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Physical principles of CEUS and sonoporation

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Ultrasonic imaging is becoming the most popular medical imaging modality, owing to the low price per examination and its safety. A B-mode ultrasound scan shows contrasted regions from transitions in acoustic impedance, *i.e.*, transitions in tissue type, in the form of brighter pixels. However, blood is a poor scatterer of ultrasound waves at clinical diagnostic transmit frequencies, which lie between 1 and 40 MHz. For perfusion imaging, markers have been designed to enhance the contrast in B-mode imaging. These so-called ultrasound contrast agents consist of microscopically small gas bubbles encapsulated by biodegradable shells. Contrast-enhanced ultrasound (CEUS) represents a significant advancement in the evaluation of angiogenesis in digestive cancers. Particularly, for the study of focal liver lesions, CEUS has been widely used for detection and characterisation of malignancy. The unique feature of CEUS of non-invasive assessment in real-time liver perfusion throughout the vascular phases has led to a great improvement in diagnostic accuracy of ultrasound, but also in guidance and evaluation of responses to therapy. Currently, CEUS is part of the state-of-the-art diagnostic work-up of focal liver lesions, resulting in safe and cost-effective patient management. In this presentation, the physical principles of ultrasound contrast agent microbubble behaviour and their adjustment for drug delivery including sonoporation are described. Furthermore, an outline of clinical imaging applications of CEUS is given.

Ultrasound

The sound that humans can perceive lies within the frequency range 20 Hz–20 kHz. Ultrasound is by definition all sound higher than 20 kHz. The ultrasound frequencies utilised in medical imaging are mainly in the range 1–40 MHz. Such high frequencies cannot be transmitted through air but can be transmitted satisfactorily through solid or fluid materials. An ultrasonic transducer serves a dual function as both transmitter and receiver of ultrasound. A signal generated by an ultrasonic transducer typically consists of a pulse of a few μ s with a certain centre frequency. Part of this signal propagates through target tissue, part is reflected by macroscopic tissue structures, part is absorbed by tissue, and part is scattered by structures in the tissue smaller than the acoustic wavelength. Only a small portion of the transmitted acoustic energy is received by the transducer, but this portion is used to build an ultrasonic image. The received signal is the superposition of specular reflections at tissue boundaries and echoes from tissue backscattering. Current real-time two-dimensional imaging capabilities are in excess of 30 frames per second.

The quality of a B-mode scan is expressed by the contrast-to-noise ratio, which is defined as the absolute difference of the signal-to-noise ratio in target tissue and the signal-to-noise ratio in surrounding tissue (noise). On clinical ultrasound devices, the intensity of the ultrasonic field is generally adjusted with a switch for the

mechanical index instead of the acoustic amplitude. The mechanical index is defined by $MI = \frac{p^-}{\sqrt{f}}$, where p^-

is the maximum value of peak negative pressure anywhere in the ultrasound field, measured in water but reduced by an attenuation factor equal to that which would be produced by a medium having an attenuation coefficient of $0.3 \text{ dB cm}^{-1} \text{ MHz}^{-1}$, normalized by 1 MPa, and f is the centre frequency of the ultrasound normalised by 1 MHz. For $MI < 0.3$, the acoustic amplitude is considered low. For $0.3 > MI > 0.7$, there is a possibility of minor damage to neonatal lung or intestine.¹ These are considered moderate acoustic amplitudes. For $MI > 0.7$, there is a risk of cavitation if an ultrasound contrast agent containing gas microspheres is being used, and there is a theoretical risk of cavitation without the presence of ultrasound contrast agents.¹ The risk increases with MI values above this threshold.¹ These are considered high acoustic amplitudes.² On commercial scanners, the MI has been limited to 1.9 for medical imaging.³ At higher MI, the contrast-to-noise ratio increases.

The density and compressibility parameters of blood cells hardly differ from those of plasma. Therefore, blood cells are poor scatterers in the clinical diagnostic frequency range. Since imaging blood flow and measuring organ perfusion are desirable for diagnostic purposes, markers should be added to the blood to differentiate between blood and other tissue types. Such markers must have resonance frequencies in the medical ultrasonic range. The resonance frequencies of encapsulated microbubbles lie slightly higher than those of free gas bubbles, but clearly well within the clinical diagnostic range, too. Based on their acoustic properties, microbubbles are well suited as an ultrasound contrast agent.

At low-amplitude driving pressures, ultrasound contrast agent oscillate linearly, but at high-amplitude driving pressures, they oscillate nonlinearly. Detection strategies have been developed to discriminate acoustic signals generated ultrasound contrast agent microbubbles from other acoustic signals such as specular reflections and

tissue scattering. These strategies are the reason that CEUS is suitable for the detection of blood presence. The ten most common detection strategies include coded excitation, harmonic power Doppler, phase inversion and power modulation.^{4,5} All single-pulse and multi-pulse imaging detection strategies make use of the nonlinear behaviour of microbubbles.^{4,5}

Other types of nonlinear behaviour than asymmetric oscillations have been stated in Table 1, which gives an overview of the nonlinear phenomena that have been observed with ultrasound contrast agents, the type of ultrasound contrast agent in which they have occurred, and the minimum acoustic regime required. Of influence on the occurrence of these phenomena are a) the ultrasonic parameters: transmit frequency, acoustic amplitude, pulse length, pulse repetition rate and transmit phase; b) the ultrasound contrast agent composition: the composition of the shell, the bubble sizes, the size distribution and the gas; c) the physical properties of the medium: viscosity, surface tension, saturation.

