Microbubble ultrasound contrast agents: a review

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Abstract: The superior scattering properties of gas bubbles compared with blood cells have made microbubble ultrasound contrast agents important tools in ultrasound diagnosis. Over the past 2 years they have become the focus of a wide and rapidly expanding field of research, with their benefits being repeatedly demonstrated, both in ultrasound image enhancement, and more recently in drug and gene delivery applications. However, despite considerable investigation, their behaviour is by no means fully understood and, while no definite evidence of harmful effects has been obtained, there remain some concerns as to their safety. In this review the existing theoretical and experimental evidence is examined in order to clarify the extent to which contrast agents are currently understood and to identify areas for future research. In particular the disparity between the conditions considered in theoretical models and those encountered both *in vitro*, and more importantly *in vivo* is discussed, together with the controversy regarding the risk of harmful bio-effects.

Keywords: microbubble ultrasound contrast agents

1 INTRODUCTION

Ultrasound represents the safest, fastest and least expensive method of scanning for many types of medical diagnosis. Compared with techniques such as magnetic resonance imaging, however, image quality is often inferior, and methods for improving image contrast are therefore highly desirable. Microbubbles have become well established over the past 20–30 years as the most effective type of contrast agent particle (CAP) available for ultrasound radiography. They have been successfully employed with a wide range of imaging techniques, and their potential for therapeutic applications is currently under investigation. However, while a fairly substantial amount of theoretical and experimental research has been conducted, their behaviour is by no means fully understood.

It is known that the effectiveness of microbubble CAPs is due to their dynamic response to the application of an ultrasonic field. As a result of their compressibility they undergo volumetric oscillation (pulsation) and consequently scatter much more energy than rigid spheres of the same size would do. There is, in addition, a fortuitous coincidence between the size of CAP able to pass through human capillaries ($< 8\ \mu m$) and that which is resonant at the frequencies typically used in ultrasonic imaging (1–10 MHz). The amplitude of CAP oscillations (and hence the scattering effect) is thus maximized. Depending on the intensity of the applied acoustic field and the characteristics of the CAP, oscillations may be linear or non-linear and the scattered signal may thus contain both fundamental and harmonic components. The latter are potentially very useful as they can enable echoes from CAPs to be distinguished from those due to tissue (Fig. 1). Ultra-harmonic and subharmonic components have also been detected and may likewise be exploited for imaging [**1**]. A further feature of CAP behaviour of interest for medical applications is their response to high intensity ultrasound. This has been variously described as 'stimulated acoustic emission' [**2**] or 'power enhanced scattering' (PES) [**3**] and is thought to be due to disruption of individual CAPs.

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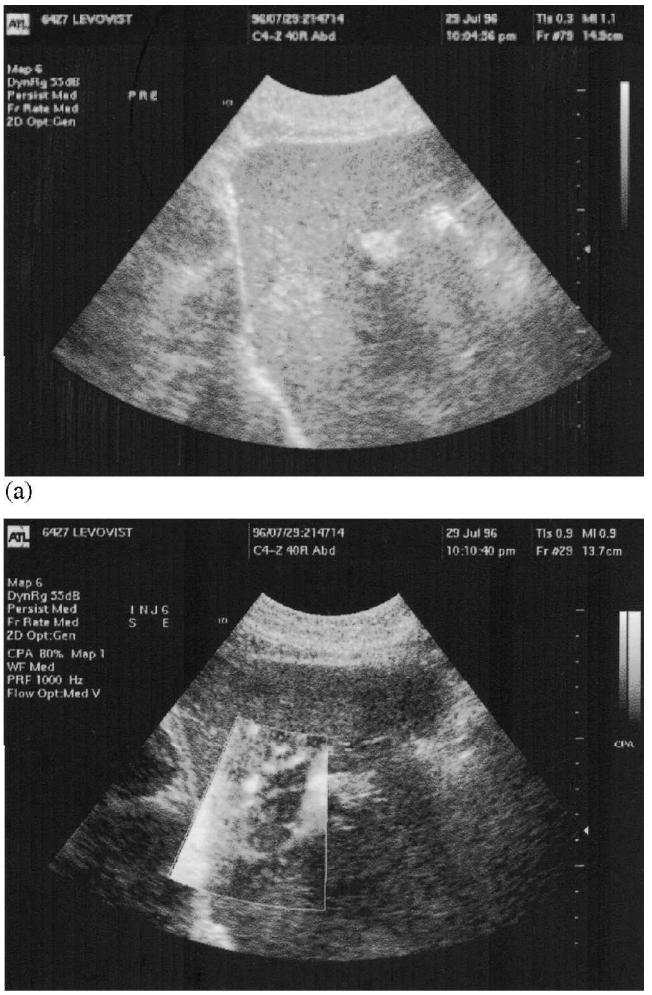
2 CHARACTERIZATION OF CAP BEHAVIOUR: MODELLING

2.1 Dynamic response

The majority of the existing theory applies to the case of a single spherical free gas- or vapour-filled bubble in an unbounded volume of liquid. The three most common forms of the equation of motion describing the bubble's behaviour are:

(a) the Rayleigh–Plesset [or Rayleigh–Plesset–



(a)

(b)

Fig. 1 Ultrasound image of a neuroendocrine tumour in the liver (a) before and (b) after contrast enhancement in power Doppler mode (photographs courtesy of Professor Peter Dawson)

Noltingk–Neppiras–Poritsky (RPNNP)*] equation, which describes the radial oscillations of a spherical, ideal gas bubble in an infinite incompressible fluid;

(b) the Keller or Trilling equation, which considers a compressible liquid;

(c) the Gilmore equation, which allows for non-linear propagation of the incident sound waves.

