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Proteomic Analysis of Plasma Membrane Proteins in an *In Vitro* Blood-Brain Barrier Model

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1. Introduction

Although several cell types have important regulatory roles in the induction and maintenance of a properly functioning blood-brain barrier (BBB) [Abbott et al., 2006; Armulik et al., 2010], it is clear that brain capillary endothelial cells (BCECs) constitute the barrier *per se* in histological terms. In the central nervous system's blood vessels, BCECs are closely interconnected by tight junctions and form a continuous, circular tube lining the basal membrane in which pericytes are embedded. The basal membrane surface is itself covered by a continuous sleeve of astrocyte endfeet (Fig. 1). The BBB is one of the most important physiological structures in the maintenance of brain homeostasis.

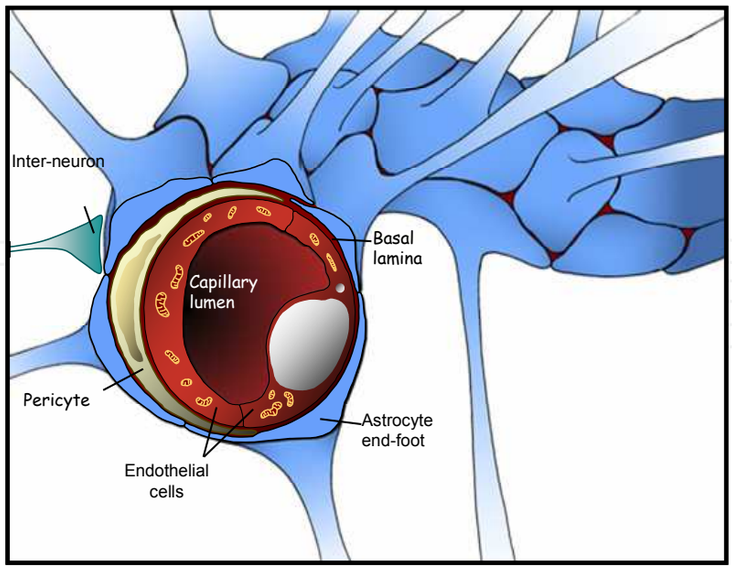


Fig. 1. Brain capillary endothelial cells constitute the core of the BBB. The endothelial cells are surrounded by a tubular sheath of astrocyte end-feet. Pericytes are embedded in the basal lamina (between the endothelium and the astrocyte end-feet). Reprinted from [Pottiez et al., 2009a], with permission from Elsevier).

The BBB is a dynamic, regulatory interface that controls the molecular and cellular exchanges between the bloodstream and the brain compartment [Abbott et al., 2010]. The BCECs' barrier function depends on the acquisition and maintenance of characteristic features (referred to as the "BBB phenotype"), such as the absence of endothelial fenestrae, decrease in the number of endocytosis vesicles, the reinforcement of tight junctions and changes in the expression pattern of certain proteins. Overall, these physiological characteristics condition cell polarisation and permeation, transendothelial electrical resistance and a number of metabolic, receptor-based and transport functions. The latter mainly rely on the properties of the BCECs' plasma membrane (PM). Relevant information regarding the lipid composition of the whole cell and of the apical and basolateral PMs has been reported [Tewes & Galla, 2001]. The latter authors demonstrated that each PM shows a unique lipid composition; the apical PM is enriched in phosphatidylcholine, whereas the basolateral PM is enriched in sphingomyelin and glucosylceramide. It has also been observed that co-culture with glioma C6 cells is able to induce a more *in vivo*-like fatty acid pattern in BCEC-based BBB models, although the intensity of these changes did not reach *in vivo* levels [Kramer et al., 2002]. Given the vital physiological functions performed by membrane lipids this aspect merits further investigation. In contrast, the PM's protein moieties have been extensively studied. The protein composition of the PM is determined by the balance between membrane protein sorting, internalization and recycling. Briefly, biosynthesized PM proteins are translocated from the endoplasmic reticulum to the Golgi apparatus, where they undergo posttranslational modifications. Proteins are then sorted to the apical or basal membrane of polarized cells. Some PM proteins are subsequently internalised and sequestrated in lysosomes and then degraded or recycled to the cell surface; endocytic adaptor proteins may have a pivotal role in this process [Howes et al., 2010; Kelly & Owen, 2011; O'Bryan, 2010; Reider & Wendland, 2011]. Plasma membrane proteins are involved in many BBB functions, including (i) cell-extracellular matrix interactions, (ii) the cell-cell junctions (especially tight junctions) that impede paracellular transport and polarise the cells, (iii) the molecular transport systems that regulate the exchange of nutrients and enable the passage of signalling molecules across the BBB and (iv) cell signalling via the expression of PM receptors [Leth-Larsen et al., 2010].

1.1 Plasma membrane proteins

Integral PM proteins are polypeptides whose particular physicochemical properties enable insertion into the lipid bilayer and interaction with both the extracellular environment and/or the intracellular compartment. In all transmembrane polypeptides examined to date, the membrane-spanning domains are α-helices or multiple β-strands. Most integral proteins span the entire phospholipid bilayer with one or more membrane domains. The domains may have as few as four amino acid residues or as many as several hundred. The integral insertion of proteins into the PM means that the side chains of buried amino acids have Van der Waals interactions with the fatty acyl chains and shield the peptide bond's polar carbonyl and imino groups. Indeed, integral proteins containing membrane-spanning α-helical domains are composed mainly of uncharged hydrophobic amino acids. These properties probably make spanning regions more resistant to proteolysis by the trypsin enzyme used in most proteomics protocols. However, hydrophobic helices are often flanked by positively charged amino acids (i.e. lysine and arginine) thought to stabilize the helix by neutralizing the helix's dipole moment and interacting with negatively charged phospholipid head groups. The second class of transmembrane proteins displays a radically

different structure in which several β strands form a barrel-shaped structure with a central pore. These strands contain predominantly polar amino acids and no long hydrophobic segments. Nevertheless, the outward-facing side groups on each of the β-strands are hydrophobic and interact with the membrane lipids' fatty acyl groups, whereas the side chains facing the inside are mainly hydrophilic [Lodish et al., 2000]. Interestingly, several posttranslational modifications that do not occur in the cytosol (such as disulphide bond formation and glycosylation) enhance the stability of PM or secreted proteins prior to their exposure to the extracellular milieu. Overall, these particularities can dramatically decrease the PM proteins' sensitivity to trypsin digestion. Newly synthesized proteins can also be targeted to the PM via the covalent attachment of a lipid anchor. Indeed, some proteins bind to the PM's cytosolic surface via a covalently attached fatty acid (e.g. palmitate or myristate) or isoprene group (e.g. a farnesyl or geranyl group, whereas proteins from the PM's outer leaflet are tethered some distance out from the surface by a glycosylphosphatidylinositol (GPI) anchor [Paulick & Bertozzi, 2008].

1.2 Proteomics of the plasma membrane

Traditionally, mass spectrometry (MS)-based identification methods, chromatography and common cell biology techniques can be combined to form powerful tools for the proteomic mapping of PM proteins. Although major technical progress in MS continues to be made [Savas et al., 2011], the extraction, purification, separation and analysis of PM proteins remains problematic due to the latter's low abundance, poor solubility in aqueous solution and micro-heterogeneity [Santoni et al., 2000]. It is now clear that the development of complementary approaches is a prerequisite for the comprehensive analysis of PM proteins, including protein isolation and enrichment strategies that best preserve certain functional states and minimize the loss of transient and/or peripherally associated non-transmembrane proteins [Helbig et al., 2010], (Fig. 2). Polarized cells are present in many different organs and so their PMs have heterogeneous morphological and functional domains. Conventionally, PM proteomics can be performed with either cells cultured in suspension or adherent cells. Fig. 2 illustrates the importance of choosing the right method for the isolation of PMs and membrane sub- and microdomains and summarizes the different methods used in PM proteome analysis. The analysis can be divided into three experimental steps, all of which are challenging: (i) PM protein enrichment, (ii) separation and quantification and (iii) identification [Sprenger & Jensen, 2010].

