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### NOVEL THERAPEUTIC APPLICATION OF MICROBUBBLES FOR TARGETED DRUG DELIVERY

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#### **ABSTRACT:**

Blood-brain barrier impermeability presents a problem while treating malignant and degenerative disorders of the central nervous system, such as brain cancer, Alzheimer's and Parkinson's disease. The protective blood-brain barrier blocks many drugs from acting on brain cells. A method for delivering drugs directly to the affected area may increase the effectiveness of chemotherapy in brain tumors and reduce its toxic effect on healthy cells. The new innovative technique that uses ultrasound and drug-laden "microbubbles" to deliver concentrated chemotherapy drugs to the inner lining of blood vessels. Microbubbles destruction has been proposed as an innovative method for noninvasive delivering of drugs and genes to different tissues. They are used to carry a drug until a specific area of interest is reached, and then ultrasound is used to burst the microbubbles, causing site-specific delivery of the bioactive materials. The microbubbles as drug carriers have an average size less than that of red blood cells, i.e. they are capable of penetrating even into the small blood capillaries and releasing drug and genes under the action of ultrasound field. Targeting ligands are attached to the surface of the microbubbles (i.e. targeted-microbubbles), which have been widely used in cardiovascular system and tumor diagnosis.

This review focuses on the characteristics of microbubbles that give them therapeutic properties and some important aspects of ultrasound parameters that are known to influence microbubble-mediated drug delivery. In addition, current studies involving this novel therapeutic application of microbubbles will be discussed.

**KEY WORDS:** Microbubbles, Doppler signal, Ultrasound field, Angiogenesis, Sonothrombolysis

#### **1.1. INTRODUCTION:**

Ultrasound-mediated microbubbles destruction has been proposed as an innovative method for noninvasive delivering of drugs and genes to different tissues. Microbubbles are used

to carry a drug or gene until a specific area of interest is reached, and then ultrasound is used to burst the microbubbles, causing site-specific delivery of the bioactive materials. Furthermore, the ability of albumin-coated microbubbles to

adhere to vascular regions with glycocalix damage or endothelial dysfunction is another possible mechanism to deliver drugs even in the absence of ultrasound. This review focuses on the characteristics of microbubbles that give them therapeutic properties and some important aspects of ultrasound parameters that are known to influence microbubble-mediated drug delivery. In addition, current studies involving this novel therapeutical application of microbubbles will be discussed (Jeane M Tsutsui et al., 2004).

Microbubbles have diameters from 1  $\mu\text{m}$  to 10  $\mu\text{m}$  and a thin flexible or rigid shell composed of albumin, lipid, or polymer confining a gas such as nitrogen, or a perfluorocarbon. These microbubbles can cross the pulmonary capillaries and have a serum half-life of a few minutes. Microbubbles in the 1-10  $\mu\text{m}$  range have their resonance at the frequencies used in diagnostic ultrasound (1–15MHz). Smaller bubbles resonate at higher frequencies caused by this coincidence, they are such effective reflectors.

The intrinsic compressibility of microbubbles is approximately 17,000 times more than water, and they are very strong scatterers of ultrasound. Under acoustic pressure the vibrating bubble radius may have a conventional linear response or a harmonic non-linear response. Microbubbles usually increase the Doppler signal amplitude by up to 30 dB. Encapsulated gas microbubbles are well known as ultrasound contrast agents for medical ultrasound imaging. Nonetheless, not only do these microbubbles help to image, but they can also be used as drug/gene carriers. The microbubbles as drug/gene carriers have an average size less than that of red blood cells, i.e. they are capable of penetrating even into the small blood capillaries and releasing drug and genes under the action of ultrasound field. The application of ultrasound and microbubbles to targeted drug and gene delivery has been the subject of intense experimental research. Under exposure of sufficiently high-amplitude ultrasound, these targeted microbubbles would

rupture, spewing drugs or genes, which are contained in its encapsulating layer, to targeted cells or tissues. Recently, targeting ligands are attached to the surface of the microbubbles (i.e. targeted-microbubbles), which have been widely used in cardiovascular system and tumor diagnosis and therapy. In this paper, the characterization of novel targeted ultrasonic contrast agents or microbubbles and their potential applications in drug delivery or gene therapy are reviewed (Yiyao Liu<sup>a, b, c</sup>, et al., 2006).