Most microbubble CAPs are coated with either a surfactant or a solid shell to counter the effects of surface tension and hence increase their longevity (Fig. 2 and Table 1). Encapsulated microbubbles have been modelled by de Jong *et al.* [**4**] and de Jong and Hoff [**5**] by incorporating experimentally determined elasticity and friction parameters into the RPNNP model, and more rigorously by Church [**6**] using linear visco-elastic constitutive equations to describe the shell. In both cases it was assumed that the shell would dominate the response and the possibility of coupling between the gas and the shell was ignored.

The results of these analyses have shown, in accordance with experimental results, that the presence of the shell increases both the damping and the resonant frequency of the CAP, compared with those of an equivalent free bubble. Only viscous damping effects were included explicitly in Church's model. In subsequent work, Frinking and de Jong [**7**] attempted to take account of the acoustic re-radiation, and thermal damping effects as well, but again by means of experimentally determined 'lumped' parameters. Recently Allen and Roy [**8**, **9**] adopted a more analytical approach in considering the effects of linear and non-linear fluid viscoelasticity upon the motion of a single unencapsulated gas bubble.

The equations of motion referred to above are all non-linear and solutions therefore must be obtained either by using linear approximations for small amplitude oscillations [**6**, **10**, **11**], or by using numerical methods [**7**, **12**]. The linear solutions are of limited use since it tends to be the harmonic, i.e. non-linear, response, which is of interest for imaging applications. Analytical solutions for CAP oscillations generating second and higher harmonics have been derived by de Jong *et al.* [**13**] and Church [**6**] but are not suitable for large-amplitude behaviour nor for modelling phenomena such as PES.

Numerical methods provide a more complete description of CAP behaviour but are of course susceptible to errors. Their usefulness is therefore restricted to certain regimes. In discussing the predictability of non-linear motion it should be noted that *chaotic* behaviour of (larger) free bubbles has been both anticipated theoretically from the Keller and the Gilmore equations and observed experimentally [**14**]. To date, this has not been the case for CAPs, but such work would be helpful for determining the conditions under which CAP response could, at least in theory, be predicted.

It has been shown that, in practice, CAP oscillations may be far from spherically symmetric [**15**]. A loss of symmetry may be due to the presence of a nearby surface or another CAP, as will be discussed below, or some other external force. Analyses of non-spherical oscillations of free gas bubbles were made by Lord Rayleigh in 1879 [**16**] and subsequently developed by Lamb [**17**]. More recent studies have been conducted (see, for example, references [**18**] to [**21**]). The stiffening effect of the encapsulating shell and the small size of microbubble CAPs would tend to make them more resistant to distortion compared with free bubbles. Countering this, however, would be the fact that any inhomogeneities in the shell, such as non-uniform thickness, would encourage asymmetric behaviour. Any treatment of non-spherical CAP oscillations would thus differ considerably from the free-bubble analysis because of the need to take account

* The acronym was coined by Lauterborn to recognize the different contributions to the final model.

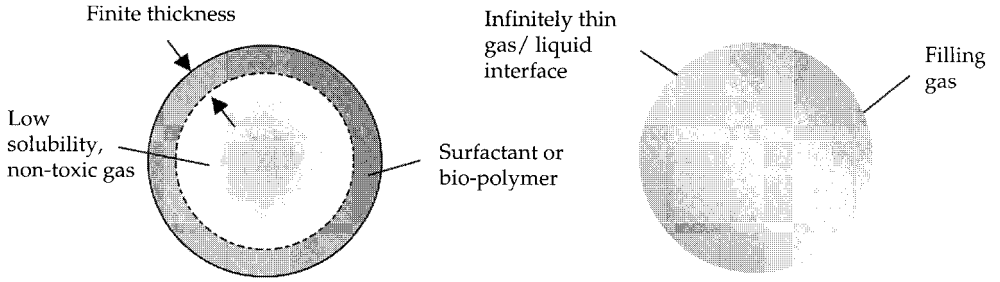


Fig. 2 Schematic illustration of the differences between contrast agent particles (CAPs) and bubbles

Table 1 Properties of selected ultrasound contrast agents

Contrast agent	Filling gas	Encapsulating shell material
Albunex®	air	sonicated serum albumin
Optison™	octafluoropropane	cross-linked serum albumin
Quantison™	air	spray-dried serum albumin
MP1950	decaflurobutane	phospholipid
Aerosomes™	perfluorocarbon gas	phospholipid
Filmix™	air	phospholipid
Bisphere™	air	polymer
Sonazoid™	perfluorocarbon gas	phospholipid

of the shell. As yet, no such treatment appears to have been carried out.

2.2 Acoustic response

The scattering of ultrasound by microbubble CAPs may be treated in two ways. Frinking and de Jong [**7**] and Church [**6**] modelled the CAP as a spherical pulsating source, using the variations in CAP radius calculated from the RPNNP equation to derive the scattered pressure waveform and/or equivalent scattering cross-section. This treatment is based on the assumptions that the CAP diameter is very small with respect to the wavelength*, that spherical symmetry is maintained and that the amplitude of CAP oscillations is quite low. It also neglects the passive component of the scattered field, i.e. that due simply to the presence of the CAP regardless of whether it is pulsating. This has been shown to be negligible for free bubbles [**22**] but may not be for stiffer encapsulated CAPs.

