Effect of Electromagnetic Field on Vesicular Endocytosis and Blood-Brain Barrier Permeability of Saquinavir-Entrapping Cationic Solid Lipid Nanoparticles

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Abstract: In the present study, cationic solid lipid nanoparticles (CSLNs) were fabricated as carriers for the transport of saquinavir (SQV), an anti-human immunodeficiency virus agent, across the blood-brain barrier (BBB) exposed to electromagnetic field (EMF). The immunocytochemical staining revealed that uptake of SQV-entrapping CSLNs by human brain-microvascular endothelial cells (HBMECs) was via vesicular endocytosis. Amount of vesicular clathrin in HBMECs during CSLN uptake was enhanced by EMF exposure. However, apparent variation in the expression of P-glycoprotein was not observed by treatment of CSLNs and/or EMF. As compared with molecular SQV, the BBB permeability of SQV-entrapping CSLNs became 17fold. The permeability of molecular SQV across HBMEC monolayer was slightly increased by exposure to amplitude modulation (AM) and frequency modulation (FM) EMF. It was worth to note that synergistic effect on improvement in the BBB permeability of SQV was obtained by combination of the present CSLNs and the appropriate EMF exposure.

Key-Words: Cationic solid lipid nanoparticle; Electromagnetic field; Vesicular endocytosis; Blood-brain barrier; Permeability; Saquinavir

1 Introduction
The central nervous system is regulated by three barriers for material exchange with the circulation system. Among the three barriers, the blood-brain barrier (BBB) actively mediates the brain microenvironment because BBB contacts directly with the brain cells. Brain-microvascular endothelial cells (BMECs), play a central role in BBB, and in BMECs, transmembrane proteins such as occludins and claudins form intercellular tight junctions (TJs). TJs not only restrain paracellular permeability but also exhibit polarized apical-basal properties of BMECs. Hence, merely small water-soluble molecules may transfer through TJs. For TJ proteins, the major function of occludins is related to integral TJ strands, and expressions of claudin 3 and claudin 5 are responsible to large trans-endothelia electrical resistance (TEER). A high density of TJs with high complexity also causes a large TEER. Thus, TEER becomes a simple analytical technique for quantitative measurement of TJs. On the other hand, the majority of substances across BBB are via transcellular pathways, which include transcellular lipophilic pathways, transport proteins, receptor-mediated transcytosis (RMT), and adsorptive-mediated transcytosis (AMT). For nanoparticulate carrier system, the mechanisms for drug delivery across BMECs are particle-BMEC interaction via RMT and release via AMT. During RMT and AMT, vascular structure emerges. In the transport of low density lipoprotein (LDL) across BBB, gold-labeled LDL in vesicles was identified by electron microscope to demonstrate RMT mechanism. Moreover, uptake of long-chain fatty acids was mediated by vesicles covered with clathrin. Note that the clathrin-covering vesicles are a trait for RMT, not for AMT. For drug efflux transporters, P-glycoprotein (P-gp), belonging to multidrug resistance transporter, has been verified to be present in lumen-facing side of BMECs. The removal of cyclosporin A and vincristine from the brain was asserted to be related to the P-gp efflux performances. In a brain perfusion model, transport of paclitaxel across BBB was inhibited by P-gp, and the BBB permeability of paclitaxel was significantly enhanced by entrapment in nanoparticles. Exposure to electromagnetic field (EMF) yielded increased BBB permeability [1]. For EMF with 10 mW/cm² and 2.54 GHz or 2.8 GHz, 2-hour exposure affected distribution of horseradish peroxidase (HRP) in the brain; however, variation in the BBB structure
by EMF was temporary. Also, by exposure to EMF with 2.54 GHz, no extravasation of Evans blue-serum albumin complex into the brain parenchyma was observed, indicating no BBB disruption. For EMF with frequency of 217 Hz, carrier frequency of 900 MHz and pulse duration of 580 μs, exposure exceeding 10 min caused an increased rate of fluid-phase endocytosis by ca. 1.5fold.