## 1.2. How microbubbles works:

Microbubbles work by resonating in an ultrasound beam, rapidly contracting and expanding in response to the pressure changes of the sound wave. By a fortunate coincidence, they vibrate particularly strongly at the high frequencies used for diagnostic ultrasound imaging. This makes them several thousand times more reflective than normal body tissues. In this way they enhance both grey scale images and flow mediated Doppler signals. As well as being useful in itself, the resonance that microbubbles produce has several special properties that can be exploited to improve diagnoses. Just as with a musical instrument, multiple harmonic signals—or overtones—are produced. Ultrasound scanners can be tuned to "listen" to these harmonics, producing strong preferential imaging of the microbubbles in an image. The selective excitation produced can also destroy microbubbles relatively easily, an effect that can be useful both in imaging and in emerging therapeutic applications (Martin J K Blomley et al., 2001).

## 2.1. Imaging and therapy:

Ultrasound is extremely sensitive to the presence of microbubbles. In fact, cavitation imaging and other techniques are capable of detecting a single microbubble. On a molecular basis, in terms of sensitivity to a small number of atoms of a contrast agent, ultrasound rivals nuclear medicine and optical imaging, and exceeds MRI by several orders of magnitude. It

exceeds computed tomography and standard X-ray techniques by an even greater amount.

## 2.2. Angiogenesis:

Angiogenesis, the process by which new blood vessels are developed, is seen in disease processes such as cancer and inflammation. It can be exploited therapeutically as a drug target. For example, angiogenesis can be stimulated to treat ischemic vascular and heart disease by improving collateral flow. Angiogenesis is also the target for new drugs, aimed at stopping the formation of new blood vessels, for the treatment of cancer, inflammation and retinopathy. In angiogenesis, blood flow increases, and the neovasculature generally has different architecture and hemodynamic properties from vessels in tissues not affected by angiogenesis. Ultrasound and ultrasound blood pool contrast agents such as Definity can be used to detect and potentially quantify angiogenesis. To image angiogenesis with a blood pool agent such as Definity, the contrast agent is administered intravenously, and images can be obtained with a number of different contrast-specific ultrasound pulse sequences. Such pulse sequences decrease the signal from background tissues and increase the signal from the contrast agent. Contrast-enhanced ultrasound can then clearly show the neovasculature of angiogenesis. Microbubbles may be destroyed using a higher energy ultrasound pulse, i.e. one with an increased Mechanical Index (MI). This can be used to create a square bolus arriving, for example, at the microvascular bed in a tumor. Using high-MI pulses to destroy the contrast, and then following re-entry of contrast with low-MI imaging, makes it possible to measure the rate of inflow of contrast. The rate of inflow at which perfusion reappears corresponds directly to tissue perfusion. Perfusion can then be quantified at the microvascular level. The images can be evaluated for rate of flow, perfusion, vascular architecture and morphology. Comparison of serial studies in patients under treatment with angiogenesis inhibitors (also

potentially chemotherapeutics) are likely to be useful for following the results of treatment, monitoring and modifying therapy. Currently many follow-up patients, particularly cancer patients, are studied with CT and MRI in order to assess the response to therapy. Generally, response is judged by changes in bi-dimensional measurements of tumor diameters. However, changes in angiogenesis will precede changes in tumor size. Rapid tests based on contrast-enhanced ultrasound using blood pool agents may enable more rapid assessment of response to therapies based on chemotherapy, angiogenesis inhibitors and other drugs. Favorable response to a selected therapy should be seen on angiogenesis imaging as a reduction in neovasculature formation, prior to changes in bi-dimensional tumor measurements. Development of contrast-enhanced angiogenesis imaging with ultrasound will make a cost-effective method for detecting disease and monitoring response to therapy.

## 2.3. Targeted contrast agents:

In addition to using ultrasound contrast agents for imaging blood flow, it is also possible to make targeted contrast agents that bind to selected cells, providing a more precise method of molecular imaging. Living cells can then be used as carriers for contrast agents. In this case the cells themselves can be exploited to target certain disease processes. Leukocytes and lymphocytes travel throughout the body, and migrate to sites of inflammation. The function of leukocytes and other phagocytic cells is to engulf 'foreign' particles, and they will therefore engulf the microbubbles. Phagocytosis of the microbubbles can be enhanced by incorporating certain materials, e.g. phosphatidylserine, into the coating on the microbubbles. Such agents targeting white blood cells can be used to image inflammation. Lindner has shown that white blood cells carrying microbubbles will enhance abscesses and ischemic lesions following reperfusion. Precise molecular imaging with ultrasound contrast agents may also be achieved