The second approach is to treat the CAPs as elastic spherical shells and to obtain the scattered acoustic field from a modal series solution of the linear wave equation. This was done by Ye [**23**] for thin-shelled CAPs and more recently by Allen *et al.* [**24**] for shells of various thicknesses. Their results suggest that Lamb waves excited on the shells of encapsulated microbubble CAPs produce extra, potentially useful, resonance responses in addition to the monopole response at the insonation frequency. Although this method employs an exact analytical solution rather than numerical approximation, non-linear effects are necessarily ignored. The solutions are therefore somewhat unrepresentative of CAP behaviour except at very low acoustic pressures.

Hilgenfeldt *et al.* [**22**] unified the different treatments for the case of a free gas bubble. They show that, when the bubble oscillations are of sufficiently low amplitude to be considered to be linear, the corresponding scattering cross-sections are the same as those derived from partial wave analysis for isotropic scattering. Since ultimately the analyses describe the same physical process, this would be expected and, by the same token, this should also be the case for CAPs. Again, however, the overlap between existing models would be restricted to the linear regime. The development of a unified non-linear treatment of CAP scattering is to be explored in future work by the present authors.

2.3 CAP destruction

2.3.1 Association with inertial cavitation

The destruction of free bubbles undergoing 'inertial' or 'transient' collapse has been treated extensively in the numerous studies of acoustic cavitation [**25**–**27**]. As a result, the conditions for the onset of inertial collapse and instability leading to fragmentation have been well established, in the form of threshold bubble sizes and acoustic pressures [**28**–**32**]. The few theoretical treatments of CAP destruction have been based upon these conditions, or upon calculations of the rate of gas diffusion out of the CAP [**33**]; in both cases, neglecting the effect of the encapsulating shell. Similarly, studies of PES have tended to concentrate on the behaviour of the free gas bubbles released after shell rupture, rather than the destruction process itself [**34**].

While there is little doubt as to the qualitative similarity between fragmentation of free bubbles and CAP destruction, it is less certain whether the analogy is valid mathematically. According to the equations discussed above, the collapse of a free bubble is dominated by the inertia of the surrounding fluid. CAP compression on the other hand, is dominated by the stiffness and viscosity of the shell, according to the de Jong *et al.* [**4**] and the Church [**6**] models. Similarly, while it is the stability of the gas/liquid interface that governs the

* The wavelength in blood is about 1.5 mm at 1 MHz, i.e. roughly 1000 times larger than the CAPs/bubbles.

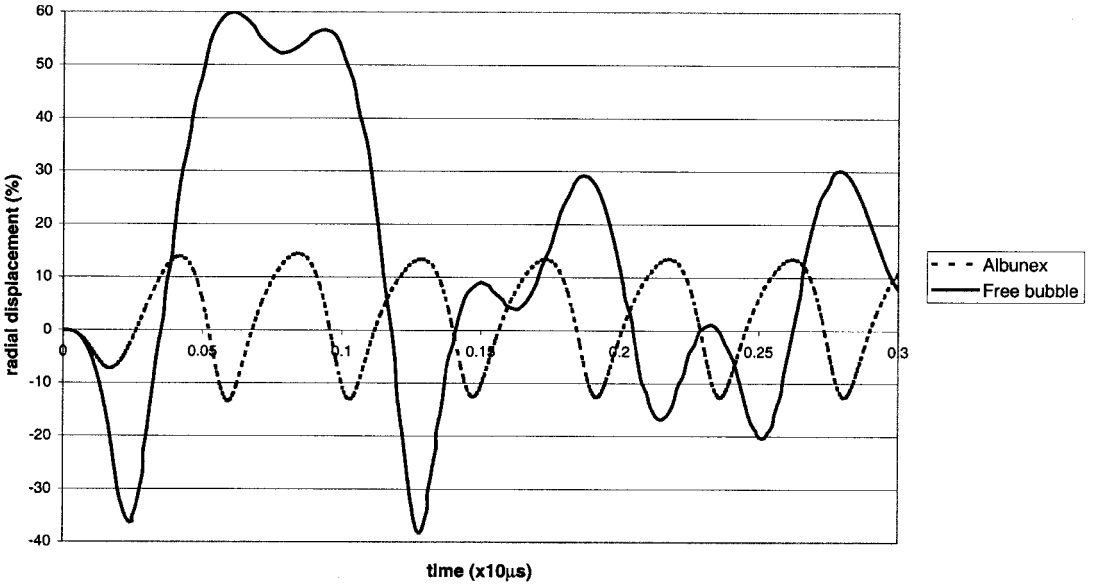


Fig. 3 Variation in radial displacement with time for CAPs insonated at 2.25 MHz and 0.3 MPa

fragmentation of a free bubble, it is the stability of the shell as a structure that is important for CAP destruction. These issues are examined more extensively in forthcoming work [**35**].