In the present study, the BBB permeability of saquinavir (SQV), a protease inhibitor for therapy of the acquired immunodeficiency syndrome, was ameliorated by introducing positively charged carriers and EMF. Low BBB-permeability SQV was entrapped in cationic solid lipid nanoparticles (CSLNs). The impact and biocompatibility of CSLNs on human BMECs (HBMECs) exposed to EMF was assayed by TEER and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-bromide (MTT). Moreover, RMT clathrin and efflux P-gp were characterized by immunocytochemical staining. Finally, HBMECs-based in vitro BBB model was applied to investigate transport of the present SQV-entrapping CSLNs with exposure to appropriate EMF.

2 Materials and methods
2.1 Reagents and chemicals
Anti-human von Willebrand factor VIII, dioctadecyldimethyl ammonium bromide (DODAB), anti-rabbit IgG with fluorescein isothiocyanate (FITC) conjugate, FITC-dextran 70000, MTT, and MTT solubilization solution were purchased from Sigma (St. Louis, MO). Mouse monoclonal to clathrin-membrane vesicle marker and mouse monoclonal to P-gp were characterized by immunocytochemical staining. HBMECs was purchased from Biocompare (South San Francisco, CA), SQV from United States Pharmacopeial (Rockville, MD), cacao butter (CB) from OCG Cacao (Whitinsville, MA), Compritol 888 ATO (CA) from Gattefosse (Codex, France), stearylamine (SA) from Fluka (Buchs, Switzerland), anti-ZO-1 from Zymed (South San Francisco, CA), Alexa Fluor® 594 goat anti-mouse SFX kit from Invitrogen (Paisley, UK), and Triton-X-100 from Acros (Geel, Belgium).

2.2 Preparation of CSLNs
SQV-entrapping CSLNs were prepared by microemulsion method. Briefly, melted 1.85% (w/w) CA, 1.85% (w/w) CB, 0.3% (w/w) SQV, and 1% (w/w) mixture of DODAB and SA were mixed with 8% polysorbate 80, 7.5% (w/w) ethanol, and 79.5% (w/w) ultrapure water at 75°C. The emulsified fluid was added into ultrapure water at 500 rpm and 3°C for 20 min. Suspended CSLNs were filtrated and centrifuged (Hitachi Koki, Tokyo, Japan) at 236500 xg for 30 min. Resuspended CSLNs with 2% (w/v) D-mannitol were refrigerated (Sanyo, Osaka, Japan) at -80°C and lyophilized (Eyela, Tokyo, Japan) over 24 h. Particle diameter of CSLNs was obtained by a Zetasizer 3000 HS A with photo correlation spectroscopy (Malvern, Worcestershire, UK). For fluorescent CSLNs, SQV was replaced by FITC-dextran 70000. 0.5% (w/v) SQV-entrapped CSLNs in Dulbecco’s phosphate buffered saline (DPBS) containing 0.05% sodium azide were degraded by agitation with a baths-reciprocal shaker at 60 rpm and 37°C over 24 h.

2.3 Evaluation of HBMEC monolayer
2.3.1 Cultivation of HBMECs
HBMECs were cultured by the method described previously with modifications [2]. Briefly, HBMECs were seeded on a human fibronectin (HFN)-treated culture dish in endothelial cell medium (ECM) containing 5% fetal bovine serum (FBS), 1% penicillin/streptomycin solution, and 1% endothelial cell growth supplement and placed in a humidified, 37°C CO2 incubator (NuAire, Plymouth, MN).

2.3.2 Staining of tight junctions
Immunohistochemical staining of tight junctions (TJs) were described previously with modifications [3]. Briefly, 14-day cultivated HBMECs were exposed to the standard amplitude modulation (AM) EMF with square wave, power of 5 mW, modulation of 20 MHz, depth of 100%, and frequency of 915 MHz. The electromagnetic signals were generated by a radiofrequency synthesizer (Hameg, Mainhausen, Germany) connected to a cylindrical copper coil, as illustrated on the top of Fig.1.

![Fig.1. Schematic representation of transport system across the in vitro BBB exposed to EMF.](image-url)
HBMEC film was desiccated by formalin, incubated with anti-ZO-1 and anti-rabbit IgG with FITC conjugate in the CO₂ incubator. Fluorescent images of TJ s were obtained by argon laser and FITC filter for excitation at 458 nm and emission at 488 nm under an inverted phase contrast fluoromicroscope (TE2000-U, Nikon, Tokyo, Japan).

