Medical applications of nanobubbles

Echogenic bubble formulations have wide applications disease diagnosis and therapy. Therefore, nanobubbles were prepared and the contrast agent function was evaluated in order to study the nanosized bubble’s property for ultrasonic imaging. For this purpose Coumarin-6 as a model drug was loaded into nanobubbles to investigate the drug delivery potential to cells. The results showed that the nanobubbles composed of 1% of Tween 80, and 3 mg/ml of lipid worked well as an ultrasonic contrast agent by presenting a contrast effect in the liver region in vivo. The drug-loaded nanobubbles could enhance drug delivery to cells significantly, and the process was analyzed by sigmoidally fitting the pharmacokinetic curve. It can be concluded that the nanobubble formulation is a promising approach for both ultrasound imaging and drug delivery enhancing.

The ability of oxygen-loaded chitosan bubbles to exchange oxygen in the presence or in the absence of Ultrasound is studied. Results shows that Oxygen delivery is enhanced by sonication and both frequency and time duration of Ultrasound affected the exchange kinetics.

Nanobubbles with measured dimensions of 30 nm wide and 1 nm high on a hydrophobic Au (111) surface in air-saturated water can be observed using tapping mode atomic force microscopy with a Q-control system. The existence of these nanobubbles implies that smaller, unstable ones may be present on a single extended hydrophobic surface or in water confined to hydrophobic pores on the nanometer scale. This leads to propose an idea that a direct obstruction of the pore of a ligand-gated ion channel can arise from the deposition of anaesthetic molecules at a gating region with a relatively large hydrophobic inner surface area in the pore.

From the viewpoint of safety, non-viral vector systems represent an attractive gene delivery system for gene therapy. However, the transfection efficiency of non-viral vectors in vivo is generally very low. Microbubbles, utilized as imaging agents for diagnostic echocardiography, could promote gene delivery into cells when combined with ultrasound exposure. Researchers therefore developed novel liposomal bubbles (Bubble liposomes) containing the lipid nanobubbles of perfluoropropane which is used as ultrasound imaging agent. These Bubble liposomes were smaller in diameter than conventional microbubbles and induced cavitation upon exposure to ultrasound. These results suggested that cavitation of these Bubble liposomes could be an efficient approach for delivering plasmid DNA into cells. In addition, in in vivo gene delivery, the combination of Bubble liposomes and ultrasound provided more effective gene delivery than conventional lipofection methods, further suggesting that Bubble liposomes could be effective as a non-viral vector system in in vivo gene delivery.

Bubble liposomes (liposomes which entrap an ultrasound imaging gas) may constitute a unique system for delivering various molecules efficiently into mammalian cells in vitro. In this study, Bubble liposomes were compared with cationic lipid (CL)-DNA complexes as potential gene delivery carriers into tumor in vivo. The delivery of genes by Bubble liposomes depended on the intensity of the applied ultrasound. Transfection efficiency plateaued at 0. 7 W/cm2 ultrasound intensity. Bubble liposomes efficiently transferred genes into cultured cells even when the cells were exposed to ultrasound for only 1 s. In addition, Bubble liposomes could introduce the luciferase gene more effectively than CLDNA complexes into mouse ascites tumor cells and

solid tumor tissue. This research conclude that the combination of Bubble liposomes and ultrasound is a minimally-invasive and tumor specific gene transfer method in vivo.

In dendritic cell (DC)-based cancer immunotherapy, it is important that DCs present peptides derived from tumor-associated antigens on MHC class I, and activate tumor-specific cytotoxic T lymphocytes (CTLs). However, MHC class I generally present endogenous antigens expressed in the cytosol. Therefore one innovative approach capable of directly delivering exogenous antigens into the cytosol of DCs; i. e., a MHC class I-presenting pathway is developed. In this study, the effect of antigen delivery using perfluoropropane gas-entrapping liposomes (Bubble liposomes, BLs) and ultrasound exposure on MHC class I presentation levels in DCs, as well as the feasibility of using this antigen delivery system in DC-based cancer immunotherapy is investigated. DCs were treated with ovalbumin (OVA) as a model antigen, BLs and Ultrasound exposure. OVA was directly delivered into the cytosol but not via the endocytosis pathway, and OVA-derived peptides were presented on MHC class I. This result indicates that exogenous antigens can be recognized as endogenous antigens when delivered into the cytosol. Immunization with DCs treated with OVA, BLs and Ultrasoand exposure efficiently induced OVA-specific CTLs and resulted in the complete rejection of E. G7-OVA tumors. These data indicate that the combination of BLs and Ultrasound exposure is a promising antigen delivery system in DC-based cancer immunotherapy.

Interleukin-12 (IL-12) gene therapy is expected to be effective against cancers because it primes the immune system for cancer cells. In this

therapy, it is important to induce IL-12 gene expression in the tumor tissue. Sonoporation is an attractive technique for developing non-invasive and non-viral gene delivery systems, but simple sonoporation using only ultrasound is not an effective cancer gene therapy because of the low efficiency of gene delivery. Researchers solve this problem by combining ultrasound and novel ultrasoundsensitive liposomes (Bubble liposomes) which contain the ultrasound imaging gas perfluoropropane, this is an effective gene delivery system, and that Bubble liposome collapse (cavitation) is induced by ultrasound exposure. In this study, they assessed the utility of this system in cancer gene therapy using IL-12 corded plasmid DNA. The combination of Bubble liposomes and ultrasound dramatically suppressed tumor growth. This therapeutic effect was T-cell dependent, requiring mainly CD8+ T lymphocytes in the effector phase, as confirmed by a mouse in vivo depletion assay. In addition, migration of CD8+ T cells was observed in the mice, indicating that the combination of Bubble liposomes and ultrasound is a good non-viral vector system in IL-12 cancer gene therapy.

Nanobubbles has combine properties of polymeric drug carriers, ultrasound imaging contrast agents, and enhancers of ultrasound-mediated drug delivery has been developed. At room temperature, perfluorocarbon nanodroplets stabilized by the walls made of biodegradable block copolymers. Upon heating to physiological temperatures, the nanodroplets convert into nano/microbubbles. The phase state of the systems and bubble size may be controlled by the copolymer/ perfluorocarbon volume ratio. Upon intravenous injections, a long-lasting, strong and selective ultrasound contrast is observed in the tumor volume indicating nanobubble

extravasation through the defective tumor microvasculature, suggesting their coalescence into larger, highly echogenic microbubbles in the tumor tissue. Under the action of tumor-directed ultrasound, microbubbles cavitate and collapse resulting in a release of the encapsulated drug and dramatically enhanced intracellular drug uptake by the tumor cells. This effect is tumor-selective; no accumulation of echogenic microbubbles is observed in other organs. Effective chemotherapy of the MDA MB231 breast cancer tumors has been achieved using this technique.

In the treatment of acute ischemic stroke recanalization is the main goal. Thrombolysis with recombinant tissue plasminogen activator (rtPA) is efficient in humans, but shows significant problems including slow and incomplete recanalization and frequent bleeding complications. Limited therapeutic window (the first three hours after onset) is the major limitation resulting in reach too few patients. Therefore, adjunctive therapies extending the reperfusion time window, increasing efficacy and reducing side effects of rtPA are needed. Ultrasound augmentation of rtPA-mediated thrombolysis is suggested to overcome some of these problems, but low-frequency ultrasound (less than 1 MHz) is not safe and high frequency ultrasound (2 MHz) is not much effective. Researchers suggest that normobaric hyperoxia (NBO) may increase the efficacy of ultrasound and rtPA combination in addition to its own efficacy in acute ischemic stroke. Briefly, NBO increases arterial partial oxygen pressure (pO2) significantly up to 6-fold. Increase of pO2 results in an increase of dissolved oxygen in the blood according to Henry’s law. Enhanced dissolved oxygen increases gas nuclei formation around and inside of the clot, and decreases the Blake threshold. Under

ultrasound field, these small gas nuclei form nanobubbles which fuel inertial cavitation as substrates, and therefore increase the clot fragmentation and lysis. This hypothesis has not been tested so far. The combination of rtPA, therapeutic ultrasound and NBO may be more efficacious than rtPA alone or its combination with ultrasound as acute stroke treatment modality, because each has different and probably additive mechanism of action.

A new method of optically guided controlled release was experimentally evaluated with liposomes containing a molecular load and gold nanoparticles (NPs). NPs were exposed to short laser pulses to induce transient vapor bubbles around the NPs, plasmonic nanobubbles, in order to disrupt the liposome and eject its molecular contents. The release efficacy was tuned by varying the lifetime and size of the nanobubble with the fluence of the laser pulse. Optical scattering by nanobubbles correlated to the molecular release and was used to guide the release. The release of two fluorescent proteins from individual liposomes has been directly monitored by fluorescence microscopy, while the generation of the plasmonic nanobubbles was imaged and measured with optical scattering techniques. Plasmonic nanobubble-induced release was found to be a mechanical, nonthermal process that requires a single laser pulse and ejects the liposome contents within a millisecond timescale without damage to the molecular cargo and that can be controlled through the fluence of laser pulse.

Dextran nanobubbles were prepared with a dextran shell and a perfluoropentan core in which oxygen was stored. To increase the stability polyvinylpirrolidonewas also added to the formulation as stabilizing agent. Rhodamine B was used as fluorescent marker to obtain fluorescent

nanobubbles. The nanobubble formulations showed sizes of about 500 nm, a negative surface charge and a good capacity of loading oxygen, no hemolytic activity or toxic effect on cell lines. The fluorescent labelled nanobubbles could be internalized in Vero cells. Oxygen-filled nanobubbles were able to release oxygen in different hypoxic solutions at different time after their preparation in in vitro experiments. The oxygen release kinetics could be enhanced after nanobubble insonation with ultrasound at 2. 5 MHz. The oxygen-filled nanobubble formulations might be proposed for therapeutic applications in various diseases.

When targeted, Rhodamine-labeled echogenic liposomes (Rh- ELIP) containing nanobubbles are delivered to the arterial wall, and whether 1 MHz continuous wave ultrasound is given, it enhance the delivery profile of the drug. Aortae excised from apolipoprotein-E deficient (n= 8) and wild-type (n= 8) mice were mounted in a pulsatile flow system through which Rh-ELIP were delivered in a stream of bovine serum albumin. Half the aortae from each group were treated with 1-MHz continuous wave ultrasound at 0. 49 MPa peak-to-peak pressure, and half underwent sham exposure. Ultrasound parameters were chosen to promote stable cavitation and avoid inertial cavitation. A broadband hydrophone was used to monitor cavitation activity. After treatment, aortic sections were prepared for histology and analyzed by an individual blinded to treatment conditions. Delivery of Rh-ELIP to the vascular endothelium was observed, and sub-endothelial penetration of Rh-ELIP was present in five of five ultrasound-treated aortae and was absent in those not exposed to ultrasound. However, the degree of penetration in the ultrasound-exposed aortae was variable. There was no evidence of

ultrasound-mediated tissue damage in any specimen. Ultrasound-enhanced delivery within the arterial wall was demonstrated in this novel model, which allows quantitative evaluation of therapeutic delivery.

The advent of microbubble contrast agents has enhanced the capabilities of ultrasound as a medical imaging modality and stimulated innovative strategies for ultrasound-mediated drug and gene delivery. To enhance their multifunctional contrast and delivery capacity, it is critical to reduce bubble size to the nanometer range without reducing echogenicity, this can be achieved by using the surfactant Pluronic, a triblock copolymer of ethylene oxide copropylene oxide coethylene oxide into the formulation. Five Pluronics (L31, L61, L81, L64 and P85) with a range of molecular weights (Mw: 1100 to 4600 Da) were incorporated into the lipid shell either before or after lipid film hydration and before addition of perfluorocarbon gas. Results demonstrate that Pluronicâˆ’lipid interactions lead to a significantly reduced bubble size. Among the tested formulations, bubbles made with Pluronic L61 were the smallest with a mean hydrodynamic diameter of 207. 9 ± 74. 7 nm compared to the 880. 9 ± 127. 6 nm control bubbles. Pluronic L81 also significantly reduced bubble size to 406. 8 ± 21. 0 nm. We conclude that Pluronic is effective in lipid bubble size control, and Pluronic Mw, hydrophilicâˆ’lipophilic balance (HLB), and Pluronic/lipid ratio are critical determinants of the bubble size. The results have shown that although the bubbles are nanosized, their stability and in vitro and in vivo echogenicity are not compromised. The resulting nanobubbles may be better suited for contrast enhanced tumor imaging and subsequent therapeutic delivery.

To fabricate the nanobubble-based contrast agent, the researchers first ultrasonicate a mixture of Span 60 and polyoxyethylene 40 stearate and then use differential centrifugation to isolate the relevant subpopulation from the parent suspensions.

Doppler enhancement

Excellent power Doppler enhancement was found in vivo in renal imaging following intravenous injection of the team’s nanobubble contrast agent. The tiny bubbles are small enough to leak through the vascular pores of the tumour and accumulate in the tumour tissue by means of passive targeting due to the higher permeability of tumour blood vessels compared with normal tissue vasculature. After extravasations, an increased acoustic signal is obtained from the accumulated nanobubbles, providing strong ultrasound contrast imaging.

Nanobubble accumulation

In addition to diagnostic applications, the nanobubbles show great potential as ultrasound-mediated drug-delivery vehicles to facilitate drug release and extravascular delivery.

Nanobubbles (NB) with ultrasound (US) to permeabilize cancer cells and potentiate the cytotoxicity of anti-cancer drugs (cisplatin and 5-FU). Researchers used 293T human kidney, MCF7 human breast adenocarcinoma, EMT6 murine mammary carcinoma and colon 26 murine rectum carcinoma cells. Cytotoxicity was evaluated with MTT assay. Under optimal conditions, NB (albumin or lipid, 10% v/v) combined with US (frequency: 945 kHz, duty

ratio: 20-80%, pressure: 0. 96 MPa) produced significant cytotoxicity not seen with either US or drug alone. Increasing the duty ratio up to 80% further increased cytotoxicity. From the observation of rapid collapse of nanobubbles with US, we hypothesised that sub-nanobubbles (cavitation bubbles) are produced by the collapse of nanobubbles and shock waves generated from the cavitation bubbles lead to the transient membrane permeability, followed by entry of plasmid DNA or drugs. To investigate the mechanisms of molecular delivery with shock waves, we performed molecular dynamics (MD) simulations of the interaction of the shock wave impulse with a lipid bilayer and subsequently increased the fluidity of each molecule of the layer. These changes in bilayer may be important factors to enhance drug susceptibility of cancer cells.

Novel biocompatible nanobubbles were fabricated by ultrasonication of a mixture of Span 60 and polyoxyethylene 40 stearate (PEG40S) followed by differential centrifugation to isolate the relevant subpopulation from the parent suspensions. Particle sizing analysis and optical microscopy inspection indicated that the freshly generated micro/nanobubble suspension was polydisperse and the size distribution was bimodal with large amounts of nanobubbles. To develop a nano-sized contrast agent that is small enough to leak through tumor pores, a fractionation to extract smaller bubbles by variation in the time of centrifugation at 20g (relative centrifuge field, RCF) was suggested. The results showed that the population of nanobubbles with a precisely controlled mean diameter could be sorted from the initial polydisperse suspensions to meet the specified requirements. The isolated bubbles were stable over two weeks under the protection of

perfluoropropane gas. The acoustic behavior of the nano-sized contrast agent was evaluated using power Doppler imaging in a normal rabbit model. An excellent power Doppler enhancement was found in vivo renal imaging after intravenous injection of the obtained nanobubbles. Given the broad spectrum of potential clinical applications, the nano-sized contrast agent may provide a versatile adjunct for ultrasonic imaging enhancement and/or treatment of tumors.

Functionalised nanoparticles have been proposed as potential agents for non-invasive therapies where an external source such as a laser or an electro-magnetic wave is used to heat targeted particles for either drug release or malignant cell damage. It is desirable to have intracellular reactions to minimise the damage to health cells. However, it is still debatable from the thermal response point of view, whether intracellular hyperthermia is better than extracellular delivery due to conventional ideas of localisation of heat by nanoparticles. This work conducts an analytical study on the heating of a single nanoparticle by a pulsed laser and reveals the potential role of the formation of nanobubbles around heated particles. The rapid formation and contraction of bubbles around heated nanoparticles, associated with the propagation of pressure waves, could bring thermal-mechanical damage to surrounding cells at a dimension much larger than that of a nanoparticle.

Apomorphine is a dopamine receptor agonist for treating Parkinson’s disease. However, its clinical application is limited by its instability and the need for frequent injections. The aim of the present work was to develop acoustically active perfluorocarbon nanobubbles (PNs) for encapsulation of

both apomorphine HCl and base forms to circumvent these delivery problems. The PNs were prepared using coconut oil and perfluoropentane as the inner phase, which was emulsified by phospholipids and cholesterol. The morphology, size, zeta potential, and drug release of the PNs were characterized. The particle size ranged from 150 to 380 nm, with differences in the oil or perfluorocarbon ratio in the formulations. Atomic force microscopy confirmed oval- or raisin-shaped particles and a narrow size distribution of these systems (polydispersity index = 0. 25-0. 28). The stability experimental results indicated that PNs could protect apomorphine from degradation. Evaporation of the PNs at 37 degrees C was also limited. Apomorphine HCl and base in PNs showed retarded and sustained release profiles. Ultrasound imaging confirmed the echogenic activity of PNs developed in this study. The apomorphine HCl release by insonation at 1 MHz showed enhancements of two- to fourfold compared to the non-ultrasound group, illustrating a possible drug-targeting effect. On the contrary, apomorphine base showed a decreased release profile with ultrasound application. Apomorphine-loaded PNs showed promising stability and safety. They were successful in sustaining apomorphine delivery.

Drug delivery in polymeric micelles combined with tumor irradiation by ultrasound results in effective drug targeting, but this technique requires prior tumor imaging. A technology that combined ultrasound imaging with ultrasound-mediated nanoparticle-based targeted chemotherapy could therefore have important applications in cancer treatment. Mixtures of drug-loaded polymeric micelles and perfluoropentane (PFP) nano/microbubbles stabilized by the same biodegradable block copolymer were prepared. Size

distribution of nanoparticles was measured by dynamic light scattering. Cavitation activity (oscillation, growth, and collapse of microbubbles) under ultrasound was assessed based on the changes in micelle/microbubble volume ratios. The effect of the nano/microbubbles on the ultrasound-mediated cellular uptake of doxorubicin (Dox) in MDA MB231 breast tumors in vitro and in vivo (in mice bearing xenograft tumors) was determined by flow cytometry. Statistical tests were two-sided. Phase state and nanoparticle sizes were sensitive to the copolymer/perfluorocarbon volume ratio. At physiologic temperatures, nanodroplets converted into nano/microbubbles. Doxorubicin was localized in the microbubble walls formed by the block copolymer. Upon intravenous injection into mice, Dox-loaded micelles and nanobubbles extravasated selectively into the tumor interstitium, where the nanobubbles coalesced to produce microbubbles with a strong, durable ultrasound contrast. Doxorubicin was strongly retained in the microbubbles but released in response to therapeutic ultrasound. Microbubbles cavitated under the action of tumor-directed ultrasound, which enhanced intracellular Dox uptake by tumor cells in vitro to a statistically significant extent relative to that observed with unsonicated microbubbles (drug uptake ratio = 4. 60; 95% confidence interval [CI] = 1. 70 to 12. 47; P = . 017) and unsonicated micelles (drug uptake ratio = 7. 97; 95% CI = 3. 72 to 17. 08; P = . 0032) and resulted in tumor regression in the mouse model. Multifunctional nanoparticles that are tumor-targeted drug carriers, long-lasting ultrasound contrast agents, and enhancers of ultrasound-mediated drug delivery have been developed and deserve further exploration as cancer therapeutics.

Recently, there have been numerous reports on the application of non-thermal ultrasound energy for treating various diseases in combination with drugs. Furthermore, the introduction of microbubbles and nanobubbles as carriers/enhancers of drugs has added a whole new dimension to therapeutic ultrasound. Non-thermal mechanisms for effects seen include various forms of energy due to cavitation, acoustic streaming, micro jets and radiation force which increases possibilities for targeting tissue with drugs, enhancing drug effectiveness or even chemically activating certain materials. Examples such as enhancement of thrombolytic agents by ultrasound have proven to be beneficial for acute stroke patients and peripheral arterial occlusions. Non-invasive low intensity focused ultrasound in conjunction with anti-cancer drugs may help to reduce tumor size and lessen recurrence while reducing severe drug side effects. Chemical activation of drugs by ultrasound energy for treatment of atherosclerosis and tumors is another new field recently termed as “ Sonodynamic therapy”. Lastly, advances in molecular imaging have aroused great expectations in applying ultrasound for both diagnosis and therapy simultaneously. Microbubbles or nanobubbles targeted at the molecular level will allow medical doctors to make a final diagnosis of a disease using ultrasound imaging and then immediately proceed to a therapeutic ultrasound treatment.

Today there exists only one FDA-approved treatment for ischemic stroke; i. e., the serine protease tissue-type plasminogen activator (tPA). In the aftermath of the failed stroke clinical trials with the nitrone spin trap/radical scavenger, NXY-059, a number of articles raised the question: are we doing the right thing? Is the animal research truly translational in identifying new

agents for stroke treatment? This review summarizes the current state of affairs with plasminogen activators in thrombolytic therapy. In addition to therapeutic value, potential side effects of tPA also exist that aggravate stroke injury and offset the benefits provided by reperfusion of the occluded artery. Thus, combinational options (ultrasound alone or with microspheres/nanobubbles, mechanical dissociation of clot, activated protein C (APC), plasminogen activator inhibitor-1 (PAI-1), neuroserpin and CDP-choline) that could offset tPA toxic side effects and improve efficacy are also discussed here. Desmoteplase, a plasminogen activator derived from the saliva of Desmodus rotundus vampire bat, antagonizes vascular tPA-induced neurotoxicity by competitively binding to low-density lipoprotein related-receptors (LPR) at the blood-brain barrier (BBB) interface, minimizing the tPA uptake into brain parenchyma. tPA can also activate matrix metalloproteinases (MMPs), a family of endopeptidases comprised of 24 mammalian enzymes that primarily catalyze the turnover and degradation of the extracellular matrix (ECM). MMPs have been implicated in BBB breakdown and neuronal injury in the early times after stroke, but also contribute to vascular remodeling, angiogenesis, neurogenesis and axonal regeneration during the later repair phase after stroke. tPA, directly or by activation of MMP-9, could have beneficial effects on recovery after stroke by promoting neurovascular repair through vascular endothelial growth factor (VEGF). However, any treatment regimen directed at MMPs must consider their pleiotropic nature and the likelihood of either beneficial or detrimental effects that might depend on the timing of the treatment in relation to the stage of brain injury.

Combining diagnostic and therapeutic processes into one (theranostics) and improving their selectivity to the cellular level may offer significant benefits in various research and disease systems and currently is not supported with efficient methods and agents. We have developed a novel method based on the gold nanoparticle-generated transient photothermal vapor nanobubbles, that we refer to as plasmonic nanobubbles (PNB). After delivery and clusterization of the gold nanoparticles (NP) to the target cells the intracellular PNBs were optically generated and controlled through the laser fluence. The PNB action was tuned in individual living cells from non-invasive high-sensitive imaging at lower fluence to disruption of the cellular membrane at higher fluence. We have achieved non-invasive 50-fold amplification of the optical scattering amplitude with the PNBs (relative to that of NPs), selective mechanical and fast damage to specific cells with bigger PNBs, and optical guidance of the damage through the damage-specific signals of the bubbles. Thus the PNBs acted as tunable theranostic agents at the cellular level and in one process that have supported diagnosis, therapy and guidance of the therapy.

The main goal in the treatment of acute ischemic stroke is prompt arterial recanalization. Thrombolysis with recombinant tissue plasminogen activator (rtPA) is efficient in humans, but shows significant problems including slow and incomplete recanalization and frequent bleeding complications. Limited therapeutic window (the first three hours after onset) is the major limitation resulting in reach too few patients. Therefore, adjunctive therapies extending the reperfusion time window, increasing efficacy and reducing side effects of rtPA are needed. Ultrasound augmentation of rtPA-mediated thrombolysis is

suggested to overcome some of these problems, but low-frequency ultrasound (less than 1 MHz) is not safe and high frequency ultrasound (2 MHz) is not much effective. Researchers suggest that normobaric hyperoxia (NBO) may increase the efficacy of ultrasound and rtPA combination in addition to its own efficacy in acute ischemic stroke. Briefly, NBO increases arterial partial oxygen pressure (pO2) significantly up to 6-fold. Increase of pO2 results in an increase of dissolved oxygen in the blood according to Henry’s law. Enhanced dissolved oxygen increases gas nuclei formation around and inside of the clot, and decreases the Blake threshold. Under ultrasound field, these small gas nuclei form nanobubbles which fuel inertial cavitation as substrates, and therefore increase the clot fragmentation and lysis. This hypothesis has not been tested so far. The combination of rtPA, therapeutic ultrasound and NBO may be more efficacious than rtPA alone or its combination with ultrasound as acute stroke treatment modality, because each has different and probably additive mechanism of action.