2.3.2 Importance of transients

When the RPNNP equation was solved numerically by Leighton [**36**] to include the initial transient oscillations, it was found that their magnitude was sufficient in theory both to enhance the stable cavitation of bubbles close to resonant size, and to trigger the unstable collapse of smaller bubbles. In both cases the estimated internal temperatures and pressures reached during compression would have been sufficient to cause sonoluminescence. This concurred with Leighton's previous observations of this phenomenon in situations where bubbles were excited from rest, e.g. by intermittent pulsation*, and would hence experience transient oscillations. In a subsequent paper [**37**], high-speed photography was used to obtain both qualitative and quantitative confirmation of the theoretical results.

On the basis of these results it would seem plausible to suggest that transient oscillations might also play a significant part in determining CAP behaviour. They might, for example, provide an explanation for PES. However, when a numerical solution† of the Church model was obtained by the present authors, there was found to be little difference between the transient and steady state oscillations even at high insonation pressures and over a wide range of frequencies covering resonance. The contrast between the behaviour of a CAP and a free bubble is demonstrated in Fig. 3. Thus the influence of transients would seem to be relatively minor in the case of CAPs, although this conclusion is clearly contingent upon the validity of the assumptions underlying the Church model. These results are also discussed further in forthcoming work [**35**].

2.4 Population response

It is the more complex behaviour of a population of particles that must be considered when modelling ultrasound contrast agents under diagnostic conditions. In predicting the scattered signal from a bubble or CAP population it has generally been assumed (see, for example, reference [**6**]) that the bubbles are sufficiently far apart for multiple scattering to be negligible and that the overall response is simply the sum of individual bubble responses. However, as will be discussed in Section 3.4, a number of phenomena have been observed experimentally, which can only be explained in terms of the bubble population as a whole.

Studies have been made of the interactions between free bubbles in an acoustic field since Bjerknes [**38**] and Bjerknes [**39**]. They identified the existence of an attractive 'secondary radiation' force between the bubbles arising from the pressure gradient set up around each of them as a result of energy dissipation. If bubbles are insonated for sufficient time at a high enough pressure, they will form clusters as a result of secondary radiation forces. As a result, the mechanical and hence the acoustic response of the individual bubbles/CAPs will be altered [**40**–**42**]. The occurrence of multiple scattering will also be increased and a more sophisticated treatment

* Assuming the pulse repetition rate was sufficiently low so as to allow the bubbles time to settle between pulses so that transients would be excited on the next pulse.

† A fourth-order adaptive step Runge–Kutta routine was used with the parameters given for Albunex® CAPs by Church [**6**].

therefore required in order to predict the final scattered signal. A number of multiple-scattering models have been developed (see, for example, reference [**43**]). However, the validity of each is limited to fairly low concentrations of bubbles/CAPs and linear oscillations.

Ye *et al.* [**44**] studied multiple scattering in bubbly liquids (specifically spherical free air bubbles in water) via numerical investigations based on solutions of the fundamental wave equation. They have shown that acoustic localization (i.e. confinement of the waves close to the transmitting source) can occur in bubble fields accompanied by 'an amazing collective behaviour of the bubbles'. This takes the form of a phase transition, which leads to effective cancellation of the wave. The authors do not refer to contrast agents specifically in their work, but it would seem likely that their findings would be relevant for high-concentration suspensions, such as those discussed in Section 3.4. Their analysis is, however, still restricted to linear bubble/CAP behaviour.

Recently Chin and Burns [**45**] have developed a model, which may be more suitable for CAP populations. They simulate the population response by considering a sample volume (length = longest echo expected = 4 × imaging pulse length), containing a random suspension of N bubbles with a size distribution based on a real contrast agent. The received echo is still obtained by summing individual bubble echoes but these are first scaled by the normalized beam profile and time-shifted according to bubble axial position. The single-bubble response is calculated from a modified Trilling equation* and allowance is made for variations in local incident pressure on and off the beam due to interference of the scattered signal. The presence of a shell, thermal damping effects and the possibility of non-linear propagation through the surrounding tissue (i.e. the presence of harmonics in the incident field) are neglected.

There is another important aspect of modelling a CAP population, which has been identified, although not undertaken, by several workers [**45**, **46**]. This involves the changes in the population and hence in the acoustic response, which take place with time. These changes may be due to a number of processes including mechanical damage in the heart, phagocytosis†, attachment to capillary walls and effects of pressure fluctuations and variations in blood gas content. The relative importance of these processes, and thus the length of time for which contrast enhancement may be maintained, will depend upon individual CAP characteristics.

* This was modified to allow for the presence of a sound field which was not considered by Trilling and because his casting of the equations was not suitable for solution by computer.

† Envelopment by certain types of white blood cell (phagocyte). The survival of microbubble CAPs after being phagocytosed is utilized in harmonic imaging of the liver, but it is assumed that they are ultimately destroyed either by the respiratory burst to which they are subjected or by gradual dissolution. The implications of their indefinite persistence may warrant further investigation, however.

2.5 Interaction with boundaries

Just as the interactions between individual bubbles/CAPs need to be taken into account, so too do the interactions between bubbles/CAPs and nearby boundaries, e.g. the wall of a blood vessel. The presence of a boundary will modify both the incident and the scattered acoustic fields. This can have a significant effect upon the dynamic and acoustic response of the bubble/CAP. Reflection of scattered sound may give rise to multi-pole effects. The reason for this may be envisaged if the reflections are modelled as having originated at an 'image' source, on the opposite side of the boundary from the bubble/CAP [**47**]. The phase and amplitude of the reflected signal, relative to those radiated by the bubble/CAP, are dependent upon the nature of the boundary. The pulsation of the bubble/CAP and the reflected pressure wave will cause the surrounding fluid to be accelerated to and fro. Owing to the difference in its density between the compressed and expanded states the CAP/bubble may experience a net force pushing it towards or away from the boundary (again depending upon the nature of the boundary).