2.3.3 Determination of TEER
TEER of HBMEC monolayer was assessed by the methods described previously with modifications [1]. Briefly, HBMECs were seeded on Millicell® polycarbonate (PC) membranes and cultivated in the CO₂ incubator over 14 days. ECM containing 0.0125% (w/v) CSLNs was applied to the HBMEC culture for 4 h, and TEER was determined by the Millicell electrical resistance system (Millipore, Bedford, MA). HBMEC film was also exposed to the standard AM EMF, and TEER was detected in fresh ECM.

2.3.4 Estimation of cytotoxicity
Cytotoxicity of HBMECs was analyzed by the methods described previously with modifications [2]. Briefly, HBMECs were treated with 0.0125% (w/v) CSLNs and/or exposure to the standard AM EMF. HBMECs were reacted with MTT in the CO₂ incubator. After MTT removal, MTT solubilization solution was employed. 200 μL of solution specimen was detected by a UV-visible spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 570 nm.

2.4 Characterization of endocytosis
2.4.1 Staining of vesicular clathrin
HBMECs were cultivated with 0.0125% (w/v) fluorescent CSLNs in the CO₂ incubator. HBMEC culture was exposed to the standard AM EMF or the standard frequency modulation (FM) EMF with square wave, power of 5 mW, modulation of 20 MHz, deviation of 400 kHz, and frequency of 915 MHz. Cultured HBMECs were fixed by methyl alcohol, treated with Triton-X-100, reacted with monoclonal anti-clathrin heavy chains, and Alexa Fluor® 594 goat anti-mouse SFX kit. The relations between vesicular clathrins and CSLNs were observed under a confocal laser scanning microscope (CLSM, LSM 510, Zeiss, Oberkochen, Germany) for red wavelength at 550 nm and green wavelength at 488 nm.

2.4.2 Staining of P-gp
The treatments for HBMECs with CSLNs and/or EMF were the same as those for clathrin except antibodies. HBMECs were reacted with monoclonal anti-P-gp JSB-1, anti-human von Willebrand factor VIII, Alexa Fluor® 594 goat anti-mouse SFX kit, and anti-rabbit IgG with FITC conjugate. The images of P-gp and CSLNs were analyzed by the CLSM.

2.5 Transport of SQV across the in vitro BBB
An in vitro BBB transport system was described in the previous study with modifications [4]. Schematic representation of the system exposed to EMF was illustrated in Fig.1. Amount of transported SQV was assessed by a high performance liquid chromatography (Jasco, Tokyo, Japan) connected with a UV-visible spectrophotometer (UV-2075 Plus, Jasco, Tokyo, Japan) at 239 nm using two high pressure pumps (PU-2080 Plus, Jasco, Tokyo, Japan) impelling mobile phase containing gradient of acetonitrile. The permeability coefficient of SQV across HBMEC monolayer, $P_{\text{HBMEC}}$, was evaluated by formulas taking mass resistance into account [3].

3 Results and discussion
3.1 TJ s among HBMECs
Fig.2 shows the image of immunocytofluorescent staining of TJ s exposed to the standard AM EMF. As revealed in this figure, structured green spiderweb among round-shaped black HBMECs, indicating that the apparent characteristics of TJ s and the main trait of BBB were preserved. EMF exposure did not mutilate the integrity of TJ s, which were composed of transmembrane proteins such as claudin, occludin, and other junctional adhesion molecules. Zonula occludens (ZO) including ZO-1, ZO-2, and ZO-3 could bind to claudin and occludin, and connect with intracellular actin, forming a fixed inseparable contact among adjacent HBMECs.

Fig.2. Typical fluorescent image of TJ s among HBMECs exposed to the standard AM EMF.

3.2 Effect of CSLNs and EMF on TEER
Fig.3 shows the effect of composition of cationic lipids on TEER. TEER represented confluent level of the in vitro HBMEC monolayer and quantitatively analyzed the feature of TJ s. In the present HBMEC monolayer without CSLNs, TEER was 163 ± 3.7 Ω·cm². As revealed in Fig.3, TEER reduced by the treatment with CSLNs. This was because the
electricity of the HBMEC monolayer was neutralized by CSLNs. In a study of transport of positively charged poly(amidoamine) (PAMAM) dendrimers across Caco-2 monolayer, TEER reduced as time and uptake of PAMAM increased. As displayed in Fig.3, an increase in SA level yielded an increase in TEER. This was because CSLNs with higher amount of SA caused a lower electrostatic effect, rendering lower impact on the HBMEC monolayer. Exposure to EMF led to a slightly reduced TEER. This might be resulted from charge regulation on the membrane of HBMECs [5], rendering higher cellular conductivity. For transformed HBMECs, TEER of the monolayer cultured over 4 to 7 days was greater than 120 Ω⋅cm², suggesting sufficient confluence for in vitro analysis.