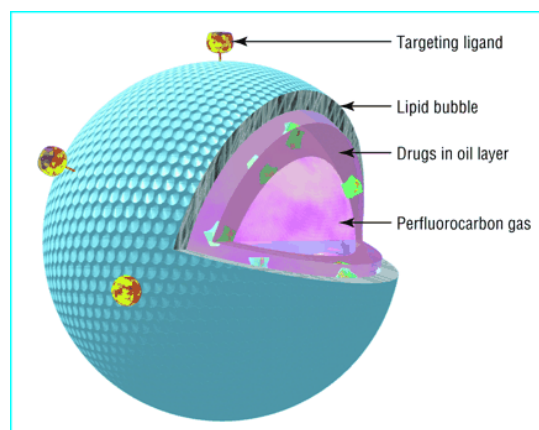
by incorporating targeting ligands into the surface of the microbubble. The targeting ligands may be in the form of modifications in the coating material stabilizing the microbubbles, or specific molecules attached to the surface of the microbubbles. In our group we have developed a number of different targeted microbubble preparations for molecular imaging. Broadly speaking, these comprise agents for targeting the vasculature, and agents for targeting structures that lie beyond the vasculature. For targets within the vasculature, micron-sized microbubbles are probably adequate. For targets beyond the vasculature, it is generally best for the particles to be small, certainly less than 500 nm diameter, and preferably much smaller. Blood vessels are lined by endothelial cells which have fenestrations that generally permit passage of molecules and very small particles from the intravascular into the extravascular space. In the brain, however, there are tight junctions between these endothelial cells, creating the blood brain barrier (BBB). The BBB prevents passage of most molecules and particles into the central nervous system (CNS). Outside the CNS, however, the fenestrations of the endothelial barrier allow passage of very small particles, and this can be exploited for molecular imaging. Nanoparticles

bearing targeting ligands can then be used for targeted molecular imaging and therapy. While microbubbles typically have diameters of the order of 1 to 2 microns, and are sometimes as small as a few hundred nanometers, other materials, e.g. perfluorocarbon (PFC) emulsions, can be much smaller in diameter: down to the range of 100 nm diameter, and potentially even smaller. Microbubbles are mainly suitable for targeting vascular targets that are expressed on endothelial cells or on other cells exposed to circulating blood. A size smaller than several hundred nanometers diameter (e.g. perfluorocarbon droplets) is desirable if the particles are to extravasate from the vasculature to target cell surface epitopes on epithelial cells and others. This can be achieved with nanobubbles and PFC emulsions (E. Unger et al., 2003).

Injecting a gas into the circulation may seem potentially hazardous, but extensive clinical experience has shown that the tiny volume of air or gas given (under 200  $\mu$ l) is not dangerous, and the safety of microbubbles compares well to that of conventional agents in radiography and magnetic resonance imaging (Martin J K Blomley et al., 2001).

#### Targeted microbubbles:

Figure 1 depicts a targeted microbubble.



**Fig. 1. A targeted microbubble: a gas microbubble is covered in a lipid membrane in which targeting ligands have been incorporated.**

The microbubble has bioconjugates incorporated into the membrane stabilizing the microbubble. The bioconjugate has a polyethyleneglycol tether between the ligand and the lipid anchor inserted into the microbubble. MRX-408 is a recently developed microbubble incorporating bioconjugates targeted to the GPIIb/IIIa receptor of activated platelets.

### **3.1. Intravascular applications:-**

#### **3.1.1. Angiogenesis:**

In addition to thrombosis, there are other intravascular targets for molecular imaging with ultrasound. Certain integrins such as  $\alpha V\beta 3$  are expressed in angiogenesis. Lindner and others have shown that microbubbles targeted to  $\alpha V\beta 3$  can be used to measure the temporal expression of this integrin in association with angiogenesis. A contrast agent targeted to endothelial-based markers of angiogenesis might be used to improve diagnosis and treatment of disorders affected by angiogenesis. Other endothelial-based targets such as P-selectin might be exploited to develop targeted imaging agents for detecting inflammation.