Consequently spherical symmetry can no longer be assumed for wall motion. This has implications, for example, for the stress distribution in the encapsulating shell of a CAP and is extremely important for free bubbles undergoing inertial collapse. Shape instability leading to involution of the bubble may occur, with the formation of a liquid 'microjet' [**48**, **49**] (Fig. 4). As far as the present authors are aware, this phenomenon has not been observed for CAPs but, should this be the case, the potential for damaging effects would be considerable. This is discussed later.

Recent studies of free bubbles near boundaries have been made by Zhong *et al.* [**50**] and Krasovitski and Kimmel [**51**]. In the latter study, three cases for bubble–boundary interaction were identified:

(a) single bubble bounded in one direction, in one

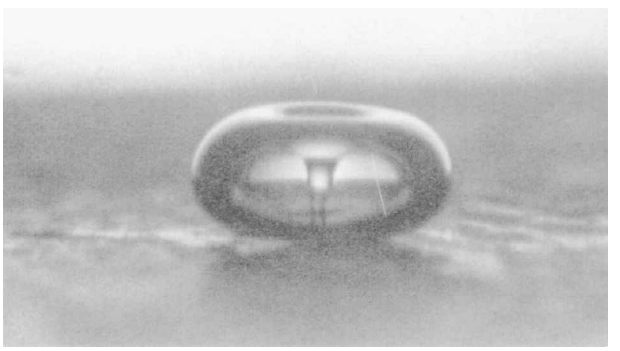


Fig. 4 Formation of a microjet during the collapse of a free gas bubble close to a boundary. Reproduced with permission from Coleman *et al.* [**135**] (© 1987 World Federation of Ultrasound in Medicine and Biology)

dimension (within one diameter of an infinite plane rigid surface bounding an otherwise infinite fluid);
(b) single bubble bounded in two directions in one dimension (between two parallel infinite plane rigid surfaces);
(c) single bubble bounded in all directions in all dimensions (i.e. enclosed as in a cell).

The first case and the subsequent involution of the bubble to form a microjet have been treated quite extensively in the references given above, and more recently by Zhang *et al.* [**52**], Chahine [**53**] and Sato [**54**]. Krasovitski and Kimmel provide an analysis of the second case, which predicts the formation of two microjets, one towards either surface as the bubble collapses and divides. High jet tip velocities were forecast during bubble involution, but the simulation was not valid for the period during which the jets would impinge upon the surfaces. A threshold was defined for jet formation in terms of initial bubble radius, distance between the walls, liquid surface tension and insonating frequency. No experimental validation of the results was carried out and, as with most of the studies for case (a), the analysis was concerned with rigid planar walls and incompressible inviscid irrotational fluids. Similarly, only unencapsulated single bubbles were considered.

The third case and the case of bubble confinement in a tube do not appear to have been treated theoretically for conditions relevant to CAP enhanced examinations. Studies of phagocytosis have tended to treat the surrounding cell as a region of increased viscosity rather than a boundary [**55**]. Geng *et al.* [**56**] carried out both theoretical and experimental investigations of the damping and frequency dependence of confined bubble oscillations but only for relatively large (1–3 mm) free bubbles driven by audible frequencies 150–200 Hz. The potential for exciting shape oscillations falls with decreasing bubble size [**26**] making it inappropiate to extrapolate from the results of Geng *et al.* to micron-sized bubbles and especially to encapsulated CAPs.

3 CHARACTERIZATION OF CAP BEHAVIOUR: EXPERIMENT

3.1 Acoustic response

Church [**6**] did not undertake experimental validation of his model but the results of Hoff *et al.* [**11**] showed good agreement between the measured and expected attenuation for low incident pressure amplitudes, frequencies between 1 and 8 MHz and dilution factors between 1 : 2500 and 1 : 250 (i.e. fairly low concentrations). In contrast, de Jong *et al.* [**57**] found that there were considerable discrepancies between their experimental and theoretical results for the non-linear behaviour of Albunex® (i.e. harmonic generation) at higher acoustic pressures. These were attributed to a number of factors including invalidity of the assumptions made regarding the CAP size distribution, the nature of the incident field, inadequacy of linear theory for describing harmonic response and pressure dependence of model parameters.

Frinking and de Jong [**3**] and Frinking *et al.* [**34**] showed that the RPNNP model could not accurately predict scattering above a certain level of incident pressure (about 200 kPa) which they identified as the threshold for PES. Similarly Shi and Forsberg [**46**] found that none of the existing models were capable of predicting effects such as the subharmonic and ultra-harmonic generation which they had observed at high acoustic pressures. Forsberg *et al.* [**58**] found discrepancies between model predictions and *in vivo* experiments using different CAP surfactant coatings/gases. These were attributed to the neglect of multiple scattering, changes in CAP sizes, uncertainty in gas/blood parameters and interaction between CAP coatings and blood components, leading to alteration of surface properties. The inadequacy of their model for the range of shell materials tested may also have been a factor, however. Evidence for multiple-scattering effects is discussed below.