Fig.4 shows the influence of composition of cationic lipids on the viability of HBMECs. As indicated in this figure, the cellular viability was about 98% for the case of HBMECs treated with CSLNs. Also, the cellular viability was about 94% for the case of HBMECs treated with CSLNs and exposed to EMF. For the above two cases, the cellular viability was nearly independent on SA content in CSLNs. The present EMF caused little lethal effect on HBMECs. Also, the highest death rate was 2% when yeasts exposed to EMF with radiofrequency of 900 MHz and AM of 217 pulses per second [6]. However, uptake of nanoparticles by HBMECs was more obvious by EMF exposure [1].

In an investigation on viability of murine peritoneal cells by the influence of lipids, it was concluded that CA caused no apparent toxic effect, DODAB yielded noticeable cytotoxicity, and stearic acid generated highest toxic level. In an investigation on viability of mouse J774 macrophages, mouse 3T3 fibroblasts, and human HaCaT keratinocytes, it was observed that soybean oil-SA system resulted in significant reduction in viability and stearic acid-SA system led to even higher cytotoxicity. For degraded CSLNs, an increase in SA level caused a decrease in viability of HBMECs, as displayed in Fig.4. This was because SA was more toxic to HBMECs than DODAB. In a study on Tween 80- and Span 20-stabilized CSLNs composed of CA and cetylpalmitate as internal lipids, cytotoxic influence on African green monkey kidney fibroblast-like cell line COS-1 of one-tailed cationic lipid such as SA was comparatively high and cytotoxicity of two-tailed lipid such as DODAB was in the acceptable range.
3.4 Vesicular endocytosis of CSLNs by HBMECs

Fig. 5 shows the relation between endocytosed CSLNs and vesicles. As exhibited in Fig. 5 (a), red clathrins circled vesicular periphery and the green CSLNs were situated inside the vesicles. These images suggested that the uptake of CSLNs by HBMECs could be resulted from RMT of vesicles. The reasons of the RMT-related behavior were elaborated below. Drug carriers mimicked LDL particles by covering a film of polysorbate 80 with the anchorage of apolipoprotein E (apo E). Hence, CSLNs with an external layer of polysorbate 80 were endocytosed by HBMECs via LDL receptors, i.e., the RMT mechanism. Note that apo E, a fragment of lipoprotein, could be recognized by LDL receptors. As presented in Fig. 5 (b) and (c), uptake of CSLNs exposed to EMF could also invoke the RMT mechanism. When neutrophils exposed to pulsing EMF with low frequency and low power, density and function of A3 adenosine receptors were enhanced [7]. Besides, EMF with low intensity radiofrequency affected binding between ligands and receptors on cellular membrane, rendering alteration in the distribution of membrane proteins. As exhibited in Fig. 5, EMF could induce additional vesicles in HBMECs, inferring that the functions of LDL receptors and the RMT-related behavior could be promoted by appropriate EMF exposure.

3.5 Expression of P-gp on HBMECs during uptake of CSLNs

Fig. 6 shows the distribution of P-gp and CSLNs on HBMECs. Red patches and green spots were P-gp and CSLNs, respectively. As indicated in Fig. 6 (b), (c), and (d), uptake amount of CSLNs followed the order of AM EMF exposure > FM EMF exposure > without EMF, which was consistent with the result of Fig. 4. Also, amount of P-gp expressed on HBMECs were almost irrelevant to the EMF exposure and the uptake amount of CSLNs. Note that P-gp is a membrane protein. The typical state of cellular membrane at physiological temperature could be categorized as liquid crystal. If thermal effect of EMF led to increase in local temperature, the liquid-crystal membrane became mobile, rendering protein dislocation and detachment. These images implied that the present EMF conditions caused no such thermal effect and no evident injury to the HBMEC-based BBB model.

