REVIEW

Blood-brain- barrier co-culture models to study nanoparticle penetration: focus on co-culture systems

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ABSTRACT   The blood-brain barrier, as a physical, active transport and metabolic barrier represents the main obstacle in the treatment of central nervous system diseases. The field of nanoparticle delivery systems is rapidly developing and nanocarriers seem to be promising for drug delivery or targeting to the brain. For testing the toxicity, uptake and transcellular transport of nanoparticles culture models of the blood-brain barrier are widely used, including immortalized brain endothelial cell lines, primary brain endothelial cells in static or dynamic culture conditions, and in co-culture systems with glial cells and/or pericytes. This mini-review gives a brief summary of blood-brain barrier co-culture models that were used for testing nano-carriers, the types of different nanoparticle systems that were examined on blood-brain barrier models, and the advantages, limitations and suitability of the blood-brain barrier models for nanoparticle penetration studies.   **Acta Biol Szeged 59(Suppl.3): (2015)**

Introduction

The blood-brain barrier, a dynamic interface separating the brain from systemic circulation, is the major entry route for therapeutic compounds to the central nervous system. Most drug candidates developed for neurological diseases have limited access to their brain targets because of the blood-brain barrier. This barrier is composed of brain microvascular endothelial cells, surrounded by pericytes, astrocytic endfeet, perivascular microglia and neurons (Zlokovic 2008). The cross-talk between these cells constitutes a functional unit which is crucial for the formation and maintenance of the blood-brain barrier. The main roles of the blood-brain barrier are creation of ionic homeostasis for neuronal functions, the supply of the brain with nutrients, protection from toxic insults and communication between the periphery and the central nervous system (Abbott et al. 2010).

The blood-brain barrier is the most important defense system against xenobiotics, pathogenic microorganisms and other insults from the systemic circulation. However, the same mechanisms that protect the central nervous system also limit drug delivery to the brain. These mechanisms include (i) the restricted paracellular pathway regulated by complex interendothelial tight junctions, (ii) low level of pinocytosis resulting in minimal non-specific transendothelial transport and (iii) active efflux transporters (Abbott 2010). The growing group
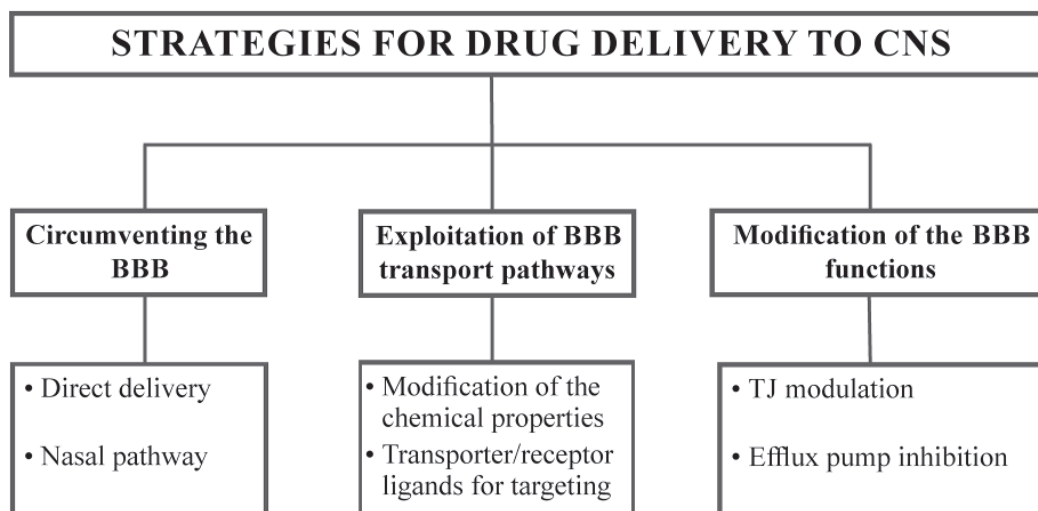
of drug efflux transporters at the blood-brain barrier includes P-glycoprotein, brain multidrug or brain cancer resistance protein, several members of the multidrug resistance proteins and organic anion transporting polypeptides (Redzic 2011). The low level of paracellular flux and transendothelial vesicular trafficking result in a transport barrier for drugs which are hydrophilic or have a molecular mass >400 dalton (Pardridge 2012). On the other hand brain penetration of lipophilic drugs is limited by the presence of efflux transporters at the luminal membrane of brain endothelial cells. Due to these reasons the blood-brain barrier prevents the brain entry of potential new neuropharmaceuticals including biopharmacons, nucleic acids and peptide or protein drugs. Improving drug delivery to the brain is considered essential for the future therapy of neurological disorders therefore novel drug delivery methods or systems are needed which take into account the structure and complex roles of the blood-brain barrier.

