration (100 μM) and incorporated into the CXCL12 solution. The inhibitor markedly reduced the CXCL12 signal (80% for 20 nM CXCL12; Fig. 2D). To confirm this specificity for CXCL12/CXCR4 interaction, we prepared LVP from HEK293T cells previously transfected with specific siRNA for CXCR4 (siLVPX4), as described [24]. We observed 87.66 ± 5.37% signal reduction compared to the signal for wt particles (Fig. 2E). The signal was comparable to that in control flow chambers. The results indicated that LVPX4 immobilized on the sensor chip bore CXCR4, and that interaction with CXCL12 was mediated specifically by this receptor.

3.4. Regeneration and reusability

Regeneration of covalently immobilized CXCR4 without damage to its physical-chemical properties is essential for repeated use of the sensor chip. A regenerable sensor surface ensures reliability of the system, which requires complete ligand-particle complex dissociation with no significant loss of assay sensitivity. In addition, reusability and precision are important performance parameters for sensors [30], as they indicate sensor capacity to reproduce measurements. To estimate sensor surface activity during measurements, we tested several regeneration solutions (5–100 mM HCl, 100 mM NaOH, 50–200 mM NaCl, acetic acid); 5 mM HCl regenerated the surface completely and permitted its prolonged use. CXCL12 (20 nM) injection/regeneration cycles over a period of several days showed <12% variation in the CXCL12 response (Fig. 2E); CXCR4 withstood at least 150 regeneration cycles with no notable signal loss compared to initial binding values. We found no marked loss of ligand/receptor complex activity or damage to the physical properties of the SAM in four weeks of continuous operation.

To evaluate SPR assay precision, we compared sensor chips from several batches of metalized gold slides. Comparison of intra- and inter-immobilization variation on different days and gold chips showed no clear differences using the same or different batches of LVPX4 (Table 1). Intra-immobilization variation was

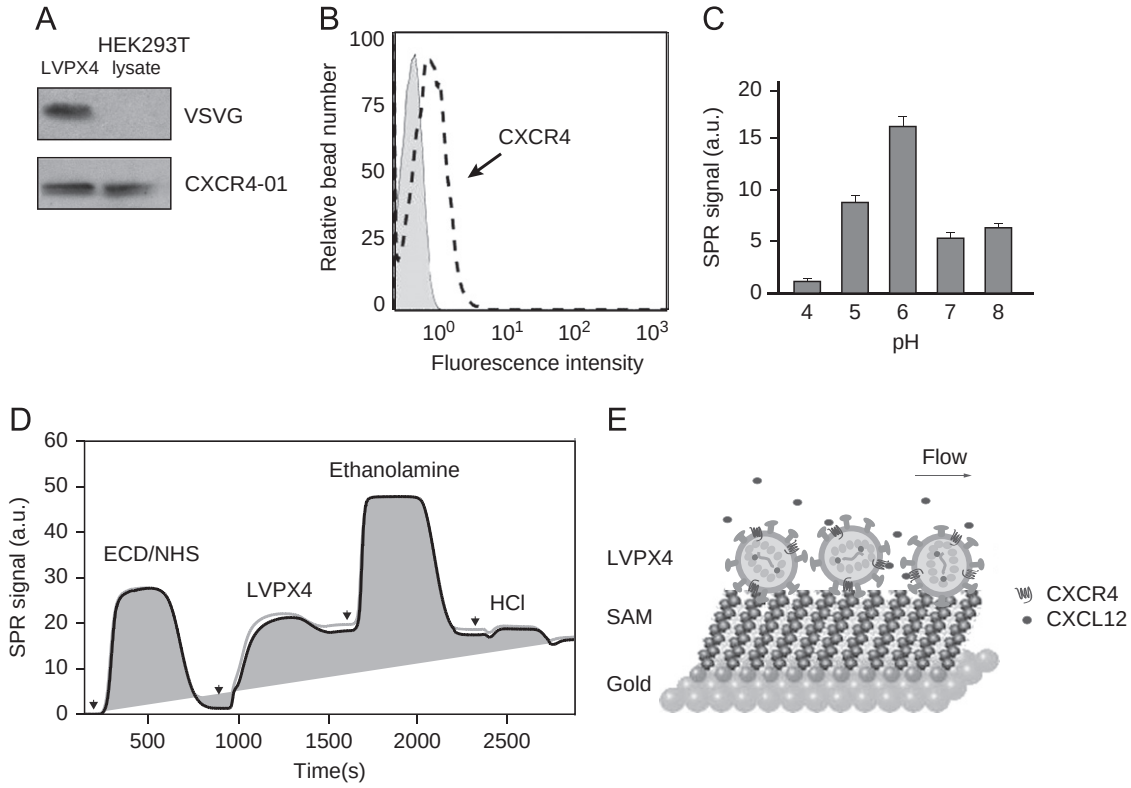

Fig. 1. CXCR4 expression on the lentiviral particle. (A) HEK293T cell lysate and LVPX4 were analyzed in Western blot with anti-CXCR4 (39 kDa) and –VSVG (57 kDa) as a structural viral protein control. (B) CXCR4 expression in LVPX4 was analyzed by flow cytometry using latex bead-coupled LVPX4 with anti-CXCR4-01 antibody (dotted line) and isotype-matched control (gray). One representative experiment is shown of four performed. (C) SPR signals produced by LVPX4 immobilization to the inactivated chip surface using a pH 4–8 range of acetate solutions of four performed (mean ± SD). (D) Representative immobilization of LVPX4 on the gold surface. The figure shows a sensorgram of LVPX4 (~300,000 TU/ml) in both biosensor flow cells. SPR signal in arbitrary units (a.u.) and time (sec). (E) Scheme showing alkanethiol self-assembled monolayer (SAM) formed on the gold sensor surface, and lentiviral particles (LVPX4) coupled covalently via amine groups. In the running buffer (PBS-T), we injected the CXCL12 chemokine. Arrow indicates flow direction.

calculated after analysis of three replicates per CXCL12 dilution in three independent assays; inter-immobilization variation was determined by analysis of three different chips, using three experiments per chip for each CXCL12 concentration. Mean intra-immobilization variation was $<9.6\%$, within acceptable limits for analytical methods [31].

3.5. CXCL12 detection in urine samples

Elevated CXCL12 levels in serum, plasma or synovial fluid are detectable by ELISA in diseases such as RA [22,32] and osteoarthritis [7], indicating a possible use of CXCL12 detection as a biomarker for these diseases. CXCL12 quantification can nonetheless be distorted by the high protein concentrations in these biological fluids, whose extraction can be invasive, complicated and time-consuming. Urine is an ideal clinical sample for disease biomarker determination, as large amounts are collected simply and non-invasively, and sampling is easily repeated. In many pathological and physiological situations such as kidney disease, urinary tract infection, high blood pressure or pregnancy, urine has moderate protein levels (~100 μg/ml); in these cases, although albumin, transferrin, IgG or microglobulin can adsorb to the sensor surface and produce an SPR background signal, it is generally lower than that produced by proteins in serum or plasma. The high salt content and low pH of urine can also interfere with detection [33], although appropriate buffer choice should reduce these effects.

We assayed second morning urine samples, as the 6.8–7.5 pH range is more appropriate than low pH (<6.5) first morning urine samples. Samples were not pH neutralized, filtered or centrifuged, as these treatments reduced CXCL12 reactivity. We added exogenous CXCL12 to urine (Fig. 3A) and compared signals with those obtained for CXCL12 in PBS-T (Fig. 3B). In both cases, SPR signals increased in parallel to the increase in CXCL12 concentration, although values in urine were slightly higher (1.05 ± 0.7 SPR units) than those for PBS-T; CXCL12 concentration and SPR signal showed linear correlation (Fig. 3B). For urine samples, reusability and sensor stability were maintained for more than 120 measurement cycles over a 4-week period, with a maximum SPR signal drop of 12% relative to the initial value.

The sensing method showed linear correlation between specificity and reproducibility in the detection of CXCL12 from urine samples; we therefore evaluated CXCL12 levels from RA patients. Samples, used immediately after thawing, were injected (60 μl/min, 10 min) over chip-bound CXCR4. SPR signals were higher in urine from RA patients (0.318 ± 0.082 SPR units) than from healthy donors (0.073 ± 0.032 SPR units), indicating a 4.4-fold increase (Fig. 3C). SPR signal specificity was confirmed by addition of AMD3100 inhibitor to running buffer and urine samples. In both cases, the SPR signal was reduced (Fig. 3D), indicating that the signal was due mainly to specific CXCL12 interaction with sensor-bound LVPX4, although non-specific absorption might also contribute to the signal. In parallel, we used ELISA to determine CXCL12 levels in these urine samples. CXCL12 values were significantly higher in RA patient urine (2.093 ± 0.270 nM) compared with values for healthy individuals (0.704 ± 0.088 nM), a 3.0-fold increase (Fig. 3E). Although a larger sample number is needed to confirm the relevance and statistical significance of these findings, our data suggest the utility of this biosensing method for the detection of small molecules in urine samples.

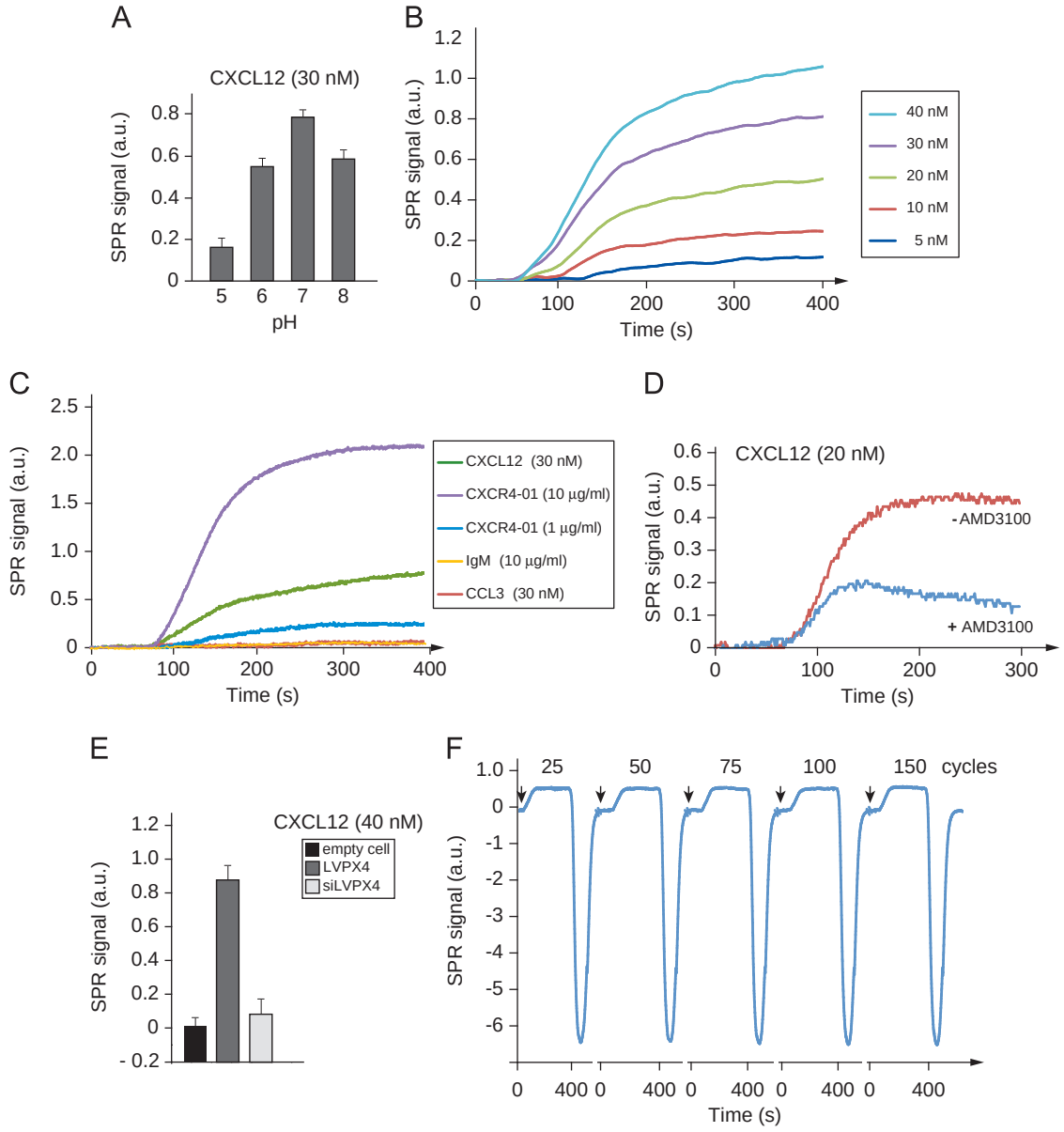

Fig. 2. Specific CXCL12 binding to LVPX4-coated chip in the biosensor. (A) SPR signal for CXL12 (30 nM) at various pH in PBS-T running buffer. A representative experiment is shown of four performed. (B) Sensorgram for CXCL12 (5–40 nM) binding to immobilized LVPX4 virions. One representative experiment is shown of four performed. (C) Sensorgrams for anti-CXCR4 (1 and 10 µg/ml), isotype-matched control (10 µg/ml), CXCL12 and CCL3 (each at 40 nM) binding to the LVPX4-coated surface, respectively. One representative experiment is shown of three performed. (D) Sensorgram for CXCL12 (20 nM) alone or in the presence of AMD3100 (100 µM), respectively. A representative curve is shown of four performed. (E) SPR signals for CXCL12 (40 nM) binding to immobilized LVPX4 and siLVPX4. Data show mean ± SD of triplicate determinations in a representative experiment of three performed. (F) Successive measurement and regeneration cycles after CXCL12 (20 nM) binding to the LVPX4 surface (to 150 completed measurements). The rows represent the injections.

Table 1
Intra- and inter-assay immobilization coefficients of variation for CXCL12 concentrations.

	CXCL12 concentration (nM)					Mean
	5	10	20	30	40	
Intra-assay variation (%RSD)						
Immobilization 1	13.36	10.90	7.19	6.86	1.32	7.93
Immobilization 2	12.86	10.79	5.69	7.97	2.05	7.87
Immobilization 3	9.43	8.88	8.85	7.02	4.52	7.74
Inter-assay variation (%RSD)						
3-immobilization period	15.67	11.16	9.75	8.59	2.83	9.60

Triplicate CXCL12 measurements on three batches of gold-surface chips. Mean of three measurements, calculated as relative standard deviation (*%RSD*).

In biological fluids, CXCL12 usually associates with other proteins. It has high affinity for the glycosaminoglycans (GAG) heparin and heparan sulfate [34], found in the urine of patients with various diseases [35]. CXCL12 binds GAG and CXCR4 via different sites, and the CXCL12/GAG complex can bind CXCR4 [36], although high heparin concentrations slightly reduce CXCL12 binding to its receptor [24]. Although heparin complexes do not markedly alter CXCL12 detection by ELISA (not shown), we cannot rule out the possibility that other GAG in urine interfere with antibody recognition [35,37]. Chemokines also undergo post-translational processing, including proteolytic modifications, glycosylation and citrullination [9,38], all of which alter their *in vitro* and *in vivo* activity. In addition, CXCL12 in solution forms dimers and/or oligomers [39]. The balance of mono- and dimeric forms varies with pH and anions, which can shift this equilibrium