3.6 Permeability of SQV across HBMEC monolayer exposed to EMF

Table 1 listed the permeability coefficients of SQV across BBB exposed to EMF for three compositions of cationic lipids in CSLNs. EMF enhanced the BBB permeability for SQV-entrapping CSLNs and molecular SQV because HBMECs exposed to EMF might slightly increase the permeability in cellular membranes. As compared with molecular SQV without EMF, the BBB permeability of SQV-entrapping CSLNs became ca. 22 fold for AM EMF and 21 fold for FM EMF, indicating synergistic effect of CSLNs and EMF. Bilayer permeability in liposomes on the basis of egg lecithin multilamellar vesicles was enhanced by exposure to EMF of 900 MHz for 5 h. However, the main reasons for considerable increase in the BBB permeability for SQV-entrapping CSLNs were the EMF-induced RMT and the particle-prevented P-gp efflux, which were evidenced by the images in Figs. 5 and 6.
The BBB permeability of AM EMF was slightly higher than that of FM EMF. These were consistent with our previous results [1]. The reasons were explained below. In a study on activity of ornithine decarboxylase (ODC) in L929 murine cells exposed to EMF of 835 MHz, modulation of 60 Hz, and sinusoids for 8 h, ODC activity of AM with depth of 23% was higher than that of FM with deviation of 60 kHz. During biosynthesis of polyamines, the key enzyme of ODC played a role in the rate-determining step. By cold injury, amount of polyamines and ODC in rat cerebrum increased and amount of abluminal and luminal endocytic pits in BMECs also increased. It was concluded that an increase in polyamines stimulated endocytosis and vesicular transport, leading to the promoted BBB permeability. Besides, polyamine-modified superoxide dismutase, insulin, albumin, and immunoglobulin G improved the BBB permeability in normal adult rats. Thus, it could be inferred that exposure to EMF activated ODC activity in HBMECs and increased amount of polyamines induced vesicular endocytosis, where AM EMF was more influential to the ODC activity in the present BBB system than FM EMF.

Table 1. Permeability coefficient, $P_{\text{HBMEC}}$ (cm/s).

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<tr>
<th>EMF</th>
<th>Sample</th>
<th>$P_{\text{HBMEC}} \times 10^6$</th>
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<tr>
<td></td>
<td>Molecular SQV</td>
<td>0.96 ± 0.19</td>
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<td></td>
<td>$P_{\text{SA/(DODAB+SA)}}$</td>
<td>0.67 15.92 ± 0.74</td>
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<tr>
<td>Without EMF</td>
<td></td>
<td>0.33 16.79 ± 0.52</td>
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<tr>
<td></td>
<td>Molecular SQV</td>
<td>1.28 ± 0.16</td>
</tr>
<tr>
<td>AM</td>
<td>$P_{\text{SA/(DODAB+SA)}}$</td>
<td>0.67 20.58 ± 0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.33 22.14 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>Molecular SQV</td>
<td>1.21 ± 0.22</td>
</tr>
<tr>
<td>FM</td>
<td>$P_{\text{SA/(DODAB+SA)}}$</td>
<td>0.67 19.46 ± 0.71</td>
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<tr>
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<td>0.33 20.82 ± 0.66</td>
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4 Conclusion

CSLNs were prepared with various components including nonionic lipids of CA and CB and cationic lipids of SA and DODAB for SQV entrapment and transport across the in vitro BBB model. TEER and cellular viability indicated that the cultivated HBMEC monolayer exposed to radiofrequency EMF was appropriate for the study on the BBB permeability of SQV-entrapping CSLNs. As compared with freshly prepared CSLNs, cytotoxicity of degraded CSLNs slightly increased and the higher the SA content, the higher the toxicity to HBMECs. EMF enhanced the formation of endothelial vesicles; however, P-gp expression was almost irrelevant to EMF exposure. As compared with molecular SQV without EMF, the BBB permeability of SQV became ca. 17fold for CSLNs, ca. 22fold for CSLNs and AM EMF, and ca. 21fold for CSLNs and FM EMF. Combination of CSLNs and EMF can be a potential strategy of the clinical treatment for the acquired immunodeficiency syndrome and other brain-related diseases.

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References:


