Chapter 2

A New Enzyme-linked Sorbent Assay (ELSA) to Quantify Syncytiotrophoblast Extracellular Vesicles in Biological Fluids

Claudia Göhner, Maja Weber, Dionne S. Tannetta, Tanja Groten, Torsten Plösch, Marijke M. Faas, Sicco A. Scherjon, Ekkehard Schleußner, Udo R. Markert, Justine S. Fitzgerald

American Journal of Reproductive Immunology: volume 73, issue 6, 2015
A New Enzyme-linked Sorbent Assay (ELSA) to Quantify Syncytiotrophoblast Extracellular Vesicles in Biological Fluids

Abstract

Problem
The pregnancy-associated disease preeclampsia is related to the release of syncytiotrophoblast extracellular vesicles (STBEV) by the placenta. To improve functional research on STBEV, reliable and specific methods are needed to quantify them. However, only a few quantification methods are available and accepted, though imperfect. For this purpose, we aimed to provide an enzyme-linked sorbent assay (ELSA) to quantify STBEV in fluid samples based on their microvesicle characteristics and placental origin.

Method of Study
Ex vivo placenta perfusion provided standards and samples for the STBEV quantification. STBEV were captured by binding of extracellular phosphatidylserine to immobilized annexin V. The membranous human placental alkaline phosphatase on the STBEV surface catalyzed a colorimetric detection reaction.

Results and Conclusion
The described ELSA is a rapid and simple method to quantify STBEV in diverse liquid samples, such as blood or perfusion suspension. The reliability of the ELSA was proven by comparison with nanoparticle tracking analysis.
A New Enzyme-linked Sorbent Assay (ELSA) to Quantify Syncytiotrophoblast Extracellular Vesicles in Biological Fluids

Introduction

Extracellular vesicles (EV) play a crucial role in general cell–cell communication. Moreover, EV have been shown to be involved in the development of diverse diseases, such as tumor growth or preeclampsia.[1,2] Preeclampsia is one of the most lifethreatening disorders during pregnancy, even in the developed world. Preeclampsia is associated with endothelial dysfunction as well as the release of syncytiotrophoblast extracellular vesicles (STBEV) into the maternal circulation.[3] The STBEV can affect immune cells, regulate coagulation, and trigger inflammatory processes on the endothelium.[4] A better understanding of the formation and function of EV would improve the understanding of related diseases. Thus, it is necessary to have specific methods to detect and quantify diverse EV. EV can be further subdivided into groups of microvesicles (100 nm to 1 µm) and exosomes (30–100 nm).[5] In general, microvesicles are produced by damaged or renewing cells, such as the syncytiotrophoblast. During the formation of microvesicles, phosphatidylserines are translocated to the outer side of the microvesicle membrane.[6,7] Consequently, phosphatidylserines can be used as specific microvesicle markers. Exosomes are formed by externalization of intracellular multivesicular bodies.[5] Due to their mode of formation, exosomes are not expected to present phosphatidylserines on their surface. However, a low amount of exosomes does expose surface phosphatidylserines.[5] Hence, it has to be taken into account that also a certain amount of exosomes could be comeasured when using phosphatidylserines as a microvesicle marker. Additionally, EV represent the molecular pattern of the parental cells. Therefore, STBEV are enriched with placental proteins like the enzyme human placental alkaline phosphatase (hPLAP), which is a broadly accepted specific placenta marker also on the STBEV-surface.[8]