1.3 Plasma membrane protein enrichment

Plasma membrane protein enrichment can be achieved either directly by extraction of membrane proteins or indirectly by pre-purification of the PM itself (or part of the PM) prior to proteome analysis. In view of the PM proteins' physicochemical properties, it is tempting to use of amphoteric agents (such as detergents) for enrichment. However, aqueous phase proteins will also be more soluble and may not necessarily be separated from the PM proteins. In contrast, the enrichment of membrane proteins based on two-phase partitioning (i.e. an aqueous phase and an organic phase) has been widely used and has proved its utility. The PM proteins can then be separated from aqueous proteins, due to the difference in hydrophobicity. Another way of directly studying the PM protein content involves its evaluation through its peptide fingerprinting. To this end, cell surface proteins undergo a "proteolytic shaving" procedure. The resulting peptides are purified, separated and then identified by liquid chromatography – tandem MS (LC-MS/MS). Although the proteolytic

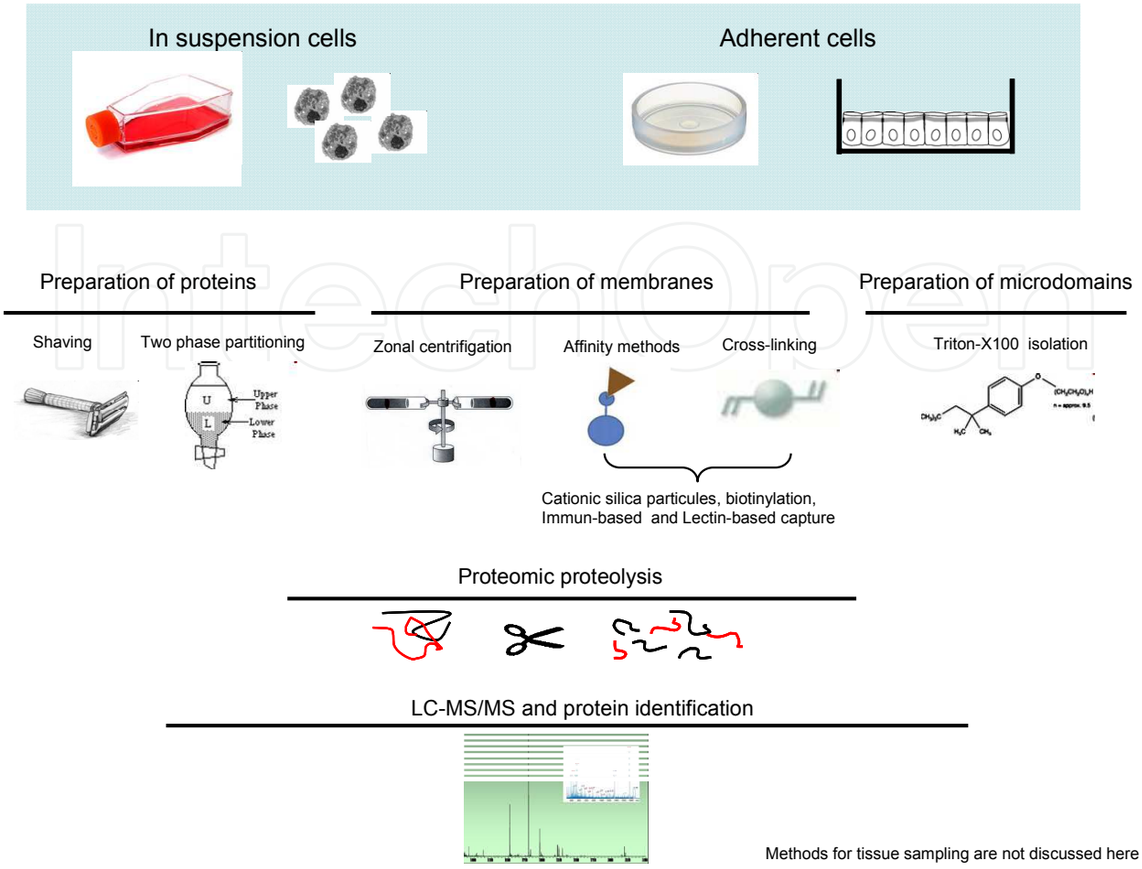


Fig. 2. A schematic drawing of complementary strategies for the comprehensive proteomic analysis of PM proteins. Approaches which best preserve certain functional states and minimize the loss of transient and/or peripherally associated non-transmembrane proteins are preferable [Helbig et al., 2010].

shaving offers many advantages in theory (because surface-exposed peptides are more water-soluble than their intrabilayer counterparts), the main drawback of this approach relates to its tendency to trigger cell lysis and thus the significant contamination of surface-exposed membrane peptides with cytosol-derived peptides. The glycosylation of PM proteins also prevents proteases from accessing the polypeptide moiety [Cordwell & Thingholm, 2010].

In view of the PM's lipid composition, membrane pre-purification and separation from soluble proteins is conventionally performed by zone centrifugation with a density gradient. Most of the PM-associated (peripheral) proteins are recovered with the integral PM protein fraction - which can constitute a drawback or an advantage. To overcome this problem, additional high-salt, high-pH washing steps can be used to form easily separable membrane sheets that lack peripheral proteins. Furthermore, plasma, mitochondrial and endoplasmic reticulum membranes all have similar densities and so membrane fractions prepared by ultracentrifugation often contain a mixture of the three [Chen et al., 2006].

In fact, the most frequently used methods for the enrichment of PMs are those based on affinity chromatography, cationic colloidal silica particles, cell biotinylation or a tissue-specific polyclonal antiserum [Agarwal & Shusta, 2009; Shusta et al., 2002]. The cell surface membrane proteins may be covalently labelled (e.g. in biotinylation) or not (e.g. with cationic silica and

antibodies). The label serves as an anchor for silica bead- or magnetic bead-based separation. Loosely PM-associated proteins can always be removed by high-salt/high-pH washing [Josic & Clifton, 2007]. Similarly, the generally glycosylated PM proteins can be affinity-purified with lectin-based chromatography media [Cordwell & Thingholm, 2010].

At a higher organizational level, the topological mapping of plasma protein complexes requires the use of chemical or photo- crosslinking prior to unavoidable cell lysis, to keep them in a close-to-native state. Crosslinkers are often homo- or hetero-bifunctional agents absorbed on the cell surface [Back et al., 2003]; after chemical or photonic triggering, polymerization leads to the formation of a network that entraps PM proteins [Cordwell & Thingholm, 2010]. The proteomic needs in this field are increasing. A recent review described a new strategy and recent progress in the field of chemical cross-linking coupled to MS [Tang & Bruce, 2010].

Last but not least, membrane enrichment can be achieved by purifying microdomain components (e.g. caveolae, rafts and tetraspannin domains) enriched in the cholesterol and sphingolipids that give these cell surface structures their concave shape. This method exploits the poor solubility of membrane microstructure lipids vis-à-vis certain detergents [Zheng & Foster, 2009] (hence the term "detergent-resistant membranes"). Indeed, cholesterol- and sphingolipid-enriched membranes are insoluble in cold, non-ionic detergents (Triton X-family, NP-40, Tween, etc.) and their low buoyancy makes them amenable to purification by density gradient centrifugation. However, the main drawback of this method relates to the detergents' ability to break up protein-protein interactions. It is important to note that membrane surface labelling and affinity purification can also be used to isolate this particular protein population.

1.4 The state of the art in BBB PM proteomics

Proteomics studies of the PM in human umbilical vein endothelial cells (HUVECs) [Karsan et al., 2005; Sprenger et al., 2004] and aortic endothelial cells [Dauly et al., 2006] have been initiated in the last decade. However, the phenotypic characteristics of these types of endothelial cell (EC) differ from those of BCECs. Hence, the use of non-brain ECs in *in vitro* BBB models is subject to debate [Cecchelli et al., 2007; Prieto et al., 2004].

To date, the very few studies to have focused on BBB EC proteomics can be divided into two distinct categories. The first category is outside the scope of the present review but is mentioned here for the sake of completeness. It concerns mid- to high-throughput proteomics initiated with *in vivo* or *in vitro* cells and that seek to answer a well-defined question (e.g. to identify the broadest possible protein expression profile in the brain microvascular endothelium [Haseloff et al., 2003; Lu Q. et al., 2008; Pottiez et al., 2010]; investigate cerebral ischemia [Haqqani et al., 2007; Haqqani et al., 2005; Haseloff et al., 2006] or evaluate a differential solubility approach for the characterization of EC proteins [Lu L. et al., 2007; Murugesan et al., 2011; Pottiez et al., 2009b]. Nevertheless, some PM proteins have been identified in the course of these high-throughput studies. The second category of truly BBB-focused PM proteomic studies arose in 2008 with the work by Terasaki et al.. These researchers used the elegant principle of isotopic dilution (see [Brun et al., 2009] for a review) to achieve the absolute quantification of 34 proteins known to be of significant interest. This list of membrane transporter and receptor proteins has recently been expanded to 114, following a human brain microvessel study [Uchida et al., 2011]. In addition to studies focusing on known BBB PM proteins, an indirect method based on a multiplex expression cloning strategy after fluorescence activated cell sorting with a tissue-specific

polyclonal antiserum has been developed [Agarwal & Shusta, 2009; Shusta et al., 2002]. The latter researchers identified a total of 30 BBB membrane proteins at the transcript level. Even though the expression of the corresponding gene products remains to be confirmed, these results constitute a considerable advance. Given that most PM proteins are glycosylated, the leverage of this post-translational modification for addressing PM proteins is tempting. However, large-scale glycoproteomics studies have only recently been reported. Indeed, a methodology based on hydrazine capture of membrane and secreted glycoproteins [Haqqani et al., 2011] revealed an enrichment in glycoprotein content (over 90%) and led to the identification of 23 new glycoproteins (i.e. not referenced as such in the Uniprot database). The full study results will doubtless be published soon.

1.5 Cell surface biotinylation

Chemical labelling of cell surface proteins is a novel methodology for the isolation of new target proteins. One of the major advantages of this approach is that the labelling reagent's chemical properties can be chosen to suit the biological structures that are being targeted. Cell surface biotinylation is a selective technology for the capture of PM proteins. This technology comprises several steps: (i) the selective labelling of proteins with a biotinylating reagent, (ii) the capture of biotinylated proteins with avidin-coated magnetic beads, resins etc. and (iii) elution and digestion (or, for increased specificity, digestion and elution) of the biotinylated proteins [Scheurer et al., 2005].

Using our *in vitro* BBB co-culture model [Dehouck et al., 1990], we have initiated a differential PM proteome approach that selects, separates and identifies BCEC cell surface proteins that are expressed differently in bovine BCECs with limited BBB functions versus those with re-induced BBB functions. This method is based on biotinylation of bovine BCECs' cell surface proteins with the reagent sulfosuccinimidyl-2-[biotinamido]ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin), in which biotin is coupled to a reactive ester group. The NHS group undergoes a nucleophilic substitution reaction with the primary amines of protein amino acids (mainly lysine residues, depending on the local pH). Due to the low dissociation constant for biotin and streptavidin, the use of a cleavable spacer arm containing a disulphide bond facilitates the release of biotinylated proteins after capture on immobilized streptavidin [Elia, 2008]. Moreover, the sulfo-NHS-ester derivatives of biotin are preferable for use in PM labelling because they are more soluble in water than NHS-esters alone. This enables reactions to be performed in the absence of polar aprotic solvents and membrane permeabilizing reagents like dimethylsulfoxyde and dimethylformamide. Furthermore, the sulfo-NHS-esters are membrane-impermeable reagents, which reduces interference from cytosolic components [Daniels & Amara, 1998; Elia, 2008]. After biotinylation and hypotonic cell lysis, biotin-labelled proteins can be captured on streptavidin-coated magnetic beads and on-bead digested by trypsin. The eluted peptides are separated with nano-liquid chromatography (nano-LC) coupled to a MALDI-TOF/TOF mass spectrometer. Proteins are then identified on the basis of the MS-fragmented peptide spectra via a protein-database search with Mascot software (Matrix Science Ltd, London, UK).

2. Materials and methods

2.1 Cell culture

Bovine BCECs were isolated and characterized as described previously [Meresse et al., 1989]. Petri dishes (diameter: 100 mm) were coated with an in-house preparation of rat tail

collagen (2 mg/mL) in ten-fold concentrated Dulbecco's Modified Eagle's Medium (DMEM) from GIBCO (Invitrogen Corporation, Carlsbad, CA, USA) and 0.4 M NaOH. The BCECs (4 x 10^5 cells/mL) were seeded and cultured in DMEM supplemented with 10% (v/v) heat-inactivated foetal calf serum, 10% (v/v) heat-inactivated horse serum (Hyclone Laboratories, Logan, UT, USA), 2 mM glutamine, 50 mg/mL gentamicin (Biochrome Ltd, Cambridge, UK) and 1 ng/mL basic fibroblast growth factor (GIBCO). The culture medium was refreshed every 2 days until confluence (after around 6 days, typically). Co-cultures were set in Transwell™ cell culture inserts (diameter: 100 mm; pore size: 0.4 mm; Corning Inc., New York, NY, USA) coated on the upper side with rat tail collagen. Endothelial cells were then seeded onto the inserts and transferred to a 100 mm Petri dish containing glial cells prepared according to Booher and Sensenbrenner [Booher & Sensenbrenner, 1972]. After 12 days of co-culture (in the same medium as mentioned above), the re-induction of BBB properties in the BCECs was checked by measuring the paracellular permeability coefficient of Lucifer Yellow carbohydrazide (Pe^{LY}) and by immunostaining the main tight junction proteins (occludin and claudin-5) and the associated intracellular scaffolding protein zona occludens 1 (ZO-1). Endothelial cell biotinylation and harvesting were performed after 12 days of co-culture.

2.2 Cell surface biotinylation and cell harvesting

Bovine BCEC biotinylation was performed by slightly modifying the previously reported method [Zhao et al., 2004]. Endothelial cells were washed three times with prewarmed (37°C) calcium- and magnesium-free PBS (CMF-PBS, pH 7.4) and gently shaken for 15 min at 37°C in CMF-PBS supplemented with 3 mg EZ-link sulfo-NHS-SS-biotin (Thermo Scientific, Cergy Pontoise, France) per Petri dish. The labelling reaction was quenched by adding 1 mL of 40 mM glycine in CMF-PBS, pH 8.0. Excess quenching buffer was removed by washing the cells twice in CMF-PBS.

The cells were harvested by adding collagenase type XI (*Clostridium histolyticum*, Sigma, Lyon, France) as described previously [Pottiez et al., 2009b]. Briefly, bovine BCECs were incubated for 15 min with 1.5 mL of a 0.1% w/v collagenase solution. The cell suspension was harvested, washed three times in PBS and pelleted at 500 x *g* for 5 min at 4°C. The cell pellets were stored at -80°C until protein extraction.

2.3 Preparation of biotinylated cell surface proteins

Bovine BCEC pellets were lysed with 800 µL of ice-cold hypotonic buffer [10 Mm HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, protease inhibitor cocktail) [Zhao et al., 2004] and incubated on ice for 30 min. The cells were lysed by dounce homogenization (50 passes) and then sonicated two times (30 W, 20 s). Unbroken cells and nuclei were pelleted from the cell homogenate by centrifugation at 1,000 x *g* for 10 min at 4 °C. Aliquots of supernatants and entire pellets were stored at -20°C prior to dot blot biotinylation control.

The KCl concentration in the supernatants was adjusted to 150 mM. An aliquot (300 µL) of streptavidin magnetic beads (10 mg beads/mL, prewashed four times with hypotonic buffer) was added to supernatants. The supernatant/bead suspensions were rotated at room temperature (RT) for 90 min and then pelleted using a magnetic plate. To obtain the biotinylated protein fraction, the resulting preparations were washed three times with 500 µL of ice-cold 1 M KCl for 15 min, three times again with 500 µL of ice-cold 0.1 M Na_2CO_3, pH 11.5 and lastly once with ice-cold hypotonic buffer for 10 min. The trypsin digestion was performed directly on the beads.

2.4 On-bead proteolysis and isolation of tryptic peptides

The on-bead proteolysis of biotinylated protein fractions was carried out overnight at 37°C in 400 µL of a proteolysis buffer containing 40 mM NH_4CO_3 (pH 8.0), 0.5 mM $CaCl_2$ and 12.5 ng/µL trypsin (Promega, Charbonnières-les-Bains, France). The enzyme reaction was stopped by heat denaturation at 100°C for 5 min. The magnetic beads were pelleted using a magnetic plate and the tryptic digest peptides were transferred into a clean microtube.

The peptides attached to the streptavidin-coupled beads were eluted from beads by means of a reduction reaction for 15 min at 60°C with 100 µL of 40 mM NH_4CO_3 (pH 8.0) containing 200 mM dithiothreitol (to disrupt the disulphide bond in the sulfo-NHS-SS-biotin). The eluate was pooled and tryptic peptides were concentrated under vacuum and immediately resolubilized in 30 µL of 0.1% TFA/10% acetonitrile/water prior to nano-LC separation.

2.5 Nano-LC-MALDI-TOF-MS/MS experiments

Separations were performed on an U3000 nano-LC system (Dionex-LC-Packings, Sunnyvale, CA, USA). After a pre-concentration step (C18 cartridge, 300 µm, 1 mm), the peptide samples were separated on a Pepmap C18 column (75 µm, 15 cm) using an acetonitrile gradient from 5% to 15% over 10 min, from 15% to 65% over 38 min and from 65% to 100% over 15 min and, lastly, 15 min in 100% acetonitrile. The flow was set to 300 nl/min and 115 fractions were automatically collected (one per 30 s) on an AnchorChip™ MALDI target using a Proteineer™ fraction collector (Bruker Daltonics, Bremen, Germany). Next, 2 µl of MALDI matrix (0.3 mg/ml α-cyano-4-hydroxycinnamic acid in acetone:ethanol:0.1% TFA-acidified water, 3:6:1 v/v/v) were added during the collection process. The MS and MS/MS measurements were performed off-line using an Ultraflex™ II TOF/TOF mass spectrometer (Bruker Daltonics) in automatic mode (using FlexControl™ 2.4 software), reflectron mode (for MALDI-TOF PMF) or LIFT mode (for MALDI-TOF/TOF peptide fragmentation fingerprint (PFF)). External calibration over the 1000-3500 mass range was performed with the $[M+H]^+$ mono-isotopic ions of bradykinins 1-7, angiotensin I, angiotensin II, substance P, bombesin and adrenocorticotropic hormone (clips 1-17 and clips 18-39) from a peptide calibration standard kit (Bruker Daltonics). Briefly, a 25 kV accelerating voltage, a 26.3 kV reflector voltage and a 160 ns pulsed ion extraction were used to obtain the MS spectrum. Each spectrum was produced by accumulating data from 500 laser shots. Peptide fragmentation was driven by Warp-LC software 1.0 (Bruker Daltonics) with the following parameters: signal-to-noise ratio > 15, more than 3 MS/MS by fraction if the MS signal was available, 0.15 Da of MS tolerance for peak merge and the elimination of peaks which appears in more than 35% of fractions. Precursor ions were accelerated to 8 kV and selected in a timed ion gate. Metastable ions generated by laser-induced decomposition were further accelerated by 19 kV in the LIFT cell and their masses were measured in reflectron mode. Peak lists were generated from MS and MS/MS spectra using Flexanalysis™ 2.4 software (Bruker Daltonics). Database searches with Mascot 2.2 (Matrix Science Ltd) using combined PMF and PFF datasets were performed in the UniProt 56.0 and 56.6 databases via ProteinScape 1.3 (Bruker Daltonics). A mass tolerance of 75 ppm and 1 missing cleavage site were allowed for PMF, with an MS/MS tolerance of 0.5 Da and 1 missing cleavage site allowed for MS/MS searching. The relevance of protein identities was judged according to the probability-based Mowse score [Perkins et al., 1999], calculated with $p < 0.05$.

2.6 Bioinformatics resources and sorting protein lists

Two FASTA sequence protein datasets were extracted from UniProt using the sequence retrieval system at the European Bioinformatics Institute [Zdobnov et al., 2002]. The first FASTA sequence dataset corresponds to the list (with 18,187 entries) of all mammalian proteins having at least one transmembrane domain (the SRS-coding criteria are as follows: [uniprot-Taxonomy:mammalia*] & [uniprot-FtKey:transmem*]). The second FASTA sequence dataset corresponds to the list (424,819 entries) of all mammalian proteins lacking transmembrane domains (the SRS-coding criteria are as follows: [uniprot-Taxonomy:mammalia*] ! [uniprot-FtKey:transmem*]). The FASTA sequence datasets were subjected to *in silico* trypsin proteolysis using Proteogest [Cagney et al., 2003] and the following command line: >perl proteogest.pl –i *filename* –c trypsin –d –a –g1.
The protein lists were compared using nwCompare software [Pont & Fournie, 2010] and classified according to the Protein Analysis Through Evolutionary Relationships (PANTHER) system [Mi et al., 2007; Thomas et al., 2003] (www.pantherdb.org). PANTHER is a resource in which genes have been functionally classified by expert biologists on the basis of published scientific experimental evidence and evolutionary relationships. Proteins are classified into families and subfamilies of shared function, which are then categorized by molecular function and biological process ontology terms.

2.7 Fluorescence microscopy

For fluorescence microscopy observations, the BCECs were biotinylated according to the above-described method, except that a non-cleavable biotinylation reagent (sulfosuccinimidyl-6-[biotinamido]-6-hexanamido hexanoate; EZ-link sulfo-NHS-LC-biotin (Thermo Scientific, Cergy Pontoise, France)) was used. Filters with BCECs were fixed for 10 min in 2% w/v paraformaldehyde at RT and washed in PBS. Biotinylated proteins were revealed by incubation with a Streptavidin-Cy3 conjugate (1:50 v/v) for 30 min. After washing with PBS, cells were incubated for 2 min with the nuclear stain Hoechst 33258 (1 µg/mL) and the filter sections were mounted in Mowiol (Merck, France). Fluorescence was visualized with a Leica DMR fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.8 Dot blots for estimating the biotinylation efficiency

Briefly, 15 µg of proteins from pellets and supernatants were dot-blotted on a nitrocellulose membrane. The membrane was incubated in blocking buffer [5% bovine serum albumin (BSA) in 20 mM Tris-HCl, 150 mM NaCl; pH 7.5, and 0.05% Tween-20 (TBS-T)] for one hour at RT and then immersed for 30 min at RT in a solution of alkaline phosphatase-conjugated avidin (1:1000 v/v in BSA/TBS-T). After three 15-min washes with TBS-T and one 10-minute wash with TBS (20 mM Tris-HCl, 150 mM NaCl; pH 7.5), the membrane was incubated with 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt/p-nitro blue tetrazolium chloride substrate solution. The reaction was stopped by rinsing with deionised water during gentle shaking. The membrane image was acquired at 300 dpi with a Umax Scanner (Amersham Biosciences, Orsay, France) and stored in a Tagged Image File format.

3. Results and discussion

3.1 Confirmation of BBB-like properties

Once primary capillary ECs are isolated *in vitro*, they rapidly lose some of their BBB functions. The cells' barrier properties were restored by a 12-day co-culture in which bovine

BCECs were seeded on the upper side of a filter placed in a Petri box and glial cells were seeded on the underside (see the Materials and Methods for details). Re-induction of BBB properties was confirmed by the fact that Pe^{LY} for bovine BCECs with re-induced BBB functions (0.6 $x10^{-3}$ cm/min) was just over half that for cells with limited BBB functions (1.0 $x10^{-3}$ cm/min). Immunostaining also confirmed the presence and localization of the main tight junction proteins occludin and claudin-5 and the associated protein ZO-1, as described elsewhere by our group [Gosselet et al., 2009; Pottiez et al., 2009b].

3.2 Assessment of the susceptibility of BCEC membrane proteins to trypsin cleavage

Prior to MS identification, membrane proteins are usually cleaved by proteolytic enzymes. Whatever the protein studied, trypsin is often considered as the enzyme of choice for proteomics, because it (i) has a specific cleavage site (on the C-terminal side of Arg- Xaa and Lys-Xaa, except when Xaa is a Pro), (ii) generates peptides of the right length for MS (in terms of sensitivity and accuracy) because the relatively high abundance of Arg and Lys (around 6%, compared with 10% for Leu, the most life abundant amino acid) and (iii) yields peptides with positive trapped charges. Due to the hydrophobic nature of PM proteins, several improvements of trypsin-based digestion methods have been especially developed to improve trypsin accessibility to proteins of interest. Most use buffers containing organic solvents (methanol, acetone, acetonitrile, etc.) or detergents (SDS, CYMAL-5, n-octylglucoside, etc.) ([Lu X. & Zhu, 2005]; see [Josic & Clifton, 2007] for a review).

Other enzymes can also be used in this essential step in proteomics [Wu et al., 2003]. Other methods involve enzyme-free, hydrolytic cleavage using various combinations of acidic conditions, cyanogen bromide and microwave irradiation [Josic & Clifton, 2007; Zhong et al., 2005]. These enzyme-free methods cleave either specifically at methionine (with an average abundance of around 2.5%) or non-specifically at any peptide bond [Zhong et al., 2005]. Clearly, it is important to choose the right cleavage method when seeking to reduce bias and erroneous conclusions in the proteomic identification of membrane proteins.

The susceptibility of mammalian PM proteins to trypsin cleavage was assessed *in silico*. The two Uniprot FASTA sequence datasets (corresponding to all known mammalian transmembrane proteins and non-transmembrane proteins, respectively) were analysed with Proteogest software. This Perl-written software performs the *in silico* trypsin digestion of all listed proteins and lists the generated peptides according to length or isotopic mass. Expression of the results as histograms (Fig. 3) shows that the overall distribution of tryptic peptides (in terms of length or isotopic mass) is essentially the same for both datasets and suggests that the susceptibility of mammalian transmembrane proteins does not differ from that of non-transmembrane proteins.

As expected, the length-based distribution of peptides matches the isotopic mass distribution. Additionally, more than 75% of the potential trypsin-generated peptides in each dataset have fewer than 30 amino acids or an isotopic mass below 3000 atomic mass units, meaning that mass measurement or mass fragmentation will give unambiguous results. Even though between 10 and 17% of the *in silico* peptides have an isotopic mass below 500 atomic mass units, more than 50% of the potentially generated peptides are located in the optimal mass range for standard mass spectrometers.

3.3 Assessment of *in vitro* biotinylation

The efficiency of *in vitro* biotinylation with the non-cleavable reagent (EZ-link sulfo-NHS-LC-biotin) was assessed by florescence microscopy. The fluorescence pattern and intensity

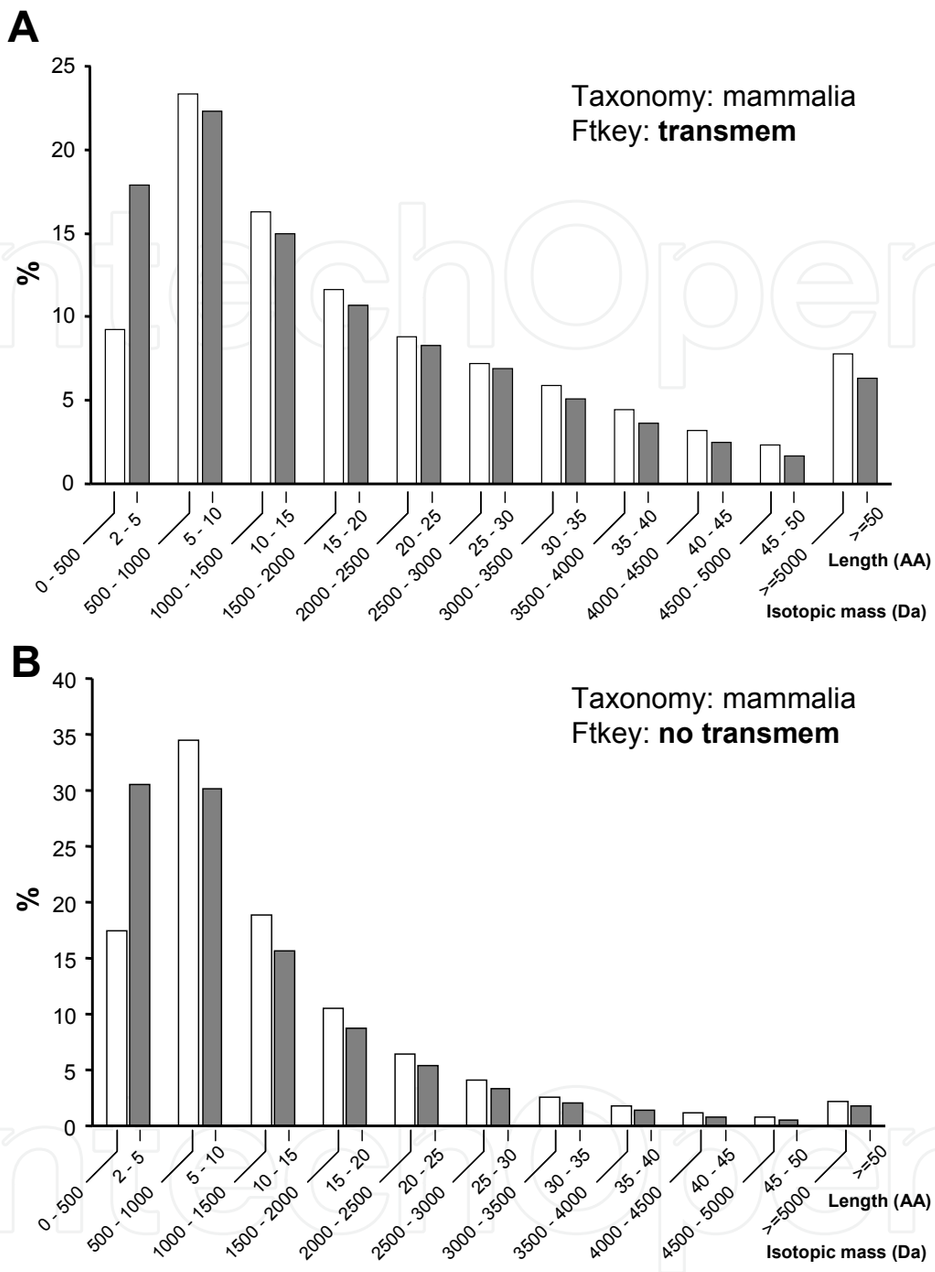


Fig. 3. Histograms of peptide counts according to the number of amino acid residues (AA) or the isotopic mass after *in silico* trypsin digestion with Proteogest [Cagney et al., 2003] of FASTA sequence datasets for (A) all mammalian proteins displaying at least one transmembrane domain (18,187 entries) and (B) all mammalian proteins lacking transmembrane domains (424,819 entries). The command line was >perl proteogest.pl –i *filename* –c trypsin –d –a –g1. The histograms show that the overall distribution of tryptic peptides (in terms of length or isotopic mass) is essentially the same in the two datasets and suggest that the trypsin susceptibility of mammalian transmembrane proteins does not significantly differ from that of non-transmembrane proteins.

did not differ significantly from one condition to another (Fig. 4) and the signal was principally located at the cell boundaries (red colour). Likewise, the EC permeabilities (deduced from the Pe^{LY} values) evolved similarly in treated and untreated cells.. Taken as a whole, these findings demonstrated that biotinylation did not affect the integrity of the BBB and did not introduce experimental bias.

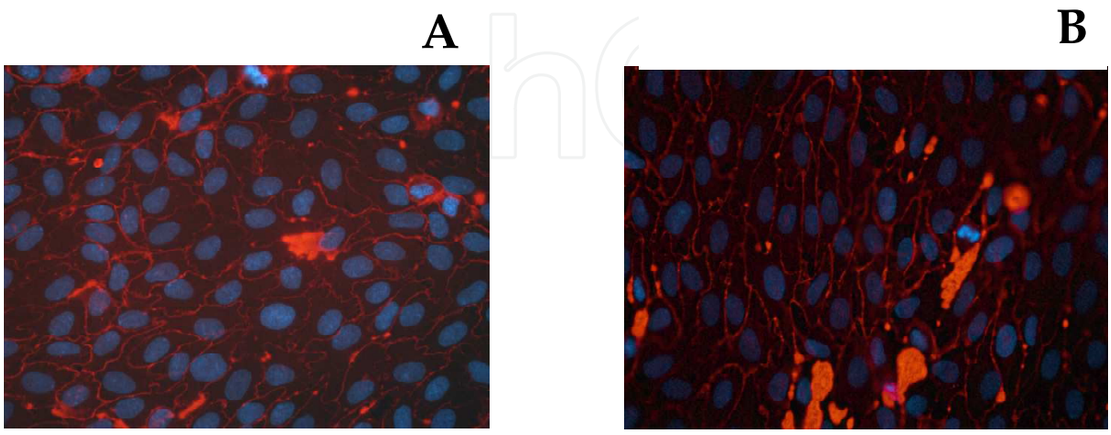


Fig. 4. Fluorescence microscopy of a bovine BCEC monolayer with limited BBB functions ("Lim. BBB", panel A) or re-induced BBB functions (Re-ind. BBB, panel B). The monolayers were biotinylated with a non-cleavable reagent (EZ-link sulfo-NHS-LC-biotin). Biotinylated proteins were revealed by incubation with a Streptavidin-Cy3 conjugate (in red), whereas nuclei were stained with Hoechst 33258 (in blue).

3.4 Nano-LC-MALDI-TOF-MS/MS maps

After *in vitro* biotinylation, adherent bovine BCECs with limited or re-induced BBB functions were detached from the extracellular matrix by collagenase treatment, in order to avoid proteolytic damage to the PM proteins. The collected cells were lysed in ice-cold hypotonic buffer and pelleted at 1,000 x *g* for 10 min at 4 °C. Biotinylated proteins in the supernatant were trapped using streptavidin-coupled magnetic beads. Elution of non-bound proteins was monitored with dot blots. Biotinylated proteins immobilised on the streptavidin-coated magnetic beads were then on-bead digested with trypsin. The resulting peptides were collected, released by reduction and concentrated prior to nano-LC-MALDI-TOF/TOF mass spectrometry analysis.

Typical chromatograms for each of the two conditions are shown in Fig. 5. As with two-dimensional gel electrophoresis, these peptide maps provide an overall, graphic representation of a sample's peptide diversity and abundance. The fact that the chromatograms for limited or re-induced BBB sample differ significantly underlines the quality of the sample preparation. Indeed, chromatograms that are too similar and/or too dense reflect inefficient labelling and purification, leading to the identification of a large set of cytosolic proteins.

3.5 Protein identification

Proteins were identified according to published guidelines [Wilkins et al., 2006] on the basis of PFF data. Briefly, the MS/MS data of all fragmented peptides were processed with the Mascot search algorithm, which compares the experimental MS/MS data to the theoretical

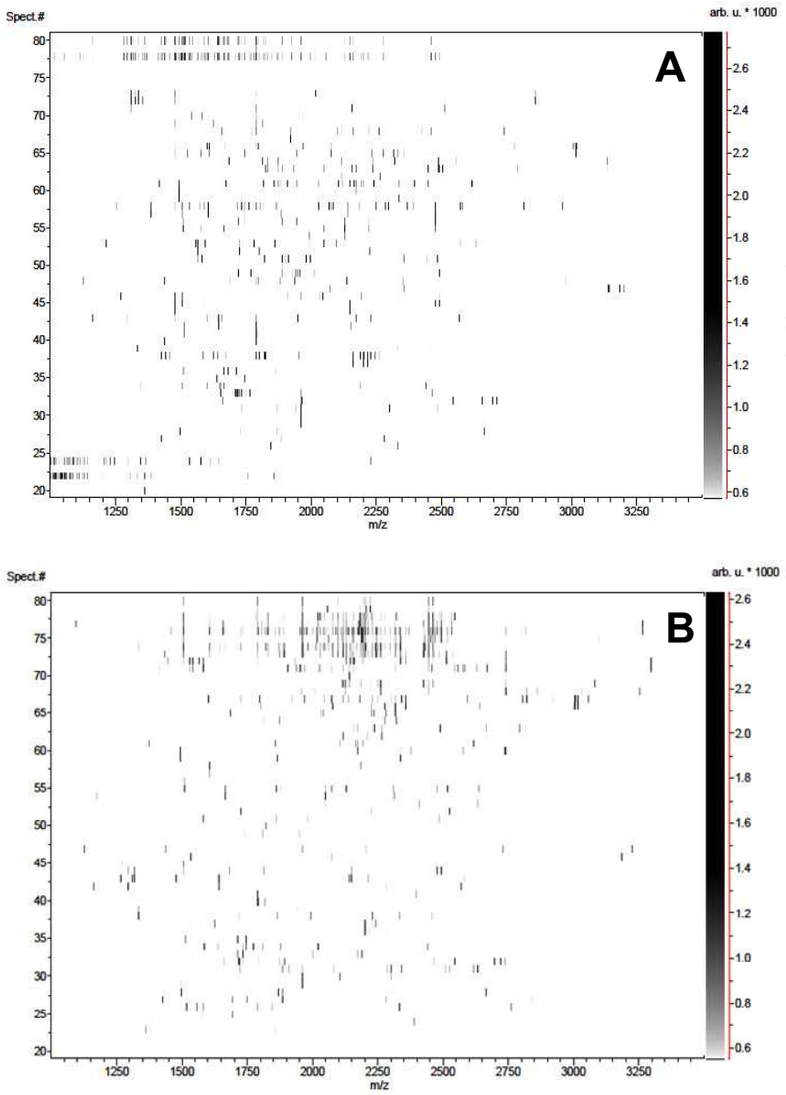


Fig. 5. Nano-LC-MALDI-TOF/TOF mass spectrometry analysis. The figure shows typical chromatograms of tryptic digests of *in vitro* biotinylated bovine BCECs monolayers with limited BBB functions (panel A) and re-induced BBB functions (panel B). The Y axis corresponds to the chromatographic retention time (expressed as a spectrum number, Spect. #), whereas the X axis displays the mass/charge (m/z) ratio of the detected peptide ions. Each peptide is characterized by its retention time (Spect. #) and molecular mass (more exactly, its isotopic distribution). Peptide abundance is grey-scale coded; the darker the signal, the more abundant the peptide.

data from the *in silico* digestion of all database-referenced proteins (or subsets of the latter). The concordance between experimental and theoretical data is then expressed as a Mascot score (-10 x log10 (p), where p is the likelihood (with 95% confidence) than the match is not due to chance). In other words, if the Mascot score for a given peptide is above the predefine threshold, the matching is not probably due to chance (and vice versa). Ultimately, the scores for each peptide matching the same protein are summed.

An illustration of the rigorousness of protein identification is shown in Fig. 6 for the sodium/potassium-transporting ATPase subunit alpha-1 precursor (AT1A1_BOVIN), a protein with 10 transmembrane domains. This catalytic subunit is located at the PM and enables creation of the electrochemical gradient required for the active transport of various nutrients. The mature form of bovine AT1A1 is composed of 1016 amino acids and has an average molecular weight of around 112 kDa. The individual identification scores of the three peptides belonging to this protein are presented in the summary box in Fig. 6. All the scores are over the chance-related threshold, demonstrating that the identification is relevant. Hence, the cumulative Mascot score of 168.6 for AT1A1 denotes unambiguous identification.

The location of the matching peptides (in bold red type) within the protein amino acid sequence shows that they are clustered in the large cytosolic region (described as "potential" in Uniprot database (aa #337 to #770)) of the Na^+/K^+-transporting ATPase subunit. Accordingly, no transmembrane domain-containing peptides served as the basis for protein identification, suggesting that the hydrophilic (i.e. cytoplasmic) regions of a given, non-denatured protein are more accessible to trypsin than their hydrophobic counterparts. Lastly, a typical MS/MS spectrum is shown in Fig. 6. The precursor ions displaying an m/z ratio of 2834.3838 atomic mass units are in-source fragmented and all the generated daughter ions had an m/z ratio below that of the parent ions. The mass differences between daughter ions allow deducing the amino acid sequence.

A typical nano-LC MS/MS analysis reported 761 fragmented peptides from samples of BCECs with limited BBB functions, whereas 957 fragmented peptides were reported for samples of BCECs with re-induced BBB functions. The efficient MS fragmentation led to the identification of 145 and 124 proteins in BCEC samples with limited and re-induced BBB functions, respectively. Sixty-three proteins were common to both conditions (Figure 7A). Following duplicate experiments on fraction-specific proteins, only 51 and 32 proteins were identified twice in BCECs with limited BBB functions and re-induced BBB functions, respectively. In all, this approach identified 211 distinct genes, of which 58 are referenced in Uniprot as coding for membrane-related proteins. Five of the 63 common proteins were PM or membrane-associated proteins, whereas 2 and 3 membrane-related proteins were identified in fraction-specific protein sets from BCECs with limited and re-induced BBB functions, respectively. Of the 15 membrane-related proteins (Figure 7B), 5 had more than one transmembrane domain (range: 2 to 17), 4 had a single transmembrane domain and 6 were lipid-anchored. Hence, the majority of membrane-related proteins were anchored to the membrane by a single domain or a lipid moiety.

3.6 Sorting protein lists

After conversion of identified proteins into their corresponding gene names via web-available bioinformatics resources, protein lists were sorted with PANTHER. The cellular locations of identified proteins (Fig. 8) were similar in the two kinds of BCEC. The protein sorting results showed that about two thirds of the identified proteins came from the cytoplasm or the PM, whereas a quarter were related to the endoplasmic reticulum, the mitochondrion, the nucleus and secreted proteins. Very few of the identified proteins belonged to the cell junction, endosome or Golgi apparatus. This ranking shows that very few proteins belonging to cytoplasm or secreted proteins (or proteins added to the cell culture medium) were recovered, despite their cellular abundance. Our findings demonstrate the efficiency of the enrichment approach used in the present study, even though only about 30 proteins came from the BCEC PM.

AT1A1_BOVIN
Sodium/potassium-transporting ATPase subunit alpha-1 precursor (EC 3.6.3.9)

Score: 168 Sequence Coverage [%]: 5.4 No. of unique Peptides: 3
MW [kDa]: 112 pI: 5. 36

m/z meas.	Δ m/z [ppm]	Score	Range	Sequence
2834.3838	-0.17	76.9	525-549	K.EQPLDEELKDAFQNAYLELGGLGER.V
1236.6960	-8.00	34.7	646-656	R.LNIPVSQVNPR.D
2464.1980	-1.80	57.0	742-764	K.QAADMILLDDNFASIVTGVEEGR.L

Matched peptides shown in **Bold Red**

```
451 ESALLKCIEV CCGSVKEMRE RYTKIVEIPF NSTNKYQLSI HKNANAGEPR
501 HLLVMKGAPE RILDRCSSIL IHGKEQPLDE ELKDAFQNAY LELGGLGERV
551 LGFCHLLLPD EQFPEGFQFD TDDVNFPVDN LCFVGLISMI DPPRAAVPDA
601 VGKCRSAGIK VIMVTGDHPI TAKAIAKGVG IISEGNETVE DIAARLNIPV
651 SQVNPRDARA CVVHGSDLKD MTPEQLDDIL KYHTEIVFAR TSPQQKLIIV
701 EGCQRQGAIV AVTGDGVNDS PALKKADIGV AMGIAGSDVS KQAADMILLD
751 DNFASIVTGV EEGRLIFDNL KKSIAYTLTS NIPEITPFLI FIIANIPLPL
801 GTVTILCIDL GTDMVPAISL AYEQAESDIM KRQPRNPQTD KLVNERLISM
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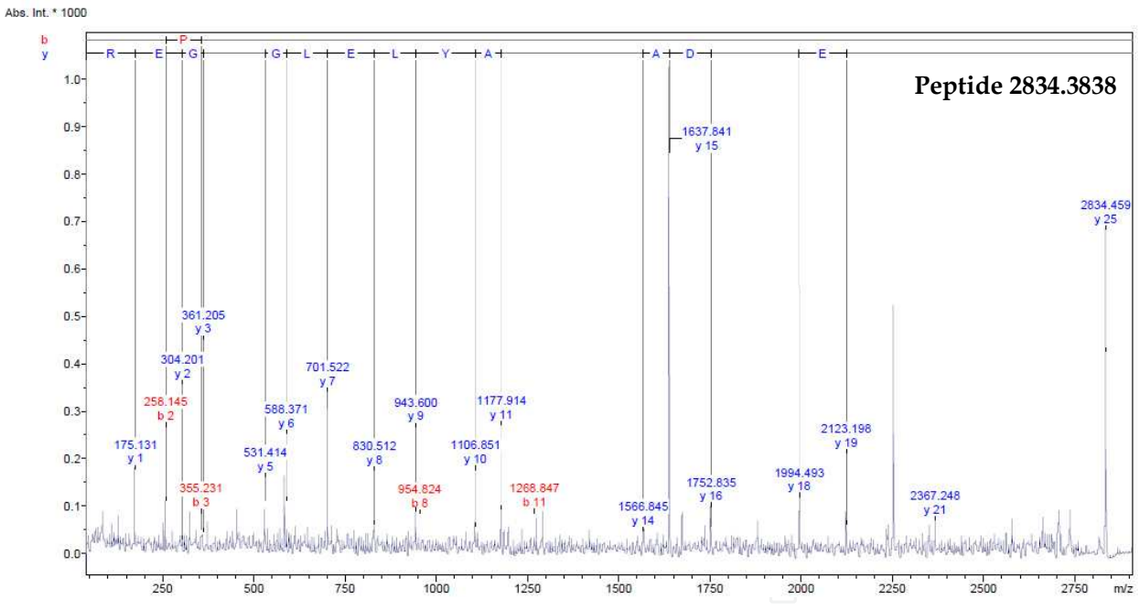


Fig. 6. Identification of AT1A1_BOVIN (sodium/potassium-transporting ATPase subunit alpha-1 precursor). The summary report describes some of the structural characteristics of the three matching peptides. The high Mascot scores correspond to an unambiguous identification. The sequences of the matching peptides are highlighted in bold red type within the AT1A1 amino acid sequence. For the sake of clarity, the amino acid sequence displayed here is truncated (ranging from amino acids #451 to #850). Lastly, the MS/MS spectrum of ionized peptides of 2834.3838 atomic mass units illustrates the amino acid sequence deduction.

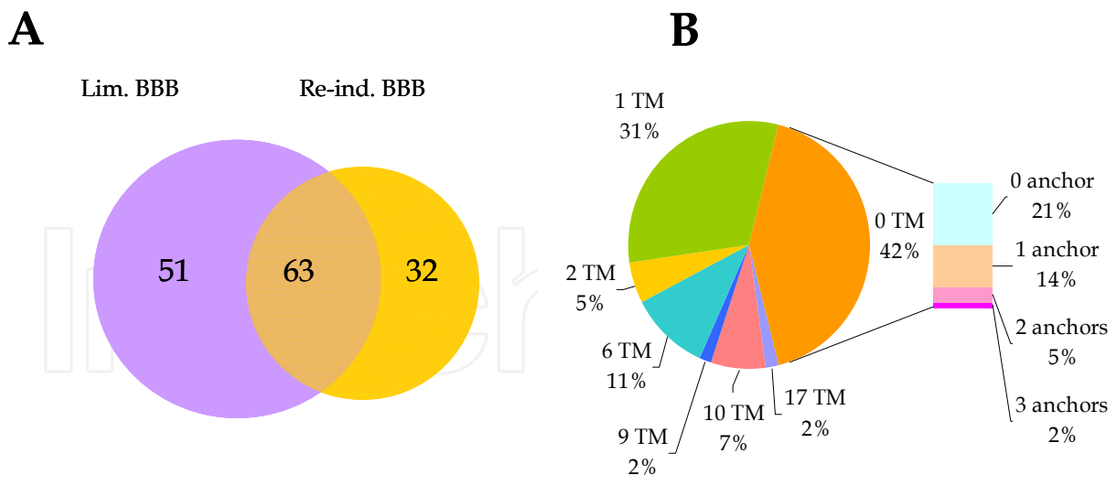


Fig. 7. Overall distribution of proteins identified using this approach. Panel A: A Venn diagram of proteins identified in BCECs with limited BBB functions and re-induced BBB functions, respectively, showing the distribution of proteins identified in both conditions and in only one condition. Panel B: The distribution of membrane-related proteins according to the number of transmembrane domains and the presence of a lipid anchor.

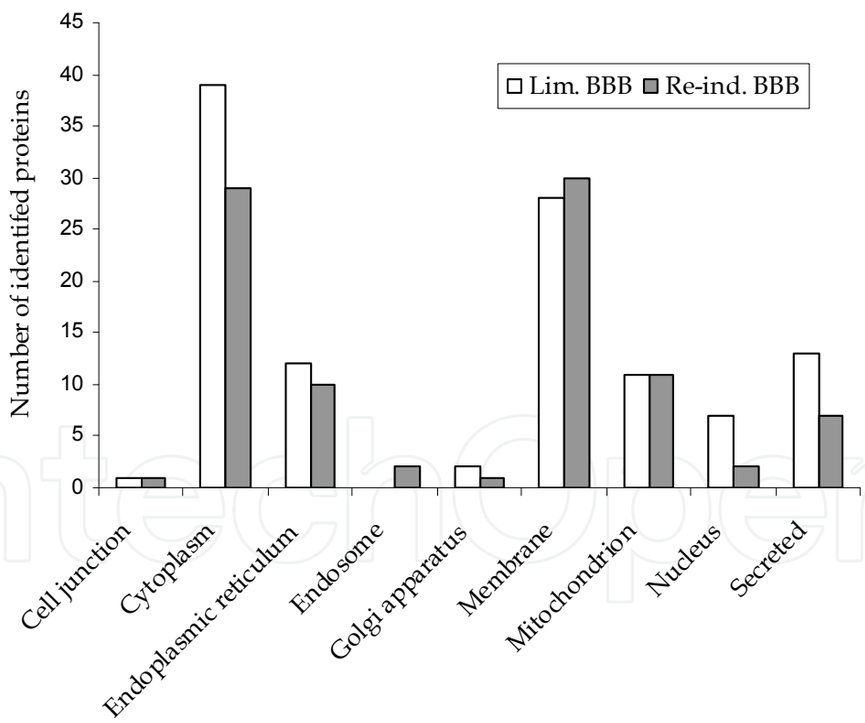


Fig. 8. Cellular location-based sorting of identified proteins. The white histogram shows the sorting results for proteins in samples of BCECs with limited BBB functions (Lim. BBB) and the grey histogram depicts the result for BCECs with re-induced BBB (Re-ind. BBB) functions. Clearly, the grey and white histograms are very similar. More than 50% of the identified proteins were related to the cytoplasm and membrane. The remaining proteins were related to the mitochondrion, the nucleus and secretory pathways.

The sorting of protein lists by molecular function is presented in Fig. 9. Proteins identified from the bovine BCECs with limited (Fig. 9A) or re-induced (Fig. 9B) BBB functions could be divided into 8 and 9 activity classes, respectively, of which 7 were common (binding, catalytic activity, enzyme regulation activity, ion channel activity, receptor activity, transporter activity and structural molecule activity). Briefly, there were twice as many proteins with catalytic or receptor activity for BCECs with re-induced BBB functions than for BCECs with limited BBB functions. In contrast, the proteins involved in binding, enzyme regulation activity and structural molecule activity were less represented (at least two fold) in BCECs with re-induced BBB functions. Interestingly, proteins displaying transcription regulation activity were only identified in BCECs with limited BBB functions; whereas lists from BCECs with re-induced BBB functions also included proteins with motor activity and antioxidant activity.

As expected for our experimental model, 59% of the 211 identified proteins were identified as bovine proteins. Indeed, certain proteins not yet reported in bovine samples were identified on the basis of inter-species sequence homologies. Given their location, this subset of proteins complements the results of our previous work, in which we used a large-scale electrophoresis- and chromatography-based approach to identify more than 430 cytoplasmic proteins [Pottiez et al., 2010]. Proteins found in both BCECs with limited and re-induced BBB functions will not be discussed further here.

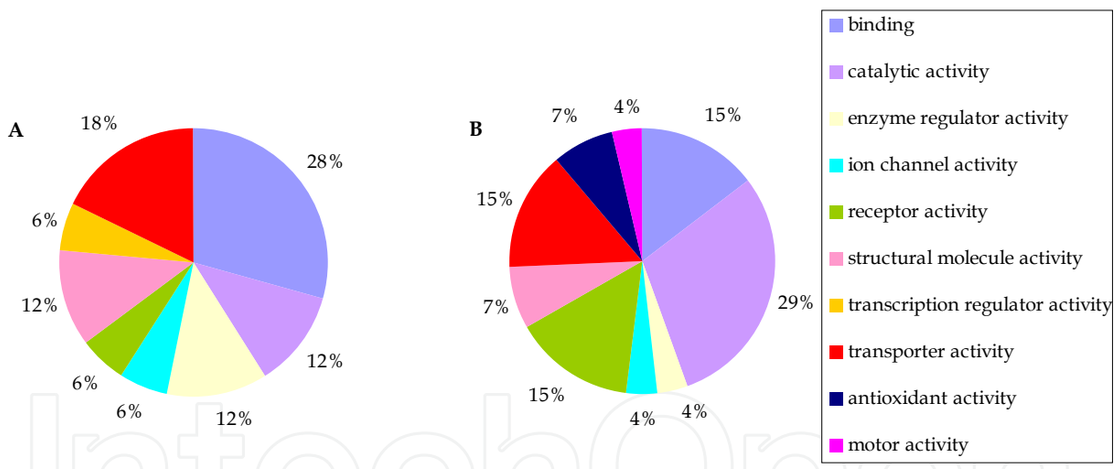


Fig. 9. Distribution of the proteins specifically identified in each condition according to their molecular function. The lists were generated under the PANTHER classification system. Proteins only identified in samples of BCECs with limited BBB function were distributed across 8 categories (Panel A), whereas those only identified in samples from BCECs with re-induced BBB functions were distributed over 9 categories (panel B).

The literature-based sorting of proteins identified in one condition only generated a lot of valuable information. A selected subset of these proteins is presented in Table 1. All of the listed proteins are found in the inner mitochondria membrane, the endoplasmic reticulum, the Golgi membrane and the vesicle membrane as well as the PM; this shows that the biotinylation reaction takes also place inside the cell, despite the experimental precautions taken. However, for the first time, we report a few PM proteins that had not previously been

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Table 1.

Lim. BBB						
Protein name	Gene name [a]	Accession number [a]	pI [b]	MW (kDa) [b]	Seq. Cov. (%) [c]	Matched peptides
Class I histocompatibility antigen	*1b04*	P30382	5.4	40.4	7.2	2
ATP-binding cassette sub-family C member 8	*Abcc8*	Q09427	9.1	177.0	1.2	2
Very long-chain specific acyl-CoA dehydrogenase	*Acadvl*	P48818	9.5	70.6	2.6	1
Cell division control protein 42 homolog	*Cdc42*	Q2KJ93	6.2	21.2	8.9	1
Cytoskeleton-associated protein 4	*Ckap4*	Q07065	5.6	66.0	2.5	1
Integrin alpha-D	*Itgad*	Q13349	5.4	126.7	2.4	2
Myosin-Ic	*Myo1c*	Q27966	9.9	121.9	4.6	3
Nitric oxide synthase, endothelial	*Nos3*	P29473	6.5	133.2	2.4	1
Platelet endothelial cell adhesion molecule	*Pecam1*	P51866	7.0	82.5	4.7	2
Spectrin beta chain, brain	*Sptbn1*	Q01082	5.3	274.4	1.0	2
Re-ind. BBB						
Protein name	Gene name [a]	Accession number [a]	pI [b]	MW (kDa) [b]	Seq Cov (%) [c]	Matched peptides
Neutral cholesterol ester hydrolase 1	*Nceh1*	Q1JQE6	6.2	46.0	3.4	1
Dysferlin	*Dysf*	A6QQP7	5.3	237.1	0.9	1
EH domain-containing protein	*Ehd2*	Q9NZN4	6.0	61.1	5.0	2
Guanine nucleotide-binding protein subunit beta-2-like	*Gnb2l1*	P63243	8.9	35.1	5.0	1
Moesin	*Msn*	Q2HJ49	5.8	67.9	3.6	1
Myoferlin	*Myof*	Q69ZN7	5.8	233.2	0.7	1
Membrane-associated progesterone receptor component 1	*Pgrmc1*	Q17QC0	4.4	21.6	4.6	1
1-acyl-sn-glycerol-3-phosphate acyltransferase alpha	*Agpat1*	Q95JH2	10.3	32.0	7.3	1
Ras-related protein Rab-35	*Rab35*	Q15286	9.4	23.0	9.0	1
Transforming protein RhoA	*Rhoa*	P61585	5.8	21.8	8.8	1
Signal peptidase complex subunit 2	*Spcs2*	Q28250	9.5	24.9	8.4	1
Translocon-associated protein subunit delta	*Ssr4*	Q2TBX5	5.4	18.8	11.0	1
Transmembrane 9 superfamily member 4	*Tm9sf4*	A5D7E2	6.2	74.3	3.0	1
Zinc transporter 1	*Slc30a1*	Q9Y6M5	6.0	55.3	3.9	1

a Gene name and accession number according to Uniprot. b Isoelectric point (pI) and molecular weight (MW). c Sequence coverage.

Table 1. List of identified plasma membrane proteins that were present only in solo-cultured (Lim. BBB)or in co-culured (re-ind. BBB) BCE

identified as such in BCECs (notably integrin alpha-D, nitric oxide synthase 3, dysferlin, myoferlin, transmembrane 9 superfamily member 4) and confirmed the presence of previously reported PM proteins (notably ATP-binding cassette sub-family C member 8, platelet endothelial cell adhesion molecule and Na^+/K^+ ATPase) [Uchida et al., 2011]. Although moesin is not a PM protein, it appears to be associated with the PM in BCECs with re-induced BBB functions (as previously reported [Pottiez et al., 2009b]).

In vivo BCECs displaying a BBB phenotype display specific endocytic trafficking that regulates (at least in part) the molecular exchanges between the blood and the brain [Abbott et al., 2010]. Interestingly, our samples from BCECs with re-induced BBB functions contained several proteins involved in cellular endocytosis, endocytic recycling, membrane trafficking and receptor internalization, such as EH domain-containing protein 2, myoferlin, dysferlin and certain cellular partners. Ferlin proteins are calcium-sensing proteins involved in vesicle trafficking and PM repair [Glover & Brown, 2007] and regulate the fusion of lipid vesicles at the PM. Myoferlin is reportedly strongly expressed in ECs and vascular tissues and was identified in a proteomics study of caveolae/lipid raft microdomains [Bernatchez et al., 2007]. Repression of myoferlin expression reduces not only lipid vesicle fusion in ECs but also protein expression levels of the vascular endothelial growth factor receptor-2 (VEGFR-2). In contrast to dysferlin, myoferlin regulates the membrane stability and function of VEGFR-2, [Sharma et al., 2010]. Dysferlin has been reported as a new marker for leaky brain blood vessels [Hochmeister et al., 2006].

Furthermore, *in vitro* myoferlin gene silencing not only decreases both clathrin- and caveolin-/raft-dependent endocytosis [Bernatchez et al., 2009] but also attenuates the expression of the angiogenic second tyrosine kinase receptor (Tie-2) [Yu et al., 2011]. In general, myoferlin appears to be critical for endocytosis events in ECs and could be a potential candidate for drug-mediated enhancement of transcytosis pathway and/or angiogenic targets. Accordingly, it has been shown that caveolin's main effect is to retain dysferlin at the cell surface [Hernandez-Deviez et al., 2008]; this inhibits the endocytosis of dysferlin through clathrin-independent pathway and therefore reinforces its PM-resealing activity. Recently, Doherty et al. have described a third interaction partner, EH domain-containing protein 2 (EHD2) [Doherty et al., 2008]. Although its role was demonstrated in myoblasts, EHD2 is an endocytic recycling protein that interacts with myoferlin to regulate lipid vesicle fusion. EHD2 binds to lipid membranes and deforms them into tubules. The protein regulates trafficking from the PM by controlling Rac1 activity [Benjamin et al., 2011] and is important for internalization of the glucose-transporter 4 (GLUT-4) [Park et al., 2004] Lastly, EHD2 is required for the translocation of a newly identified ferlin-like protein (Fer1L5) to the PM [Posey et al., 2011].

4. Conclusions

The aim of our study was to determine the distribution and the nature of PM proteins in BCECs displaying the BBB phenotype. Based in our BBB *in vitro* model, we developed a strategy for labelling these proteins (with biotin), isolating them (with streptavidin affinity chromatography) and identify them (with nano-LC MS/MS). The most frequently used methods for the enrichment of PMs are based on affinity chromatography, cationic colloidal silica particles, cell biotinylation or a tissue-specific polyclonal antiserum. We decided to use a biotinylation approach because it avoids many of the drawbacks of the other methods. For

example, proteolytic shaving offers many advantages in theory (since surface-exposed peptides are more water-soluble than their intrabilayer counterparts) but is handicapped by its tendency to trigger cell lysis and thus significantly contaminate surface-exposed membrane peptides with cytosol-derived peptides.

By using the biotinylation approach, we showed that very few cytoplasmic proteins, secreted proteins or proteins added to the cell culture medium were recovered - despite their relatively high cellular abundance. We reported on the novel identification of transmembrane and membrane-associated proteins in bovine BCECs displaying the BBB phenotype. Our findings demonstrated the efficiency of the enrichment approach used, even though only about 30 proteins came from the BCEC PM. The proteins are variously involved in cellular endocytosis, membrane trafficking and receptor internalization and may thus have significant roles in BBB function. The fact that transmembrane and membrane-associated proteins accounted for less than half the identified proteins shows how difficult it still is to isolate, solubilise and digest hydrophobic proteins of low cellular abundance. Our results suggest that the specific properties of PM proteins must be taken into account when seeking to improve biotinylation, purification and identification methods. Moreover, the glycocalyx can also impede biotinylation [Ueno, 2009]. The biotinylation targeting could probably be improved by the use of new biotin derivatives that are less likely to cross the PM.

Furthermore, the present study reports the identification of several proteins involved in cellular endocytosis, membrane trafficking and receptor internalization (such as EHD2 and myoferlin), together with their cellular partners. These proteins and the pathways of which they are a part may become new targets for increasing drug transport across the BBB.

5. Acknowledgments

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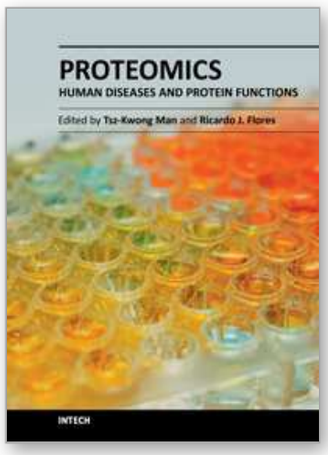
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