Drug delivery

It has been proven by numerous groups, that the cellular uptake of drugs and genes is increased, when the region of interest is under sonication, and even more so when a contrast agent is present. This increased uptake has been attributed to the formation of transient porosities in the cell membrane, which are big enough for the transport of drugs into the cell. The transient permeabilisation and resealing of a cell membrane is called sonoporation.⁶ The sonoporation-induced cellular uptake of markers with molecular weights between 10 kDa and 3 MDa has been reported in several studies.⁷⁻⁹ Schlicher *et al.* showed that ultrasound-induced cavitation facilitated cellular uptake of macromolecules with diameters up to 56 nm.¹⁰ Even solid spheres with a 100-nm diameter have been successfully delivered aided by sonoporation.¹¹ This implies that drug size is not a limiting factor for intracellular delivery.⁸ However, the pore opening times can be so short, that, if the drug is to be effectively internalized, it should be released close to the cell membrane when poration occurs.

There are two hypotheses for explaining the sonoporation phenomenon, the first being microbubble oscillations near a cell membrane, the second being microbubble jetting through the cell membrane. Based on modelling, high-speed photography, and recent cellular uptake measurements, we concluded that microbubble jetting behaviour can be excluded as the dominant sonoporation mechanism.²

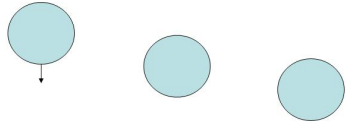
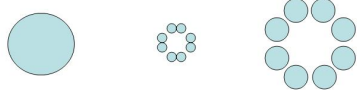
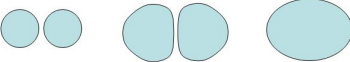
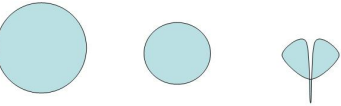
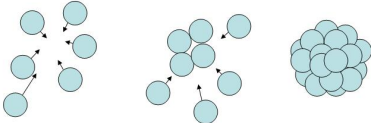
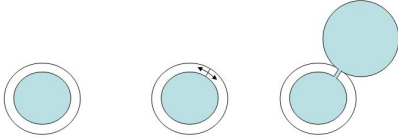
Instead of just facilitating the transient opening up of cell membranes, a microbubble might also act as the vehicle itself to carry a drug or gene load to a perfused region of interest, in which case the load has to be released with the assistance of ultrasound. Apart from mixing ultrasound contrast agent with a therapeutic agent, several schemes have been proposed to combine microbubbles with a therapeutic load. Tinkov *et al.* discriminate the following seven microbubble structure classes for drug delivery:⁹ a) attachment to the outer shell surface; b) intercalation between monolayer phospholipids; c) incorporation in a layer of oil; d) complexes with smaller particles (secondary carriers); e) physical encapsulation in a polymer layer and coating with biocompatible material; f) surface loading of protein-shelled microbubbles; g) entire volume loading of protein-shelled microbubbles.

It is a challenging task to quantify and predict which bubbly phenomenon occurs under which acoustic condition, and how these may be utilised in ultrasonic imaging. Aided by high-speed photography, our improved understanding of encapsulated microbubble behaviour will lead to more sophisticated detection and delivery techniques. In principle, in any perfused region that can be reached by ultrasound, ultrasound-directed drug delivery could be performed. However, since the ultrasonic fields used with diagnostic ultrasound scanners differ greatly per organ targeted, some regions will be far from ideal. The ultrasonic frequencies transmitted in endoscopy are much higher than the resonance frequencies of conventional ultrasound contrast agents. Therefore, for such applications, smaller carriers will have to be developed for ultrasound-directed drug delivery. In conclusion, combining ultrasound contrast agents with therapeutic substances may lead to simple and economic methods of treatment with fewer side-effects, using conventional ultrasound scanners.

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Phenomenon	Schematic representation	Microbubble classification	Acoustic regime
Translation		I, II, III, IV	L, M, H
Fragmentation		I, II	L, M, H
Coalescence		I, II	L, M, H
Jetting		I, II	H
Clustering		II, III	L, M, H
Cracking		II, III, IV	L, M, H

Microbubble shell classes: I) free or released gas; II) thin shells <10 nm; III) thick shells <500 nm; IV) very thick shells >500 nm.

Acoustic regimes: low (L) for $MI < 0.3$; medium (M) for $0.3 < MI < 0.7$; high (H) for $MI > 0.7$.