Strategies for central nervous system delivery of drugs

Several strategies have been developed to overcome or exploit the transport systems of the blood-brain barrier (Deli 2011). These strategies can be classified into three major groups summarized in Figure 1. The methods that increase drug delivery to the brain by circumventing the blood-brain barrier include direct, invasive injection to brain tissue or fluids and the use of alternative delivery pathways, like the nasal route. Intrathecal or intraventricular drug administrations are effectively used

STRATEGIES FOR DRUG DELIVERY TO CNS

Circumventing the BBB

- Direct delivery
- Nasal pathway

Exploitation of BBB transport pathways

- Modification of the chemical properties
- Transporter/receptor ligands for targeting

Modification of the BBB functions

- TJ modulation
- Efflux pump inhibition

Figure 1. Summary of the main strategies for drug delivery to the central nervous system (CNS). BBB: blood-brain barrier. TJ: tight junction.

in the clinical treatment of pain, spasticity or central nervous system infections (Cook et al. 2009), however, the slow and ineffective diffusion in brain tissue limits the efficacy of this approach especially for macromolecules (Wolak et al. 2013). A recent review summarizes novel delivery methods bypassing the blood-brain barrier or the blood-tumor barrier, among them surgically implanted polymeric matrix loaded with therapeutic agents and stem cell-mediated delivery of chemotherapeutics (Hendricks et al. 2015). At present, these techniques have marginal therapeutic benefit and several limitations such as potential perioperative complications and in the case of mesenchymal stem cells controversial data on efficacy and safety (Hendricks et al. 2015).

Intranasal delivery of small molecules or biologics to the central nervous system are widely investigated, and experimental data indicate that this alternative pathway of administration can be successfully exploited (Horvát et al. 2009; Sipos et al. 2010). Preclinical and clinical studies show that intranasal delivery of proteins, genes or stem cells is a potentially useful strategy to treat neurological diseases despite of the low amount of molecules that can reach the brain and the limited distribution to distal brain areas (Lochhead and Thorne 2012).

Another approach to enhance brain delivery of molecules is the exploitation of the transport pathways at the blood-brain barrier (Fig. 1). This can be achieved either by changing the physico-chemical properties of molecules or nanocarriers to increase their delivery across the blood-brain barrier or by specific targeting of the physiological transport pathways of the blood-brain barrier. Chemical modifications enhance brain delivery of molecules by increasing the lipid solubility or cationic charge of molecules. Lipophilic small molecules or liposomes enter the central nervous system by lipid-mediated free diffusion. High lipid solubility not only increases drug penetration across the blood-brain barrier but also uptake by peripheral tissues and sequestration in the capillary bed resulting in decreased concentration in blood and the brain (Banks 2009).

The strong negative charge created by the glycocalyx at the luminal surface of brain endothelial cells contributes to the barrier phenotype. The negative surface charge of the blood-brain barrier decreases the entry of negatively charged molecules or nanoparticles and favors the penetration of cationic ones by adsorptive-mediated transcytosis (Hervé et al. 2008).

Specific targeting can be achieved via the physiological transport pathways of the blood-brain barrier (Deli 2011). Peptides, proteins and lipoproteins reach the brain by receptor-mediated transport. Receptors for biomolecules crossing the blood-brain barrier, like insulin, transferrin or low density lipoprotein are highly expressed at the blood-brain barrier and were extensively characterized and tested for targeting large biopharmacons or nanoparticles (Pardridge 2012). The largest family of transporters at the blood-brain barrier is that of solute carriers. Several clinically used drugs, like L-DOPA, gabapentin and baclofen cross the blood-brain barrier via the large neutral amino acid transporter LAT1 (Deli 2011). Nanoparticles can be also targeted with ligands of blood-brain barrier solute carriers. As an example liposomes containing doxorubicin or methylprednisolone and targeted by gluthatione are in clinical trial phase to treat brain tumors or multiple sclerosis (Gaillard et al. 2012).

Since the interendothelial tight junctions and efflux pumps are the two major blood-brain barrier functions limiting drug access to brain, their modification can be used to increase drug penetration (Deli 2011). Opening of interendothelial

tight junctions by hyperosmolar mannitol in patients with brain tumors results in transient and reversible increase of blood-brain barrier permeability and improved delivery of chemotherapeutics to the brain (Neuwelt et al. 2008). Short chain alkylglycerols can also induce quick and reversible opening of tight junctions at the blood-brain barrier in preclinical studies and may represent a safer alternative to osmotic blood-brain barrier disruption (Hülper et al. 2013).

The extrusion of drugs by efflux pumps at the blood-brain barrier makes the treatment of brain tumors and pharmaco-resistant epilepsy especially difficult (Neuwelt et al. 2011). Efflux pump inhibitors could potentially increase the central nervous system delivery of antiepileptics or chemotherapeutics, but problems with systemic and central side-effects make this approach unrealistic for clinical treatment at present (Szakács et al. 2008). Novel pharmacological strategies are evaluated targeting the signaling cascades responsible for the upregulation of blood-brain barrier efflux transporters in epilepsy (Potschka 2012; Janigro and Walker 2014).

Co-culture models of the blood-brain barrier

Several models have been developed to examine BBB permeability. The *in silico* models use computational approaches for predicting the penetrability of new drugs and are applied to screen big compound libraries based on the physico-chemical characteristics of the molecules (Veszelka et al. 2011). The non-cell-based in vitro permeability models like parallel artificial membrane permeability assays (PAMPA) have been also proposed and evaluated for predicting passive blood-brain barrier permeability (Avdeef et al. 2015). *In silico* and PAMPA models are technically easy, quick, high throughput and low cost compared to cell-based or in vivo techniques. These models, however, cannot be used to study cellular and molecular interactions and mechanisms of transport like active influx or receptor-mediated transport, binding or metabolism. To our best knowledge *in silico* or PAMPA models were not published for modelling or testing nanoparticle transfer across the blood-brain barrier. Animal models represent the most complex physiological systems for testing drug delivery to the brain. *In vivo* experiments are valuable to examine brain and peripheral organ distribution, metabolism, excretion and toxicity of both drugs and nanoparticles, but these models are expensive, need high level of expertise and are only used for testing limited number of compounds (Veszelka et al. 2011). Cell culture-based blood-brain barrier models are middle throughput systems which proved to be versatile tools in both basic research and permeability testing of therapeutic drugs and nanoparticles (Veszelka et al. 2011; Avdeef et al. 2015). They are more complex than the *in silico* or PAMPA models but can not replace *in vivo* pharmacokinetic or pharmacodynamic studies.

The first *in vitro* blood-brain barrier model, freshly isolated brain capillaries, was developed by Ferenc Joó and his co-workers (Joó and Karnushina 1973). The development of the isolation method of brain microvessels was soon followed by a new era of culture-based blood-brain barrier models.

The restrictive paracellular barrier and efflux pumps are important characteristics of the blood-brain barrier and epithelial cell lines possessing these characteristics, originally used as test systems for gastrointestinal absorption, are also tested as surrogate blood-brain barrier models (Hellinger et al. 2012). The human intestinal epithelial cell line Caco-2, and the dog kidney epithelial cell line MDCK transfected with the human MDR1 gene provide a simple and inexpensive tool for the screening of drug candidates for passive permeability or efflux transport. Both Caco-2 and MDCK cells are utilized to investigate transcytosis of nanoparticles (Mc Carthy et al. 2015). However, the different cytoarchitecture and expression of tight junction and transporter proteins as compared to brain endothelial co-culture system limit their application as blood-brain barrier models (Hellinger et al. 2012). This consideration could be especially relevant for the selection of models to test targeted nanoparticles for central nervous system delivery.

Immortalized brain endothelial cell lines are widely used as simplified culture models of the blood-brain barrier (Veszelka et al. 2011; Avdeef et al. 2015). Among the brain endothelial cell lines the mouse bEnd3, the rat RBE4 and the human hCMEC/D3 cell lines are the best characterized and the most applied for blood-brain barrier studies. The hCMEC/D3 cell line shows decreased paracellular permeability and increased resistance values in comparison to other endothelial cell lines (Weksler et al. 2013). The uptake and transport of different nanoparticles was investigated on hCMEC/D3 cells (for review see Mc Carthy et al. 2015). Brain endothelial cell lines are easy to handle and cost effective blood-brain barrier models but their weak paracellular integrity and downregulation of blood-brain barrier -related genes (Urich et al. 2012) limit their application for drug or nanoparticle transport studies.

In most studies primary brain endothelial cell-based models are preferred to study blood-brain barrier functions as they have restrictive paracellular permeability and express various receptors, transporters and enzymes, similarly to *in vivo* conditions (Veszelka et al. 2011; Avdeef et al. 2015). Primary endothelial cells are successfully cultured from brain tissues of different species including bovine, porcine, human, mouse or rat (Deli et al. 2005; Veszelka et al. 2011). No significant differences in culture morphology or paracellular tightness can be observed between these models (Avdeef et al. 2015). Puromycin treatment not only helped to solve the problem of purity in primary brain endothelial cultures by killing contaminating cells not expressing high levels of P-glycoprotein (Perrière et al. 2005), but most probably contribute to the recent improvements of primary blood-brain barrier models
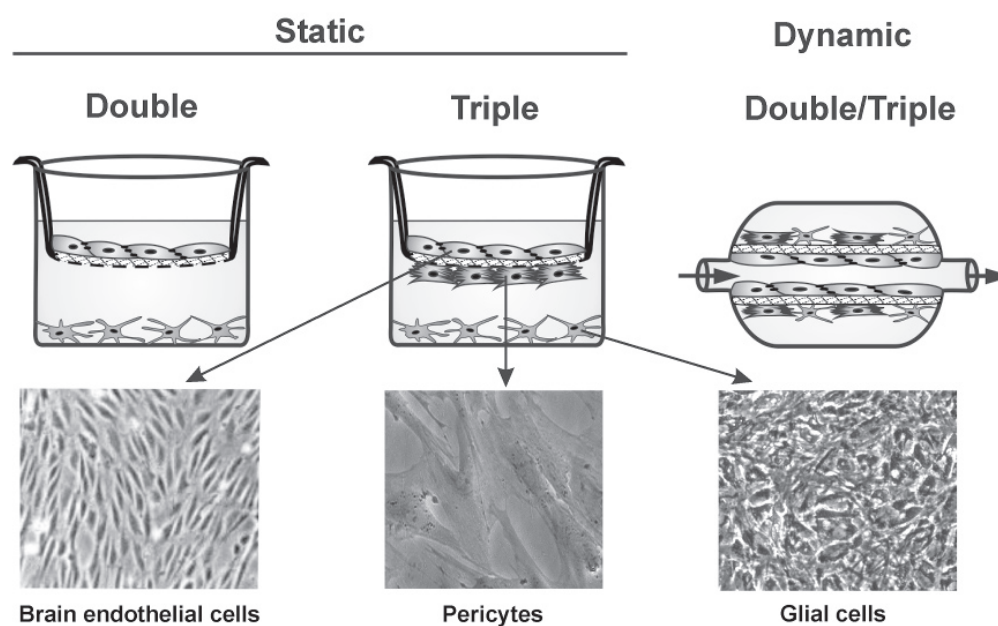
Figure 2. Co-culture models of the blood-brain barrier prepared from two (double), or three cell types (triple). For the static culture condition culture inserts, for dynamic (flow based) condition hollow fibers are used. In both systems porous membranes divide the culture compartments. The morphology of brain endothelial, brain pericyte and astroglia cells is shown on phase contrast images.

by favouring capillary brain endothelial cells expressing the highest levels of efflux pumps and tight junction proteins (Avdeef et al. 2015).