3.2 *Microbubble destruction*

Frinking and de Jong [**3**] hypothesized that the PES phenomenon was due to the disruption of CAP shells undergoing large-amplitude oscillations and the subsequent release of free gas bubbles. Since free bubbles are both less stiff and less damped than encapsulated bubbles, an increase in their response to the sound field and hence in the scattering would be expected. This effect should last until the bubbles have either dissolved under the influence of surface tension or undergone inertial collapse and fragmentation, depending on their size and the acoustic pressure. The same workers subsequently compared the scattering enhancement, its duration and the non-linear response (i.e. the harmonic spectrum) of a solution of Quantison® (air bubbles with albumin shells) exposed to high acoustic pressure, with results from an RPNNP model for free gas bubbles and predictions for their dissolution time [**34**]. They found good agreement between their theoretical and experimental results, although the actual rupture process and formation of the free bubbles was not modelled. They used their results to derive size distributions for the CAPs contributing to PES, which indicated that only a very small number (about 1 per cent) were involved. Their results also suggested that, the higher the frequency of the high amplitude 'burst' causing CAP disruption, the smaller would be the free bubbles produced. No theoretical analysis of this effect was made, however.

Klibanov *et al.* [**59**] found, by direct observation, that the destruction of insonified Albunex® CAPs tethered to a plate occurred by a process of gradual deflation. The rate of deflation increased with:

(a) reducing CAP concentration,
(b) the presence of a flow,
(c) increasing mechanical index (i.e. acoustic pressure) and
(d) increasing pulse length or repetition rate.

Two explanations were proposed for the 'self-protection' effect observed with high CAP concentrations. The first was that a layer of gas-saturated fluid would be built up around a high-concentration CAP cluster which would discourage further diffusion. This was in agreement with the finding that the protective effect was less evident in the presence of a flow (which would prevent the formation of a saturated layer), but not with the fact that only the edges of each cluster were affected*. The preferred explanation was that the mechanical response of the CAPs was modified by the secondary radiation forces generated between them and that this slowed down the diffusion process.

Dayton *et al.* [**15**] conducted further optical and acoustical investigations of tethered CAPs (with both phospholipid and albumin shells). They observed that at low acoustic pressures the CAPs underwent deflation (with the filling gas dissolving into the surrounding fluid) but that, as the pressure was increased, shell rupture and ultimately fragmentation occurred, with the release of free gas bubbles. Kudo *et al.* [**60**] and Chomas *et al.* [**33**] made similar observations of untethered CAPs both in suspension and individually flowing through a cellulose tube. The latter group identified three mechanisms for CAP disruption: static diffusion, acoustically driven diffusion and fragmentation†. They found that a much higher proportion of the CAPs was destroyed than was suggested by the results of Frinking *et al.* [**34**], with at least 70 per cent being destroyed at similar peak negative pressures. This discrepancy seems surprising at first since Chomas *et al.* were using phospholipid shelled CAPs which have been found to be more durable than those with albumin shells [**15**]. However, Frinking *et al.* were using much higher (about 300 times) concentrations of contrast agent and lower insonation frequencies (away from resonance), both of which would be expected to reduce the likelihood of CAP destruction. There were differences also, albeit perhaps less significant, between the pulse forms and test vessels used in the two studies.

Moran *et al.* [**61**, **62**] designed a tissue-mimicking phantom to enable untethered CAP behaviour to be investigated in a more realistic setting. Their results supported the Frinking *et al.* rupture-release theory for PES, showing that the phenomenon was short lived, irreversible and proportional to the number of CAPs involved. The tests were carried out using similarly high CAP concentrations and pressures. The frequencies were somewhat higher (3.5–5 MHz) but so too were the CAP shell thicknesses and thus resonance should still have been avoided. They also reported the results of some tests by CAP manufacturers (Andaris) which showed that there was no substantial change in the number and size of microbubbles before and after insonification. They interpreted this as an indication that PES was due to leakage or a change in shell permeability rather than to CAP destruction. However, if, as claimed by Frinking *et al.*, PES involves the destruction of only a very small number of CAPs, substantial changes in the population would not be expected. The manufacturer's test conditions were not specified so a definite conclusion cannot be reached. Unlike Frinking and de Jong, Moran *et al.* interpreted the reduction in scatter efficiency as an indication of the robustness of a percentage of the CAP population rather than being due to leakage of fluid into CAP shells after rupture. The optical studies by Chomas *et al.* [**33**] and others would seem to support the latter explanation, however, showing as they do the initially transparent CAPs remaining visible and spherical but becoming opaque.

Kamiyama *et al.* [**63**] also identified three categories of CAP behaviour based on measurements of echoes from CAP suspensions [Levovist® (air bubbles with a surface layer of palmitic acid)] whose concentration was sufficiently low as to enable single CAP responses to be identified. These categories could not be correlated directly with those of Chomas *et al.* but there were some broad similarities. They performed numerical simulations of free-bubble behaviour using an RPNNP model, which appeared to confirm that bubbles smaller than resonant size would collapse within the space of a single pulse and were therefore responsible for producing the observed PES or 'flash echoes'. Wei *et al.* [**64**] also suggested that resonance was not a necessary condition for CAP destruction. They identified total power absorption as the most important factor.