There are few methods currently accepted to detect and quantify STBEV. The three most common methods are flow cytometry,[9] nanoparticle tracking analysis (NTA),[10] and enzyme-linked immunosorbent assay (ELSA).[2] Flow cytometry is a common method to perform multiparametric analysis of cells and EV, although being limited by the particle size of the sample.[9,11] As reviewed by,[9] microvesicles constitute the lower size boarder for flow cytometric analysis, whereas exosomes cannot be analyzed due to their small size. But even the analysis of microvesicles is limited by high requirements on equipment and is more suitable for a characterization of the microvesicles but not for a quantification.[9,11] NTA was proven to be a suitable way of analyzing size and quantity of diverse particle-enriched suspensions. NTA measures and tracks light being scattered by particles to follow the Brownian motion of the particles in the sample, and calculates their size and concentration based on these measured variables. The laser beam can also excite fluorescent dyes or quantum dots to emit light, which can be captured by a camera. Hence, fluorescence-labeled antibodies could be used to specifically label EV like STBEV and track them by NTA. However, the method
A New Enzyme-linked Sorbent Assay (ELSA) to Quantify Syncytiotrophoblast Extracellular Vesicles in Biological Fluids

requires the dye to have a very high intensity and half-life. This restricts the applicability of fluorescence-labeled antibodies to specifically detect placenta-derived EV. To our knowledge, no marker of this nature is commercially available for NTA detection of STBEV. Additionally, the costs of purchase of the machine as well as antibodies have to be taken into account. The third widely accepted method to quantify STBEV in fluid samples is an ELSA described by the group of Redman and Sargent, which is based on an anti-hPLAP-antibody.[2] This antibody specifically binds to soluble hPLAP, and also to membranous hPLAP on the surface of the ST-BEV. Therefore, the antibody can be applied to the ELSA for catching STBEV in fluid samples. As hPLAP in fluid samples, such as blood or placenta perfusates, is not expressed exclusively on STBEV, but may also appear as soluble hPLAP, the ELSA does not discriminate between both forms. Consequently, a strict ultra-centrifugation protocol has to be followed to avoid contamination by soluble hPLAP.

Thus, research on STBEV is highly limited by the lack of specific and accepted quantification methods. For this reason, the present work aimed to develop a specific method to quantify STBEV taking into account their microvesicle character as well as their placental origin. Maternal single-sided ex vivo placenta perfusion was performed to produce an STBEV-enriched suspension, which was pre-quantified via flow cytometry. This suspension has been used for preparation of standard and reference samples for the newly developed enzyme-linked sorbent assay (ELSA).
A New Enzyme-linked Sorbent Assay (ELSA) to Quantify Syncytiotrophoblast Extracellular Vesicles in Biological Fluids

Materials and methods

Sample Acquisition and Preparation

Samples were acquired by blood donation or *ex vivo* placenta perfusion after informed consent. Blood samples were collected in 5 mL Coagulation Monovettes (Sarstedt AG & Co., Nümbrecht, Germany). The blood was centrifuged for 5 min at 65509 g, and plasma was collected as a supernatant. Placenta perfusion samples were collected by maternal single-sided or double-sided *ex vivo* placenta perfusion in a conventional perfusion cabinet (test samples) or by maternal single-sided *ex vivo* placenta perfusion on a slanted plain with spikes (production of standard suspension for the ELSA). An intact cotyledon was chosen macroscopically and fixed on the perfusion device. The maternal tissue was connected to the system by carefully introducing four buttoned cannulae into the decidua. Prior to the main perfusion, the maternal side of the tissue was pre-rinsed with perfusion suspension for 20 min in an open setup, discarding the perfused suspension containing maternal blood residues. During the main perfusion, the perfused suspension was returned to the suspension reservoir to establish a circulating setup. Perusions have been performed for up to 120 or 360 min. Samples were taken at the end of the perusions to be quantified with the described ELSA. The whole suspension of the perfusion of another placenta was collected after 300 min of perfusion, to gather and concentrate the included STBEV via ultra-centrifugation and to use them later on as quantification standards for the described ELSA (see flow cytometric pre-quantification of STBEV). The perfusion suspension was centrifuged for 5 min at 6550x g to pellet cells and large cell debris.

Perfusion suspension was prepared as follows: 1 L NCTC-135 (with L-glutamine, without phenol red, without vitamin B12, without sodium hydrogen carbonate; AppliChem GmbH, Darmstadt, Germany) was supplemented with 0.5 L Earl's buffer, 60 g bovine serum albumin (Bovine Albumin, Fraction V, MP Biomedicals LLC, Solon, OH, USA), 15 g Dextran (FP 40; Serva Electrophoresis GmbH, Heidelberg, Germany), 2 g D-Glucose (water-free; Merck KGaA, Darmstadt, Germany), 0.38 g Amoxicillin (Sigma-Aldrich Chemie GmbH, Hamburg, Germany), and 0.75 mL Heparin (Heparin-Natrium-25.000, 25,000 i.E./5 mL; Ratiopharm GmbH, Ulm, Germany). Earl's buffer consisted of 111.225 mM NaCl (Carl Roth GmbH + Co.KG, Karlsruhe, Germany), 5.365 mM KCl (Merck KGaA), 1.015 mM NaH$_2$PO$_4$ (Merck KGaA), 26.187 mM NaHCO$_3$ (Merck KGaA), 811.425 µM MgSO$_4$•7H$_2$O (Merck KGaA), 1.36 mM CaCl$_2$•2H$_2$O (Merck KGaA). The pH of the perfusion suspension was adjusted to pH = 7.4 with NaOH (Carl Roth GmbH + Co.KG, Karlsruhe, Germany) prior to filtration using a 0.8/0.2 µm filter (AcroPak™ 200 Capsules with Supor Membrane; Pall Corporation, Port Washington, NY, USA) and freezing to 20°C until usage.

Plasma and perfused perfusion suspension for STBEV standard production were processed via ultra-centrifugation in an Optima XL-100 ultra-centrifuge (Beck
man Coulter GmbH, Krefeld, Germany) using ultra-centrifugation tubes (Thinwall, Ultra-ClearTM, 14 mL, 14 x 95 mm; Beckman Coulter GmbH) for 45 min at 100,000 x g at 4°C. The supernatant was discarded carefully, and tubes were kept inverted for 30–60 s, to remove as much supernatant as possible. Pellets were resuspended in 500 µL (blood samples) or 6800 µL (STBEV standard) 1% (w/v) BSA in 0.05% (v/v) Tween 20 in PBS (Polyoxyethylene(20)-sorbitan-monolaurate, Dulbecco’s phosphate buffered saline; both Sigma-Aldrich Chemie GmbH).

**Flow Cytometric Pre-quantification of STBEV**

Pre-quantification of ultra-centrifuged STBEV standard was performed by flow cytometry using a FACS Calibur (BD Biosciences, Heidelberg, Germany) with supplementation of FITC-fluorescent BD Flow CountTM Fluorospheres (BD Biosciences) as a quantification marker. The placental origin of the analyzed microvesicles was proven by antibody-binding (mouse-anti-human placental alkaline phosphatase antibody, conjugated with lynx rapid RPECy5.5 antibody conjugation kit; both AbD Serotec, Raleigh, NC, USA). For quantification of the STBEV, the marker for hPLAP-positively stained extracellular vesicles was adjusted to a sample of unstained STBEV as a negative control for auto-fluorescence. Based on the known concentration of the flow count fluorospheres, the concentration of the STBEV was calculated and further adjusted to 6000 STBEV/mL.

**Anti-STBEV-Enzyme-linked Sorbent Assay**

Annexin V was immobilized in wells of microtiter plates (Immuno 96 MicroWellTM Solid Plates, MaxiSorp, flat bottom, Nunc; Thermo Fisher Scientific Inc., Waltham, MA, USA) by incubation of 200 µL/well 3 µg/mL Annexin V (Sigma-Aldrich Chemie Gmbh) in sodium carbonate coating buffer (pH = 9.6; 15 mM Na₂CO₃, 28.5 mM NaHCO₃, both Merck KGaA) for 20 h at 4°C. Unbound Annexin V was removed by washing once with 380 µL Tris-buffered saline (TBS; pH = 7.5; 50 mM Tris-HCl; Sigma-Aldrich Chemie Gmbh, 150 mM NaCl, Carl Roth Gmbh + Co.KG). Wells were blocked with 380 µL 4% milk (powdered milk, blotting grade, low fat, Carl Roth Gmbh + Co.KG) in TBS for 2 h at room temperature and washed three times with 380 µL/well 0.05% Tween 20 in TBS (Polyoxyethylene(20)-sorbitan-monolaurate; Sigma-Aldrich Chemie Gmbh). A 100 µL sample or standard was applied to the respective wells and incubated for 1 h at room temperature. The top-standard (6000 STBEV/mL) was generated as described under ‘sample acquisition and preparation’ and pre-quantified by flow cytometry as the number of STBEV per mL (STBEV/mL). It was applied in serial dilution of eight standard samples of 6000 STBEV/mL, 3000 STBEV/mL, 1500 STBEV/mL, 750 STBEV/mL, 375 STBEV/mL, 187.6 STBEV/mL, 93.75 STBEV/mL, or a blank of 0 STBEV/mL. Wells were washed two times with 400 µL/well 0.05% TBS-T and afterward two times with 400
µL/well TBS. Samples were quantified by a colorimetric detection reaction using an ELSA amplification system (Invitrogen Corporation, Carlsbad, CA, USA). This system contains the alkaline phosphatase substrate nicotinamide adenine dinucleotide phosphate (NADPH), which is catalyzed by the hPLAP. For this detection reaction, 50 µL/well substrate suspension was incubated at 25°C for 1 h and afterward 50 µL/well amplifier suspension was added and incubated 1 h at 25°C. Color development was measured at 495 nm using a SPECTROstar Omega UV/Vis absorption spectrometer (BMG Labtech GmbH, Ortenberg, Germany).

**Nanoparticle Tracking Analysis**

Nanoparticle tracking analysis (NTA) was performed on perfusion samples, which were also analyzed using the ELSA described in this study. For the comparison of NTA and ELSA, the perfusion samples were centrifuged at 10,000 x g for 10 min at 4°C to remove pelleted cells and cell debris. The supernatant was further centrifuged at 18,900 x g for 30 min at 4°C to pellet microvesicles. The supernatant was discarded, and the microvesicle pellet was resuspended in 1% (w/v) BSA in 0.05% (v/v) Tween 20 in PBS. For optimal resolution, all samples were analyzed in 1:1000 dilution in PBS using an LM14 NanoSight (NanoSight Malvern Instruments Company, Malvern, Worcestershire, UK) equipped with a violet laser at 405 nm for 60 s.

**Statistical Analysis**

SPSS 20 (IBM Deutschland GmbH, Ehningen, Germany) was used to perform statistical analysis applying the Mann–Whitney test for comparison of sample medians of independent samples.
A New Enzyme-linked Sorbent Assay (ELSA) to Quantify Syncytiotrophoblast Extracellular Vesicles in Biological Fluids

Results and discussion

This work aimed to develop a specific method to detect and quantify STBEV in fluid samples based on their microvesicle character (marker: phosphatidylserine) and their placental origin (marker: hPLAP). For this approach, an STBEV-enriched suspension was produced by 5-h maternal-sided ex vivo perfusion of a human term placenta. The concentration of STBEV was analyzed by an established method (flow cytometry, Fig. 2), and suspensions of defined STBEV concentrations were produced to be used as standards for the new ELSA method (Fig. 3a). The validity of the described ELSA was then tested by measuring and comparing blood samples from healthy pregnant women and non-pregnant female blood donors (Fig. 3b). The microvesicle surface marker phosphatidylserine was used to capture STBEV by binding them to immobilized Annexin V (see functional scheme of the test in Fig. 1). In the detection step, the phosphatase activity of the membranous hPLAP, which is located on the surface of the STBEV, was utilized to perform a colorimetric quantification reaction. In this reaction, the alkaline phosphatase substrate (NADPH) is cleaved by hPLAP which initiates a cyclic enzyme reaction resulting in the formation of the detectable Formazan molecule. Single-sided ex vivo placenta perfusion provided an enriched STBEV standard suspension, which was pre-quantified via flow cytometry (Fig. 2). For this pre-quantification, the placenta-derived STBEV were stained with an hPLAP specific antibody and mixed with commercially available quantification beads of known concentration. Both the quantification beads and the hPLAP-positive STBEV were tracked by flow cytometry. Comparison of the amount of both fractions (Fig. 2) allowed the recalculation of the STBEV concentration, which was further adjusted to 6000 STBEV/mL. This top-standard served as the starting concentration of the standard samples in all of our tests. It was serially diluted to give standard samples of 6000 STBEV/mL, 3000 STBEV/mL, 1500 STBEV/mL, 750 STBEV/mL, 375 STBEV/mL, 187.6 STBEV/mL, 93.75 STBEV/mL, or a blank of 0 STBEV/mL. In general, common flow cytometry still suffers from accuracy and sensitivity problems in regard to microvesicle quantification.[9,11] Because of this, the STBEV concentration achieved by flow cytometry has to be regarded as relative numbers only, still being sufficient to give a relative STBEV concentration for this standard.

These standard samples were used to plot a curve showing the typical distribution of standards applied to ELSA or comparable analysis (Fig. 3a). The standard curve started with a nearly linear slope in the range of the lower concentrations, then increased to a plateau at the higher concentrations.
A New Enzyme-linked Sorbent Assay (ELSA) to Quantify Syncytiotrophoblast Extracellular Vesicles in Biological Fluids

Fig. 1 Functional principle of the anti-STBEV-ELSA.
In the described enzyme-linked sorbent assay (ELSA), Annexin V is immobilized on the surface of microtiter plates. It works as a capturing molecule by binding phosphatidylserine on the surface of the syncytiotrophoblast extracellular vesicles (STBEV). The membranous human placental alkaline phosphatase (hPLAP) on the STBEV catalyzes a colorimetric detection reaction.

Fig. 2 Flow cytometric pre-quantification of a STBEV sample.
Four STBEV samples were independently isolated from the perfusion suspension of one maternal single-sided ex vivo placenta perfusion. STBEV were stained with Cy5.5 labeled anti-hPLAP antibodies. The histograms show the staining intensity (x-axis) and particle count (y-axis) of hPLAP-positive vesicles (a; overlay of four separate analyses, left curve shows unstained STBEV) and of FITC-fluorescent flow count beads (b; overlay of four separate analyses), which were used for calculation of the STBEV concentration. Based on these results, the STBEV concentration was adjusted for its application as the top-standard of the standard row for the described ELSA.

Fig. 3 Exemplary measurements with the STBEV-ELSA.
(a) Measurement of the standard series, which was isolated from the perfusion suspension of a maternal single-sided ex vivo placenta perfusion. (b) STBEV quantification in control blood of a non-pregnant woman (negative control) and six pregnant women at term. The bars show mean values of duplicates for the samples of pregnant women at term. The non-pregnant control was measured 12 times in total (measurement of duplicates on six different plates). Error bars indicate the standard error of the mean. *P < 0.05 (Mann–Whitney test).
Test measurements were performed with the plasma of six pregnant donors and a non-pregnant donor as negative control (Fig. 3b). All pregnant women could be distinguished significantly (P < 0.05) from the negative control. The substrate for the detection reaction is a potential target for different types of alkaline phosphatases. Furthermore, plasma samples include microvesicles from multiple cell types [7] that are also phosphatidylserine positive and will compete with STBEV for binding to the Annexin V. However, only the samples from pregnant women featured a positive signal which underlines the specificity of the test. Even samples from ex vivo placenta perfusion (Fig. 4) were successfully analyzed with the described method. As expected, samples from ex vivo placenta perfusion turned out to be highly concentrated, so dilution of these samples prior to the measurement is highly recommended in this assay.

To confirm the reliability of this ELSA, results acquired by ELSA were compared to those acquired by NTA analysis of the same samples. The results obtained by the anti-STBEV-ELSA could be verified by NTA (Fig. 4). The differences observed in the results of these methods may be due to the differences in the test systems. It is known that a very high proportion of microvesicles in blood originates from platelets and endothelial cells.[7] Thus, it also can be expected that placenta perfusates are not only enriched with placental extracellular vesicles, but also with extracellular vesicles derived from endothelial or other cells residing in the placenta (e.g., decidual cells) or residual circulatory cells (e.g., blood cells). In contrast to the NTA, the ELSA is more specific and measures only the placental vesicle fractions. This explains the discrepancies in the results of the two tests.

The test described here is comparable to a conventional ELSA, but works without the immune-active component. Therefore, our assay represents an enzyme-enhanced sorbent assay (ELSA). The exclusion of the immune-active component can reduce the susceptibility for disturbances of the test because it reduces the working steps in the assay.

There are some limitations to this method. One may argue that also other EVs, but not exclusively STBEVs, may have an phosphatase activity. As shown in Fig. 3, in non-pregnant plasma, the detected EV concentration is comparatively low, which indicates low activity of EVs other than STBEVs. The standard suspension which was applied here was produced by perfusion of only one placenta, but the distribution of hPLAP and PS on the surface of STBEV might vary between samples from diverse patients. Thus, it may be advisable to collect perfusion material of more placentae and to mix the suspensions to produce a more representative standard suspension. For this reason, the ascertained values should be regarded only as relative values and not as total vesicle counts. Also the pre-quantification of the standard via flow cytometry gives only relative STBEV quantities due to accuracy and sensitivity problems of the method regarding microvesicle analysis.[11] Furthermore, it is advisable to prepare the standard and samples, which shall be analyzed, following the same preparation protocol. If both specimens are isolat
A New Enzyme-linked Sorbent Assay (ELSA) to Quantify Syncytiotrophoblast Extracellular Vesicles in Biological Fluids

ed with the same centrifugation speeds, the quantification of the samples based on the standard will be even more reliable. Nevertheless, the test presented here revealed its usefulness for the purpose of quantifying STBEV, especially in lower ST-BEV concentration ranges. This test will also be very beneficial in functional STBEV analysis by providing a rapid, simple, and cost-efficient quantification method. The ELSA will facilitate studying STBEV and thereby also support preeclampsia research in general.

![Graph showing comparison of ELSA and NTA data](image)

**Fig. 4** Comparison of the STBEV quantification achieved via ELSA and the total microvesicle quantification achieved via NTA. Samples from *ex vivo* placenta perfusion were analyzed with ELSA and NTA. As the ELSA is based on measurement of the placenta-specific hPLAP activity on captured extracellular vesicles, it provides STBEV concentrations (x-axis). In contrast, the NTA (y-axis) measures all microvesicles, not only the ones derived from syncytiotrophoblast. The linear fit line of the comparison of the ELSA and the NTA values and the 95% confidence interval are indicated by the line and the two ambient curves.
A New Enzyme-linked Sorbent Assay (ELSA) to Quantify Syncytiotrophoblast Extracellular Vesicles in Biological Fluids

Acknowledgements

We would like to thank S. Murugappan and Dr. W.L.J. Hinrichs from the Department of Pharmaceutical Technology and Biopharmacy, University of Groningen for the provision of the NanoSight and their support. Furthermore, we would like to thank the German Research Foundation (Deutsche Forschungsgemeinschaft grant number FI1635/1-1) for financing parts of this project. C. Göhner has been partly financed by the Thuringian Ministry for Education, Science and Arts and is financed by a fellowship of the Abel Tasman Talent Program of the University of Groningen.

References