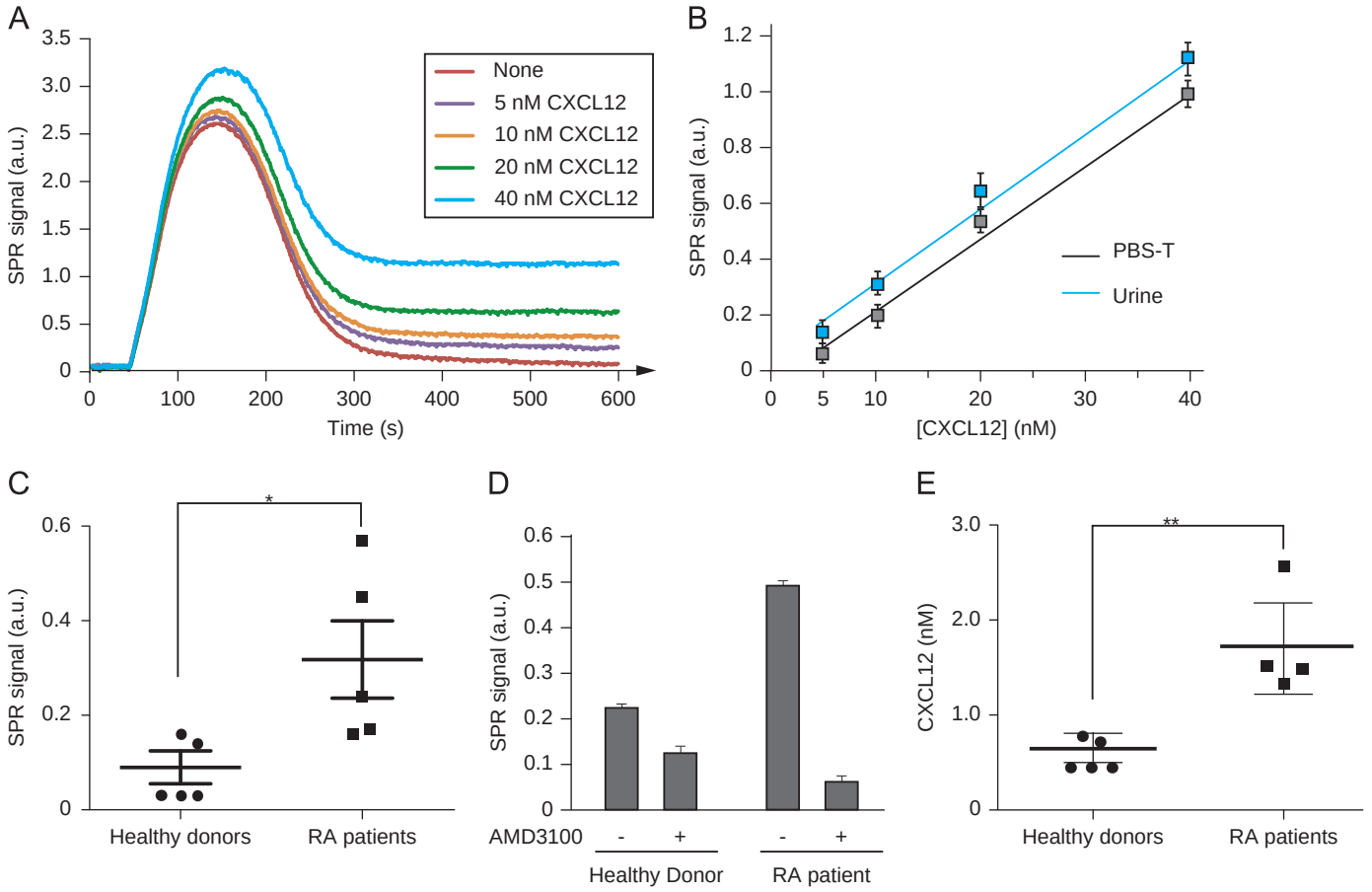

Fig. 3. CXCL12 detection in urine. (A) Sensorgram for healthy donor urine with increasing concentrations of exogenous CXCL12 (5–40 nM). One representative curve is shown of 3 performed. (B) CXCL12 standard curve in PBS-T and healthy donor urine using increasing CXCL12 concentrations (5–40 nM). The representative points are selected at 600 s One representative experiment is shown of 4 performed. (C) Comparison of SPR signal for urine samples from five healthy donors and five patients with active RA (DAS28 ≥ 5.1) (mean $\pm$ SEM, $^{*}p < 0.05$). (D) SPR signal at equilibrium from sensorgrams of healthy donors (HD) and RA patients, in absence or presence AMD3100 (100 μM). Data are expressed as the mean $\pm$ SEM of three independent experiments performed. (E) Comparison of CXCL12 concentrations (nM) in urine from five healthy donor and five RA patients, as determined by ELISA (mean $\pm$ SEM, $^{**}p < 0.01$).

toward the dimeric state [40] and thus influence chemokine function through distinct receptor interactions and signaling pathways [41]. The resulting complexes might be underestimated in ELISA, which does not discriminate among monomers, dimers or oligomers, or between active and inactive forms. In contrast, these complexes could promote higher signals in SPR, where mass is measured. Receptor-based SPR analysis allows detection of different CXCL12 species as long as they are able to interact with the receptor, and thus reflects the amount of functional isoform in urine.

4. Conclusions

We developed an effective SPR technique to detect biologically active analytes in urine, specifically CXCL12. We studied binding of this chemokine to its receptor, CXCR4, in the context of the cell membrane as presented by lentiviral particles. The assay uses HEK293T cells to generate receptor-bearing virions; use of these particles as a vehicle to immobilize CXCR4 on the sensor surface is a robust, simple and reproducible means of specific CXCL12 detection. The lentiviral-based SPR method is rapid, occurs in real time, uses non-radioactive ligands, and gives reproducible results for urine samples, with no requirement for preliminary treatment. Application of this technique to detect CXCL12 suggests that this chemokine is expressed at higher levels in urine of RA patients than in healthy controls; a larger-scale study is under way to confirm these findings and to correlate levels with RA severity. GPCR are sensitive to solubilization; detergents can render these proteins non-functional [42] and therefore useless for receptor-based studies. Although additional analyses are needed to ensure the universality of this approach, the assay allows the study of ligand interactions in the cell membrane context, which approximates *in vivo* conditions, and would facilitate screening for agonist and antagonist compounds in drug discovery research.

Acknowledgments

We thank Dr Juan Mulero (Department of Rheumatology, Hospital Puerta de Hierro, Madrid, Spain) for urine samples and C. Mark for editorial assistance. BV received a predoctoral fellowship from the Spanish Ministry of Health (FISS program). This work was supported in part by grants from the Spanish Ministry of Science and Innovation (SAF 2011-27370), the Comunidad de Madrid (Raphyme, S2010/MBD-2350), the FISS program (RD12/0009/0009) and the European Union (FP7-integrated project Masterswitch 223404).

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