Miller *et al.* [**65**] employed a simple linear model in their analysis but noted in their discussion that the free bubbles produced after shell rupture would be larger than resonant size (the resonant frequency for free bubbles being lower than for encapsulated bubbles as above). They would, however, be the right size for generating the subharmonic signals which are observed during PES. They also discussed the possibility of correlating subharmonic generation with harmful bio-effects. They argued that this would have a stronger theoretical basis than for instance correlation with second harmonics, since subharmonics were more clearly associated with inertial cavitation.

Deng *et al.* [**66**] employed a dual frequency technique (similar to that used by Frinking *et al.* [**67**]) for examining CAP behaviour, using separate low-frequency activation and high-frequency imaging pulses. They observed both linear and non-linear oscillations (up to

* The bubbles were not so close that the flow could not penetrate between them. Had this been the case, the edges of the cluster would be expected to be primarily affected.
† The former group reported similar findings but their complete results were not available at the time of publication.

the twentieth harmonic) without noting any changes in the Albunex® population (i.e. the same spectra were observed before and after the activation pulse). At higher pressure amplitudes they did observe depletion of the insonified region, initially by destruction and subsequently by streaming. This was inferred from the fact that the 'back wall' echo, which was obscured by attenuation at low pressures, became stronger as the pressure was increased and then weaker again as time progressed. They found too that there was a temporal progression in changes in radii. This was indicated by an increase in both the back scatter observed at negative pressure peaks and in the reduction in radius on positive peaks, which levelled off. They suggested that this might be due to a fatigue process causing the shells to become more fragile or pliable with each pulse and an increasing resistance to compression below certain minimum radius.

Podell *et al.* [**68**] conducted extensive tests on the stability of Optison® and concluded that the gas content of the surrounding fluid was likely to be a significant factor in CAP destruction. They found that gas deficiency in blood led to an irreversible outward diffusion of the CAP filling gas, causing the CAPs to shrink down to an undetectable size in a relatively short time even without insonation (see also [**69**]). They confirmed that the rate of diffusion was increased at low CAP concentrations and under imposed pressure, i.e. in the presence of an acoustic field or even the human heart beat. In contrast, chemical dissolution of the shell in a variety of solutions (acid, alkaline, reducing etc.) was found to be relatively negligible.

3.3 Relationship between CAP destruction and inertial cavitation

Shi *et al.* [**70**] conducted an investigation of the occurrence of inertial cavitation in the presence of a surfactant-based contrast agent (Sonazoid®). They concluded that CAP destruction at insonation pressures below 1.6 MPa was due to a relatively slow deflation process following disruption of the shell. Above 1.6 MPa, rapid destruction was observed but the acoustic response differed from that expected for inertial cavitation, being more prolonged and lacking the characteristic pressure 'spikes'. Uhlendorf *et al.* [**71**] and Chen *et al.* [**72**] also found that the thresholds for CAP rupture and inertial cavitation thresholds were different, the latter being higher. However, Church and Carstensen [**73**] reinterpreted the results of Shi *et al.* as indicating that Sonazoid® CAPs did undergo both stable and unstable inertial cavitation. This conclusion was reached on the basis of the scattered signals reported by Shi *et al.* for low insonation pressures. These indicated that the amplitude of radial pulsation (according to the relationship derived from linear theory) was more than 100 per cent of the original radius and hence satisfied the definition of stable inertial cavitation given by Flynn [**74**] (the associated deflation processes were neglected). They further suggested that the disparity between the acoustic signals corresponding to high-pressure CAP destruction and inertial cavitation was in fact due to the signal processing employed by Shi *et al.* rather than to any physical difference in these processes.

While these results may indicate that certain types of CAP do undergo inertial cavitation, they by no means resolve the matter. Intuitively, a CAP with a surfactant coating would be expected to behave in a manner similar to a free bubble, i.e. such that its collapse would be dominated by fluid/shell inertia under the right conditions. This is not the case for a CAP with a more solid shell. For as long as it maintains its integrity, the shell should always dominate the response [**35**]. That this is so in practice is indicated by the results of Dayton *et al.* [**15**] (Fig. 5). These show, optically, that albumin-shelled CAPs do not pulsate spherically even at relatively low insonation pressures. Instead the CAPs were seen to buckle during the initial compression, after which a part of the shell underwent low-amplitude expansion and contraction. Kudo *et al.* [**60**] reported similar observations. At moderate pressures the shell appeared to rupture and a free gas bubble was released, in accordance with the explanation of PES by de Jong *et al.* Clearly, once shell rupture has taken place, the resulting free bubble could undergo genuine inertial cavitation, stable or unstable, depending on its size. The implications of this in terms of damage to the surroundings are discussed later.

At high pressures a more dramatic fragmentation of the shell was observed, occurring within a single pulse, with the released gas dissolving into the surrounding fluid (although presumably free-bubble formation would also be a possibility if a less soluble gas were used). Chomas *et al.* [**33**] concluded that the process was independent of shell characteristics, having observed it for both albumin- and phospholipid-shelled CAPs. However, fragmentation of a solid or semisolid object must necessarily involve fracture of the material from which it is made. The observed process must therefore be controlled by the mechanical properties of the shell and, unless there is some rapid change, which renders the shell instantly ineffective, is thus distinct from a true fragmentary cavitation collapse. The acoustic response corresponding to rapid destruction obtained by Chomas *et al.* [**33**] also differs from that which would be expected for inertial cavitation. Without direct correlation between the acoustic and optical results or precise details of the signal processing, however, this cannot be taken as conclusive evidence.