#### **3.1.2. Vulnerable plaque:**

Another important intravascular target is vulnerable plaque. Vulnerable plaques are those that have been infiltrated by macrophages, and are undergoing inflammation (Bjørn Tore Gjertsen et al., 2002). Inflammation can lead to rupture of vulnerable plaque and formation of thrombus, as in stroke and myocardial infarct. Vulnerable plaques may lie hidden as unseen threats, liable to cause morbidity and sudden death. A noninvasive test is needed to detect vulnerable plaque. Vulnerable plaque has been successfully detected using targeted microbubbles in combination with ultrasound.

#### **4.1. Extravascular applications:**

For targeting beyond the vasculature, the particles usually need to have very small diameters. This requirement can be met by nanobubbles and perfluorocarbon emulsions.

Targeting ligands can be incorporated into these systems as described above. The ligands may then be directed to targets on the surface of cells or in the intercellular matrix outside the vasculature. Very small bubbles (i.e. those well below 500 nm diameter) can be detected with high-frequency imaging and other ultrasound techniques. When sufficient quantities of these structures are delivered to a tissue, they can act as specular reflectors and greatly increase the backscattered signal. Smaller quantities can be detected with cavitation imaging. Nanobubbles may make it possible to target, for example, epithelial cells in carcinoma. Such agents might be used to not only detect but also to treat diseases such as cancer.

#### **4.1.1. Sonothrombolysis:**

Microbubbles accelerate the rate of SonoLysis. Enhancement is greater for the targeted molecular ultrasound contrast agent, MRX-408, than for the non-targeted agent. Concentration of the cavitation nuclei into the clot via receptor-mediated interaction provides more efficient transfer of the energy from cavitation to the thrombus than in the case of non-targeted microbubbles. Microbubble enhanced SonoLysis may have potential clinical applications for rapid and safe treatment of vascular thrombosis. This could have clinical applications for treating myocardial infarction, stroke and deep venous thrombosis. SonoLysis using ultrasound at 200 kilohertz with microbubbles restored blood flow. Ultrasound without microbubbles was unsuccessful in restoring blood flow (E. Unger et al., 2003).

#### **4.1.2. Passing the blood brain barrier:**

Hynnen has shown (Jonathan Lindner, 2003) that intravenous doses of microbubbles and transcranial application of ultrasound can be used to reversibly open the blood brain barrier. This can be exploited to deliver drugs to the CNS. As the BBB is opened, co-administered drugs may then passively enter the brain. Potentially, molecular targeted agents with

ultrasound activation might be used to afford precise entry into the CNS by controlling the BBB at the molecular level (E. Unger et al., 2003).

#### **4.1.3. Increase the efficiency of cancer treatment:**

Laser-induced bubble formation around nanoparticles may play a crucial role in selective laser nanophotothermolysis of cancer cells targeted with nanoparticles. In this paper, we propose theoretically, and confirm experimentally, a new dynamic mode for selective cancer treatment that involves the overlapping of bubbles inside the cell volume. This bubbles-overlapping mode (BOM) can dramatically increase the efficiency of cancer treatment by laser-heated nanoparticles as a result of the large damage range. On the basis of nanoparticle optics below the diffraction limit and the kinetic model of bubble dynamics, we found the criteria and conditions (interparticle distance and particle size and concentration) for BOM initiation in cancer cells by laser radiation. Using MDA-MB-231 breast cancer cells, we showed that the optimal size range of the gold nanoparticles for effective laser initiation of BOM is 30–40 nm and the lower concentration limit is  $n \approx 2.44 \times 10^{11} \text{ cm}^{-3}$  (i.e. the absolute number of particles homogeneously distributed inside a tumour cell is  $n \approx 430$ ). It was demonstrated that the formation of nanoclusters on the cell surface with sizes larger than the sizes of individual nanoparticles, may further increase the efficiency of the laser treatment of cancer (V P Zharov et al., 2005).

#### **4.1.4. Prostate cancer detection:**

The diagnosis of prostate cancer is currently limited by the low sensitivity and specificity of systematic conventional grey-scale ultrasonography. We assessed contrast-enhanced colour Doppler ultrasonography by means of a microbubble ultrasound contrast agent to detect tumour vascularity and improve the diagnosis of prostate cancer. The use of a microbubble ultrasound contrast agent for

transrectal colour Doppler targeted biopsy significantly improved the detection of prostate cancer compared with systematic biopsy following conventional grey-scale ultrasonography ( $p < 0.001$ ). Contrast-agent enhanced colour Doppler imaging may allow for limited targeted biopsies (five or less), which reduces costs and morbidity (Frauscher F et al., 2001).

A new ultrasound technique involving color-enhanced Doppler imaging with microbubble contrast improves the accuracy of screening for prostate cancer. A new development in ultrasound involves the use of color Doppler imaging with microbubble contrast so that physicians are better able to determine the presence and exact location of a mass within the prostate. Doppler imaging can sense differences in velocity (i.e. blood flow versus solid tissue) and transmits these differences through different color pixels to create a picture on a screen. Microbubbles are tiny bubbles of gas that can permeate through small blood vessels without creating any harm. The microbubbles further enhance imaging by increasing the intensity of backscatter signal. Since blood vessels and blood flow are more prevalent in cancerous tissues than regular tissues, microbubbles tend to concentrate in the cancer, which is revealed on the created picture. This allows physicians to more accurately locate where biopsies should be taken. The detection rate of prostate cancer was 27% with Doppler-guided biopsies compared with 20% with conventional ultrasonography. The overall core biopsy detection rate was 13% for Doppler-guided biopsies compared with only 4.9% for conventional ultrasonography. These results indicate that Doppler-guided biopsies with microbubble contrast may enable physicians to more accurately determine the optimal location for a biopsy (The Lancet, 2001).

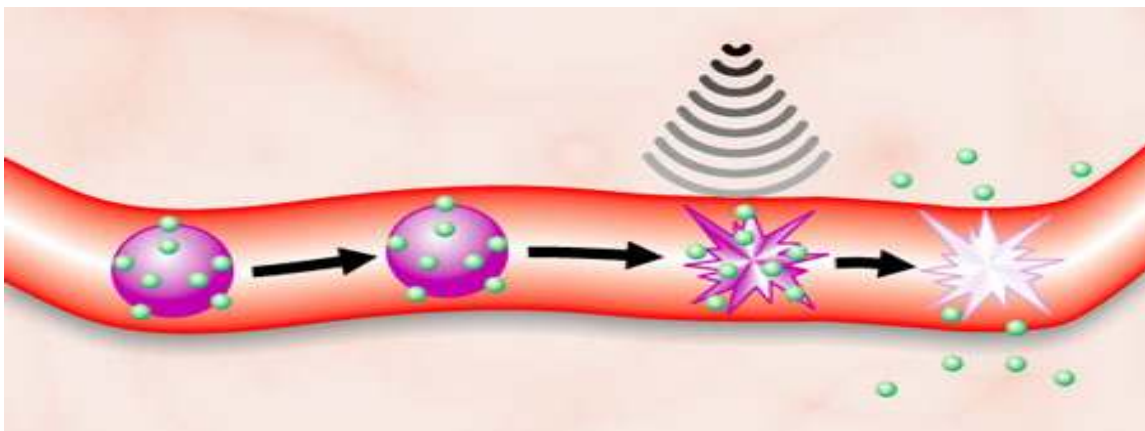
#### **4.1.5. Leukaemia Treatment:**

Acute myeloid leukaemia (AML) is a quick progressive cancer which is characterised by

neoplastic proliferation of myeloid cells. Current diagnostics of AML with prognostically and therapeutically implications includes cellular morphology, immunological markers, and in particular gene mutation analysis, cytogenetics, and response after chemotherapy. Treatments normally include high doses of cytotoxic drugs and in selected cases hematopoietic stem cell transplantation. These high intensive therapy regimen involve toxicity problems for a lot of

patients over 60 years and only 20-30% of the patients achieve long time survival. The need for new, effective and targeted treatment is therefore great.

Imaging modalities play an important role in evaluating disease extension and progression in haematological malignancies. It also has a vital role in pre-clinical trials using animal models (Fig. 2).



*Fig. 2. Gas-filled microbubbles covered with a bioactive substance pass harmlessly and uneventfully through blood vessels until they are exposed to ultrasound. Then, the bubbles burst, causing not only the release of the bioactive substance but also the opening of holes in the cells (sonoporation) that line the vessel.*