The blood-brain barrier characteristics of cerebral endothelial cells is induced *in vivo* by a cross-talk between endothelial cells of brain microvessels and the surrounding cell types, especially pericytes and glial endfeet (Abbott et al. 2010). In primary brain endothelial cell cultures, particularly during long cultivation periods or after sub-culturing loss of blood-brain barrier characteristics is observed (Deracinois et al. 2013). Astroglia cells were the first cell type to induce blood-brain barrier phenotype in brain endothelial cells, and purified astroglia or mixed glial cultures are routinely used in the next generation of blood-brain barrier models (Deli et al. 2005), the so called co-culture systems as shown on Figure 2. It is well accepted, that astrocytes tighten the paracellular barrier and induce the expression of tight junction, transporter and enzyme proteins in brain endothelial cells (Abbott et al. 2006; Deracinois et al. 2013); however, the mechanism of glial induction is still unknown. Since not only co-culture systems but also astrocyte-conditioned media are effective secreted growth factors may participate in the process (Abbott et al. 2006). Later, the inductive properties of pericytes were also confirmed in co-culture models and recently, triple co-culture models (Fig. 2) mimicking the anatomical structure of the blood-brain barrier have been described (Nakagawa et al. 2009; for review see Avdeef et al. 2015). Brain microvascular pericytes play a critical role in the development and function

of the blood-brain barrier in both physiological and pathological conditions (Winkler et al. 2011). Nakagawa and his co-workers were the first to demonstrate that pericytes also tighten the paracellular barrier of cultured brain endothelial monolayers and to establish and characterize a triple co-culture blood-brain barrier model with pericytes (Nakagawa et al. 2009).

The majority of the static *in vitro* blood-brain barrier models use culture inserts with a porous membrane (Fig. 2). The membrane pore size varies between 0.4 and 3 μm, the smaller pores are suitable for drug penetration, while the larger ones are used for nanoparticle transport studies. In co-culture models brain endothelial cells are grown on the upper surface of the inserts while pericytes or glial cells are cultured in the lower compartments (Fig. 2). This setup mimics the *in vivo* anatomy of blood-brain barrier (Abbott et al. 2010). The tightness of the co-culture blood-brain barrier models can be determined by the measurement of transendothelial electrical resistance using an electrode pair and a voltohmmeter (Benson et al. 2013). Co-culture blood-brain barrier models suitable for drug testing are characterized by high resistance values and low permeability of tracer molecules like fluorescein, sucrose, albumin, or dextrans (Deli et al. 2005; Veszelka et al. 2011; Avdeef et al. 2015). Because co-culture models from primary cultures retain morphological, functional and metabolic blood-brain barrier characteristics they are the best option for transport studies at present. However, the cell isolation technique is time consuming, needs methodological
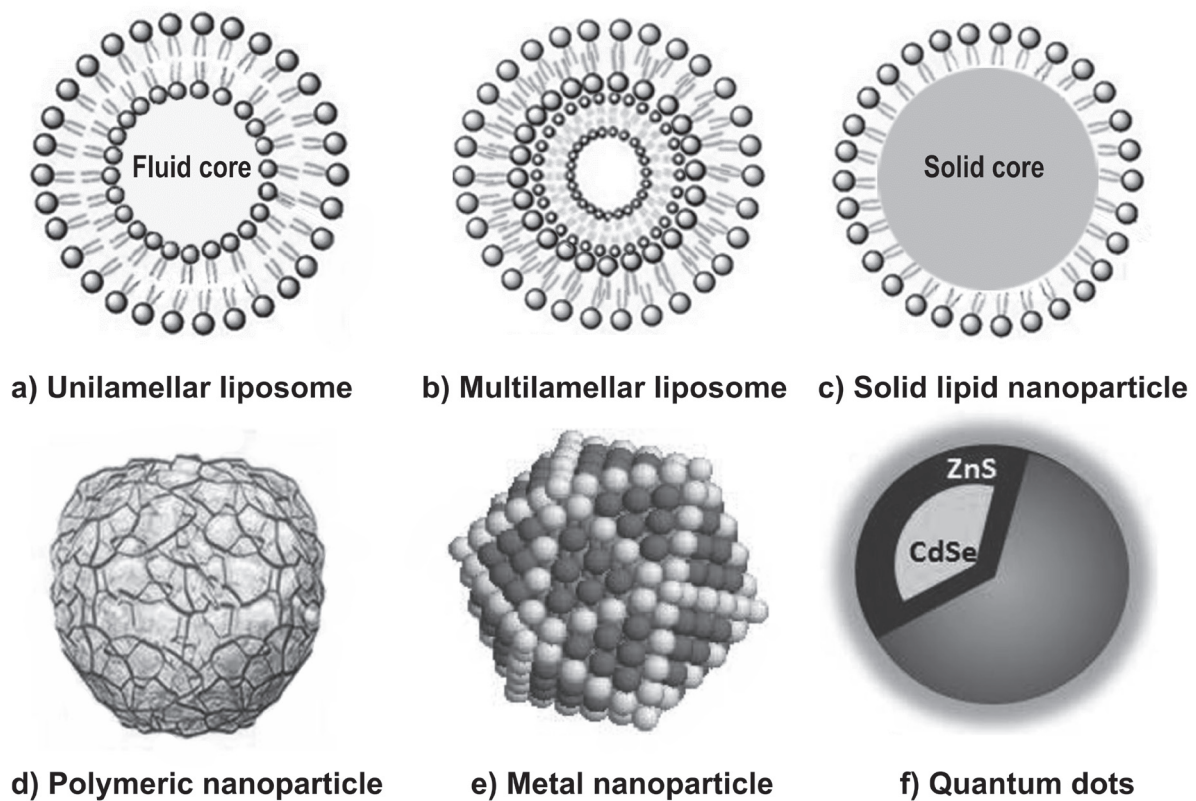
Figure 3. Schematic drawing of different types of nanoparticles.