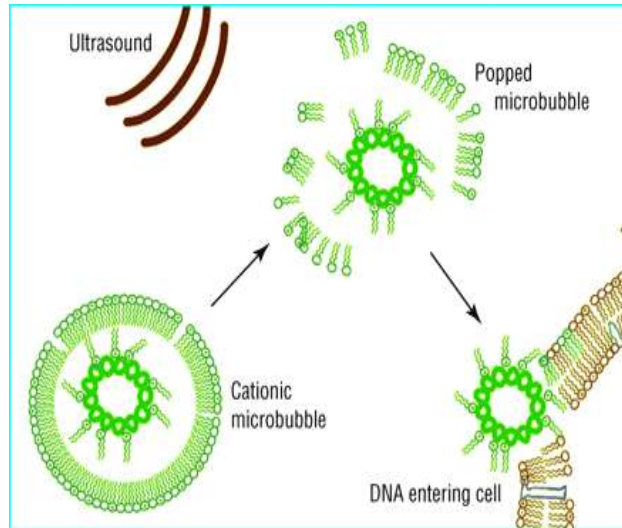
The use of different modalities has resulted in protocols overcoming the limitations of a single imaging modality. Multimodality provides a thorough view of anatomical, physiological and/or molecular processes in vivo and allows quantitative measurements and visualization of processes in specific targeted organs or tissue. This makes it a very important tool in detecting early cancer and deciding on direct individual treatment. Using sensitive and specific imaging modalities, treatment can be given before the malignant cell load become too large for available therapeutics. Since the treatment can be so exhausting, administration of direct drug delivery may allow the patient to receive disease controlling treatment with palliative intentions. Direct drug delivery also gives the opportunity to use stronger drugs than otherwise would be too

toxic for the patient (Bjørn Tore Gjertsen et al., 2002).

#### **4.1.6. Gene delivery:**

Progress in cardiovascular gene therapy has been hampered by concerns over the safety and practicality of viral vectors and the inefficiency of current nonviral transfection techniques. Ultrasound exposure (USE) enhances transgene expression in vascular cells by up to 10-fold after naked DNA transfection, and enhances lipofection by up to three-fold. We report here that performing USE in the presence of microbubble echocontrast agents enhances acoustic cavitation and is associated with approximately 300-fold increments in transgene expression after naked DNA transfections. This approach also enhances by four-fold the

efficiency of polyplex transfection, yielding transgene expression levels (Figure 3)



**Fig. 3. Gene delivery using ultrasound and microbubbles.**

approximately 3000-fold higher than after naked DNA alone. These data indicate an important role for acoustic cavitation in the effects of USE. Ultrasound can be focused upon almost any organ and hence this approach holds promise as a means to deliver targeted gene therapy in cardiovascular conditions such as such angioplasty restenosis and in many other clinical situations. The presence of gas in the gene-filled microbubble allows ultrasound energy to "pop" the bubble. An energetic wave is then created which allows the genetic material to enter surrounding cells (A Lawrie<sup>1</sup> et al., 2000). Microbubbles are currently used clinically as contrast agents in ultrasound (US) diagnostics and experimentally as drug or nucleic acid carriers. Localized delivery, drug release and tissue penetration can be achieved by local application of ultrasound leading to microbubble disruption. In cell culture, magnetic microbubbles are sedimented on target cells by magnetic force and bubble disruption and gene delivery is triggered by the application of US of 1 MHz. Biodistribution experiments upon tail vein injection in mice demonstrated the highest

nucleic acid deposition when a magnetic field was applied to a target area such as a lung lobe in combination with ultrasound. Magnetic microbubbles loaded with appropriate agents can considerably improve drug deposition and the site specificity of delivery. In particular, magnetic microbubbles may offer a combination of therapeutic intervention with molecular imaging (Dialekti Vlaskou et al., 2006).

#### **4.1.7. Blood Vessel Growth in Tumors:**

Imagine being able to quickly detect and diagnose blood vessel growth in cancerous tumors, and even predict how fast the tumors might metastasize or spread, animal models using millions of tiny microbubbles injected into the bloodstream, coupled with contrast-enhanced ultrasound, an inexpensive and widely-used technique using sound waves to "see" inside the body. They found that blood flow has a high velocity on the periphery of the tumors, but a much slower velocity inside a tumor. Blood flow velocity is a potentially important marker for tumor detection (Jonathan Lindner, 2003).



Treating malignant brain tumors can be difficult. The protective blood-brain barrier blocks many drugs from acting on brain cells. Now, a new method for delivering drugs directly to the affected area may increase the effectiveness of chemotherapy in brain tumors and reduce its toxic effect on healthy cells. Researchers at the University of California are developing an innovative technique that uses ultrasound and drug-laden "microbubbles" to deliver concentrated chemotherapy drugs to the inner lining of blood vessels. Doctors already use ultrasound to identify tumors and guide biopsy procedures. Ultrasound pulse sequences can also guide micro-packaged medications to specific parts of the body (Shortencarier M, et al., 2004).

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