expertise and co-culture models represent a low or medium throughput assay (Avdeef et al. 2015).

Blood flow-induced shear stress is an important physiological factor regulating vascular endothelial functions. Dynamic blood-brain barrier models have been developed in several laboratories. In the dynamic hollow-fiber flow model the endothelial cells are cultured on the inner side of the capillary tubes, while glial or other cells are situated on the outer side of the hollow tubes (Fig. 2). Flow-induced shear stress improves blood-brain barrier functionality by increasing the expression of tight junction, receptor, transporter and enzyme proteins in brain endothelial cells (Cucullo et al. 2011). Limitation of the system is that cell growth and cell coverage of the tube surface cannot be visualized. The hollow fiber model did not become widespread for transport studies most probably due to its complexity and the need for technical expertise (Avdeef et al. 2015). Recently new versions of the dynamic blood-brain barrier model have been developed; the microfluidic blood-brain barrier models (Avdeef et al. 2015). In these miniaturized co-culture systems brain endothelial cells and glial cells are also separated by a porous membrane, similarly to the static cultures. In blood-brain barrier chip models electrodes are built in for monitoring transendothelial electrical resistance and transparent materials allow the ob-servation of cell morphology. These integrated microfluidic devices may represent the next generation of blood-brain barrier co-culture systems.

Nanoparticles designed for drug delivery to the central nervous system

In the last 20 years the potential of nanoparticles as nanocarriers is increasingly investigated for drug delivery across the blood-brain barrier (Kreuter 2014). An ideal nanocarrier would offer (i) efficient drug delivery across the blood-brain barrier by selective targeting, (ii) protection of the therapeutic cargo from enzymatic degradation, (iii) long circulation time, (iv) self-regulated drug release, (v) avoidance of efflux transport, (vi) low immunogenicity, (vii) good biocompatibility and bioavailability (Mc Carthy et al. 2015). While no carriers fulfil all these requirements, several types of nanoparticles, especially targeted liposomes are getting close to clinical use (Gaillard et al. 2014; Mc Carthy et al. 2015). At the preclinical level nanospheres, nanocapsules and micelles made from various components, such as lipids, polymers and metals are tested for delivering test molecules or drug cargos across the blood-brain barrier. The present review aims to focus only on those types of nanoparticles (Fig. 3) which were tested on

Table 1. Properties of nanoparticle types tested on blood-brain barrier co-culture models.

Type of NP	Composition	Size	Advantage	Limitations
Liposomes	Aqueous core surrounded by phospholipid bilayers	Small unilamellar <100 nm Large unilamellar >100 nm Multilamellar >500 nm	Encapsulation of both hydrophilic and hydrophobic drugs	Poor control over release of the drug Low stability during storage
Solid lipid NPs	Hydrophobic core surrounded by triglycerides, fatty acids and waxes	10-500 nm	Low toxicity, high drug loading capacity and controlled release	Encapsulation of only hydrophobic drugs
Polymeric NPs	Natural or synthetic degradable polymers	10-1000 nm	Stability against enzymatic metabolism	Some have toxic hydrolysis by-products
Metal NPs	Gold, and titanium or iron oxides	1-100 nm	Gold and titanium NPs: inert, ultra small size, absorb and scatter near-infrared light Iron NPs: can be paramagnetic	Gold and titanium NPs: controversy on carcinogenicity Iron NPs: may induce oxidative stress
Quantum dots	Colloidal semi-conductor nanoscale crystals	2-50 nm	Fluorescent targeting and imaging, long-term visualization, extremely high stability to photobleaching	Cytotoxic in high concentrations

co-culture models of the blood-brain barrier (Table 1).

Lipid-based systems

Liposomes are vesicles consisting of one or more phospholipid bilayers (Fig. 3) made from sphingomyelin, phosphatidylcholine or glycerophospholipids (Craparo et al. 2011; Mc Carthy et al. 2015). The structure of liposomes is similar to the cell plasma membranes. Hydrophilic drugs can be encapsulated in the aqueous core, while lipophilic drugs can be entrapped in the phospholipid bilayer of liposomes. Applications of conventional liposomes are limited by the poor control of drug release, low stability during storage, and low plasma circulation time due to elimination by the mononuclear phagocyte system (Fahmy et al. 2005). Decreasing the particle size (<100 nm) or modification of the surface with polyethylene glycol (PEG) result in extended circulation time. While most liposomes are not targeted, existing liposomes were modified for drug targeting to the brain. One such example is the glutathione-targeted PEGylated liposome. This nanocarrier was investigated for the brain delivery of methylprednisolone in a rat model of multiple sclerosis (Gaillard et al. 2012) and of doxorubicin in brain tumor-bearing mice (Gaillard et al. 2014). The results indicated an increased brain uptake for both drugs in comparison to untargeted liposomes and clinical studies are in progress. Angiopeptide, a ligand for low-density lipoprotein receptor-related protein-1 also successfully targeted mitoxantrone loaded liposomes to the brain in tumor implanted mice (Orthmann et al. 2012) proving the applicability of the method.

Solid-lipid nanoparticles are usually composed of triglycerides, fatty acids and waxes (Craparo et al. 2011). They have advantages over other polymeric nanoparticles in terms of lower toxicity, higher drug loading capacity and controlled release over long-time periods. Unlike liposomes, they can encapsulate only hydrophobic drugs, which limits their use (Fahmy et al. 2005). Polysorbate 80 coating, which promotes the adsorption of apolipoprotein E, a ligand of low density lipoprotein receptor to the nanoparticle surface, enhances the uptake of solid lipid nanoparticles into the brain (Goppert et al. 2005).

Polymeric nanoparticles

Among various biopolymers poly(lactic-co-glycolic acid) (PLGA), (poly(lactic acid) (PLA), poly-ε-caprolactone (PCL), chitosan, gelatin and poly-butilcynaoacrylates (PBCA) are some of the biodegradable biopolymers used in nanocarriers for delivering drugs to the brain (Kreuter 2014). Polymeric nanoparticles are more stable than vesicular nanocarriers and can protect their cargo from enzymic degradation (Mc Carthy et al. 2015). These solid nanoparticles have been extensively studied to establish the relationship between and nanoparticle size, surface coating, charge or targeting ligands and the ability of nanoparticles to cross the blood-brain barrier (Table 2). Results with synthetic polymeric and human albumin nanoparticles indicate that they can be successfully targeted to cross the blood-brain barrier and enter the brain (Kreuter 2014). The degradation of PBCA nanoparticles by hydrolysis leads to the production of toxic metabolites, therefore PLGA, PLA or albumin nanoparticles are preferably used for brain delivery.

Metal nanocarriers

Metal nanoparticles are versatile and applied in several fields such as medical instruments and devices, water treatment, and food processing. The most investigated metal nanoparticles

Table 2. Summary of experiments testing nanoparticles on co-culture BBB models.

Nanoparticle type	Composition	Targeting ligand or coating	Cargo	Nanoparticle size (nm)	Brain endothelial cells	Co-culture cell type	Result	Ref.
Lipid NPs								
	Fluorescent magneto-liposomes	Transferrin	No	130	Primary human	Human astroglia	Increased NP permeability	Ding et al. 2014
Solid lipid NPs	Solid lipid	Anti-insulin receptor mAb	Carmustine	100-450	Primary human	Human astroglia	Increased NP uptake and permeability	Kuo et al. 2013b
	Solid lipid	Anti-insulin receptor mAb	Saquinavir	120-450	Primary human	Human astroglia	Increased NP uptake and permeability	Kuo et al. 2013a
Metal NPs								
	Fe$_3$O$_4$ (magnetic)	No	BDNF	60	Primary human	Human astroglia	Magnetically guided NP permeability	Pilakka et al. 2013
	Gold	Peptide recognizing trasferrin receptor	β-sheet breaker peptide	15	Primary bovine	Rat astroglia	Increased NP permeability	Prades et al. 2012
	TiO$_2$	No	No	25	Primary rat	Rat astroglia	NP uptake, transport and cellular toxicity	Brun et al. 2012
Polymer NPs								
	PBCA	Cationic charge, lipid coating	Albumin	60	Primary bovine	Rat glia	Increased NP transcytosis	Fenart et al. 1999
	PBCA	Polysorbate-80	Dalargin	200	Primary bovine	Rat glia	Increased BBB model permeability	Olivier et al. 1999
	PBCA	Polysorbate-80	Dalargin	300	Primary bovine	Rat astroglia	No toxicity	Kreuter et al. 2003
	PBCA	Ligand of diphtheria toxin receptor (CRM197)	Zidovudine	87, 163, 195	Primary human	Human astroglia	Increased NP uptake and permeability	Kuo et al. 2012b
	PHDCA	PEG	No	166, 171	Primary rat	Rat astroglia	Increased NP translocation	Garcia et al. 2005
	PEG-PLA	Cationic BSA	6-Coumarin	100	Primary rat	Rat astroglia	Increased NP uptake	Lu et al. 2005
	PLGA	Tween-20, BSA, Transferrin	DiI fluorescent dye	63-90	Primary bovine	Rat astroglia	Increased NP endocytosis	Chang et al. 2009
	MMA-SPM	bradykinin type II receptor agonist	Stavudine, Delavirdine, Saquinavir	48, 13, 8	Primary human	Human astroglia	Increased NP uptake and permeability	Kuo et al. 2012a
Quantu dots								
	Quantum dots	No	siRNA for MMP9	15-20	Primary human	Human astroglia	NP uptake and MMP9 gene silencing	Bonoiu et al. 2009
	Quantum rods	Transferrin	No	26 nm length, 6.5 nm width	Primary human	Human astroglia	Concentration- and time-dependent NP crossing	Xu et al. 2008
	Silica NPs and quantum dots	Amino-, carboxyl-, and PEGylated-Qdots	No	30, 100, 400	Primary rat	Rat astroglia and rat pericyte	Size- and amino group dependent NP permeability	Hanada et al. 2014

BSA: bovine serum albumin. CRM197: a ligand of diphtheria toxin receptor. mAb: monoclonal antibody. MMA-SPM: methylmethacrylate–sulfopropylmethacrylate. MMP9: matrix metalloproteinase-9. N.D.: no data. PBCA: poly(butylcyanoacrylate). PEG: polyethylene glycol. PHDCA: poly(hexadecylcyanoacrylate). PLA: poly-lactide. PLGA: poly(lactic-co-glycolic acid). Qdots: quantum dots. RMP-7 (Cereport): bradykinin type II receptor agonist.

for central nervous system delivery include gold, titanium and iron oxides (Mc Carthy et al. 2015). Gold nanoparticles are inert and can be produced in sizes as small as 1-10 nm. Their imaging is easy because they absorb and scatter near-infrared light and they are electron dense in transmission electron microscopy. Gold nanorods can deliver siRNA for gene silencing to brain endothelial cells (Bonoiu et al. 2009) and target the transferrin receptor for crossing the blood-brain barrier (Prades et al. 2012).

Iron oxide nanoparticles are investigated for various biomedical applications targeting the brain, including imaging, tumor therapy, and drug delivery (Ito et al. 2005). The superparamagnetic properties enable both the tracing of nanoparticles by magnetic resonance imaging, and targeting to selected tissues or areas with application of magnetic fields (Ito et al. 2005). Using an iron nanocarrier and magnetic guidance brain derived neurotrophic factor was delivered across a culture model of the blood-brain barrier (Pilakka et al. 2013). Uncoated iron nanoparticles may aggregate which is enhanced by external magnetic field. Surface modification of iron nanoparticles like polymer coating is necessary to prevent aggregation and vectors or magnetic guidance are needed for targeting (Ito et al. 2005).

Titanium dioxide nanoparticles can be found in various products like paints, food additives and cosmetics. Since they may pose an environmental risk their toxicity was tested on a blood-brain barrier model. Titanium dioxide nanoparticles damage the integrity of brain endothelial cell layers (Brun et al. 2012). Although metal nanoparticles are generally considered safe, the carcinogenicity of gold and titanium nanoparticles is controversial, and iron nanoparticles may induce oxidative stress.

Quantum dots

Quantum dots are colloidal semi-conductor nanocrystals (<50 nm) with excellent fluorescent properties including high brightness, stability against photobleaching, broad absorption spectra, and a tunable and narrow emission spectrum (Mc Carthy et al. 2015). The surface of the quantum dots can be easily functionalized by PEG, amino, or carboxyl groups. Among these modifications the presence of amino groups increased the brain endothelial permeability of quantum dots using a triple co-culture blood-brain barrier model (Hanada et al. 2014).

Practical considerations for the design of nanoparticles for central nervous system delivery and their testing on co-culture models of the blood-brain barrier

Uptake or transport of nanoparticles at the blood-brain barrier is dependent on physicochemical characteristics, such as size, surface charge, coating or functionalization by targeting ligands (Kreuter 2014). The size of nanocarriers developed for brain drug delivery ranges from 10 to 1000 nm (Craparo et al. 2011). No linear correlation between nanoparticle size and permeability can be established (Kreuter 2014). Using fluorescent silica nanoparticles of 30, 100 and 400 nm size the permeability coefficient of the 30 nm nanoparticles was the highest on a triple blood-brain barrier co-culture model (Hanada et al. 2014) (Table 2). This result is in agreement with data of animal experiments testing the effect of nanoparticle size on distribution to brain: accumulation of small nanoparticles (10-50 nm) in brain tissue was observed as compared to nanoparticles of >200 nm in size (Hillyer et al. 2001).

In addition to size, the surface charge also affects the uptake and transcytosis of nanoparticles. The luminal surface of brain endothelial cells contains glycocalyx residues that establish a strong negative charge which contributes to the barrier phenotype (Hervé et al. 2008). Cationic molecules or drugs have higher penetration across the blood-brain barrier mediated by adsorptive mediated transcytosis (Deli 2011). Cationization is one of the methods to increase nanoparticle delivery across the blood-brain barrier. Lipid coated cationic nanoparticles enhanced the transport of albumin in a blood-brain barrier co-culture model (Fénart et al. 1999) (Table 2). Increased brain penetration is observed for cationic nanoparticles in animal studies, too (Lu et al. 2005), but the excess positive charge also leads to the formation of a protein corona in blood and rapid removal of the particles from the circulation by the phagocyte system (Mc Carthy et al. 2015). Coating of cationic nanocarriers by neutral, hydrophilic polymers such as PEG results in enhanced stability, prolonged circulation time (Mc Carthy et al. 2015) and increased passage across a blood-brain barrier model (Garcia-Garcia et al. 2005).

Receptor-mediated transport at the blood-brain barrier is responsible for the brain penetration or clearance of peptides and proteins, like transferrin, leptin, ghrelin or low density lipoprotein (Abbott et al. 2010; Campos-Bedolla et al. 2014). This transcytotic caveolae-mediated pathway allows the specific targeting of large molecules, such as biopharmaceuticals or nanoparticles to the central nervous system (Deli 2011; Pardridge 2012). Nanoparticles targeting the transferrin receptor at the blood-brain barrier can be labeled by transferrin (Xu et al. 2008; Chang et al. 2009; Ding et al. 2014), or by a peptide sequence recognizing the receptor (Prades et al. 2012). All these particles show increased permeability across blood-brain barrier co-culture models (Table 2). Monoclonal antibodies which bind to insulin receptor increase the penetration of solid lipid nanoparticles across brain endothelial cells and the brain delivery of the drug cargo in animals (Kuo et al. 2013a, 2013b) similarly to the effect of insulin as targeting ligand for nanoparticles (Shilo et al. 2014). A blood-brain barrier receptor for membrane-bound precursor of heparin

binding epidermal growth factor-like growth factor also acts as a receptor for diphteria toxin. A safe, clinically used ligand of this blood-brain barrier receptor, CRM197 is effective to target drug-carrying polymer nanoparticles across brain endothelial cells (Kuo et al. 2012b). Coating nanoparticles by polysorbate 80 also results in enhanced penetration across the blood-brain barrier (Olivier et al. 1999; Kreuter et al. 2003). The mechanism is the adsorption of apolipoprotein E from plasma to the surface of injected nanoparticles followed by receptor mediated transcytosis via lipoprotein receptors, like low density lipoprotein receptor across the blood-brain barrier (Goppert et al. 2005; Kreuter 2014). Active targeting to the brain can be also achieved by ligands of blood-brain barrier solute carriers, like glutathione for both liposomal drug formulations (Gaillard et al. 2012, 2014) and polymeric nanoparticles (Grover et al. 2014).

Another approach is the use of tight junction modifying/opening molecules (Fig. 1), such as the bradykinin agonist RMP7, which enhances the delivery of antiretroviral drugs loaded in polymeric nanoparticles when tested on blood-brain barrier co-cultures (Kuo et al. 2012a). These data indicate that all major strategies used for drug delivery to central nervous system can be successfully applied to nanoparticle delivery or targeting. Systematic studies comparing active targeting by different ligands or different delivery strategies across blood-brain barrier models would be necessary to evaluate the efficacy of these methods.

Co-culture models of the blood-brain barrier are considered the most complex and also the most reliable for prediction of drug penetration to brain based on *in vivo-in vitro* correlations (Veszelka et al. 2011; Hellinger et al. 2012; Avdeef et al. 2015). Such correlations are not available for nanocarriers, but a couple of studies provide data on nanoparticle delivery using both animal and culture blood-brain barrier models (Mc Carthy et al. 2015). For most cases there is concordance between the results, especially, when the same species is used for both culture and animal studies and when primary cultures are used for blood-brain barrier models. Discrepancy between results arises from the use of brain endothelial cell lines as culture blood-brain barrier models, or different species for *in vivo* and *in vitro* experiments. There are two likely explanations of these contradictions: (i) brain endothelial cell lines express low levels of key blood-brain barrier transporters, like glucose transporter-1 or P-glycoprotein as compared to freshly isolated brain caillaries or primary cultures (Urich et al. 2012) and (ii) different blood-brain barrier transporter and receptor levels can be detected in different species (Uchida et al. 2011). Based on these considerations immortalized brain endothelial cell lines or epithelial cell line-based surrogate models are not recommended for testing nanoparticle transport. Such simplified systems may be used for preliminary screening but results need to be confirmed on more complex blood-brain barrier culture models. There

are no data on dynamic blood-brain barrier or chip models to test nanoparticle passage, but these systems can be useful to reveal the effect of flow on nanoparticle interaction with brain endothelial cells. In addition to uptake and transport experiments cellular toxicity tests are important applications of blood-brain barrier models and are recommended as the first step of all nanoparticle studies.

Conclusion

In summary, nanoparticle based drug delivery to brain is a rapidly expanding research field with a great therapeutic potential. Blood-brain barrier co-culture models are useful tools to test the toxicity, uptake and transcellular transport of different nanocarriers developed for central nervous system drug targeting or delivery. In addition to providing estimation for brain penetration, the mechanism of the delivery or targeting can be also revealed by these models. Active targeting of vesicular or solid nanoparticles by ligands of receptors or transporters present at the blood-brain barrier enhances brain delivery in preclinical models and clinical studies on actively targeted nanocarriers for treatment of neurological diseases including brain tumors are in progress. Culture-based blood-brain barrier models will greatly contribute to the development of novel, even more specific nanoparticle drug delivery platforms to treat central nervous system diseases.

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