The experimental results obtained by Chomas *et al.* [**33**] for CAPs with phospholipid shells indicate that their behaviour is closer to that of free bubbles. This implies in turn that the shell material is more fluidic than albumin. The phospholipid CAPs were seen to retain their

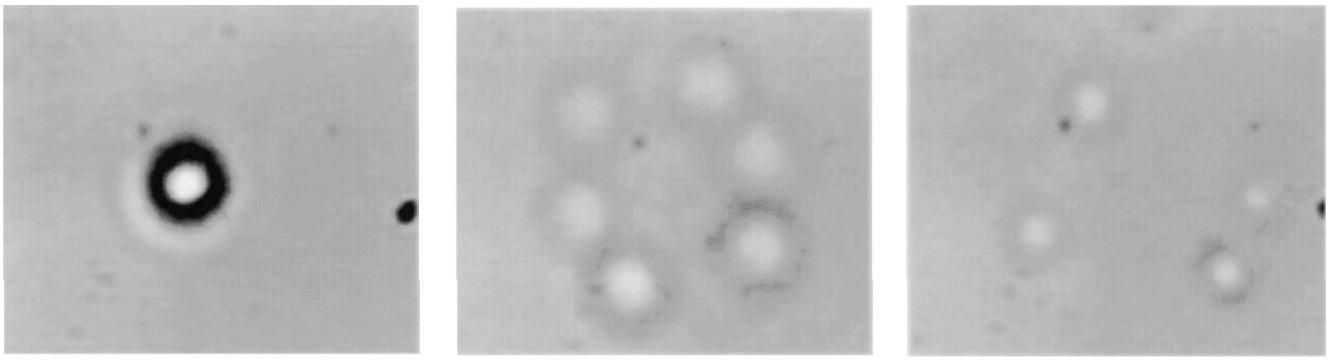


Fig. 5 Fragmentation of a 2 μm phospholipid shelled CAP at 5 MHz, 2.6 MPa. (a) initially at rest, (b) 4.16 ms after insonation, (c) 620 ms after insonation. Reproduced with permission from Chomas *et al.* [**33**] (© 2001 IEEE)

sphericity when pulsating in response to low insonation pressures. No defect formation was observed at moderate pressures, and at high pressures the CAPs fragmented into arrays of small bubbles, just as a free bubble would be expected to do. The dissolution times for the small bubbles were longer than expected. This indicated that they were subject to lower surface tension than would be experienced by a free bubble of the same size and hence that they were still, at least partially, coated with shell material.

The amplitudes of CAP pulsation oscillation were considerably smaller than would be expected for free bubbles at the same pressures, however, and would not satisfy the criteria cited by Church and Carstensen [**73**] for inertial cavitation. Likewise, Chomas *et al.* [**33**] only obtained order-of-magnitude agreement at high pressures between the predicted and experimental conditions for stability (the Plesset–Mitchell criterion). Recent work by the present authors has shown that this would in fact be expected and that free bubble analysis could not provide an accurate prediction of CAP behaviour [**35**]. It is clear from both theoretical and experimental results that a distinction must be drawn between the different types of coating when modelling CAP behaviour, and assessing any associated risks.

3.4 Population response

3.4.1 Secondary radiation force

In 1975, Crum [**75**] obtained photographic evidence of bubble clustering as a result of secondary radiation forces and carried out measurements of the relative speeds of approach. More recently, Duinevald [**76**] conducted a theoretical and experimental investigation of bubble attraction and coalescence. As predicted, he found that insonified pairs of air bubbles in pure water were attracted to one another and, up to a certain insonation pressure, the bubbles coalesced upon meeting. At higher pressures, however, coalescence was inhibited. This was thought to have been due to the increase in speed of approach of the bubble walls at higher amplitudes of oscillation, which caused the bubbles to rebound from each other before coalescence could occur. He also found that pairs of bubbles which did not coalesce tended to lose their transparency and suggested that this was due to resonance in other modes. Non-spherical behaviour was also suggested by the optical images obtained during the experiment.

Both of the above studies employed relatively large free bubbles and correspondingly low frequencies. Investigations of secondary radiation forces between CAPs have been made by Dayton *et al.* [**15**, **77**–**79**]. They confirmed the occurrence of clustering and found good agreement between the predicted and measured speeds of approach of neighbouring CAPs. While the clusters were found to be resistant to separation during insonification even at low pressures, they dispersed as soon as transmission was stopped. Thus the effects of secondary radiation forces were only found to be significant at high pulse repetition frequencies. Under these conditions, however, the CAPs were found to have increased resistance to destruction, as was the case for the tethered CAPs in the experiments described previously. The exposure geometry was such that shielding of the centre of the cluster by the outer CAPs could not explain this effect. The results therefore indicated that the dynamic response of the CAPs had indeed been altered. This conclusion was reinforced by the change in the acoustic response discussed below.

3.4.2 Multiple scattering

Numerous experimental studies of free-bubble arrays have been made, demonstrating a wide variety of collective bubble behaviour. These have been comprehensively reviewed in the articles by Neppiras [**26**], Chahine [**53**] and Leighton [**41**]. A general theoretical model accurately describing these different forms of behaviour has yet to be developed, however. Moreover the conditions under which the majority of these studies were carried

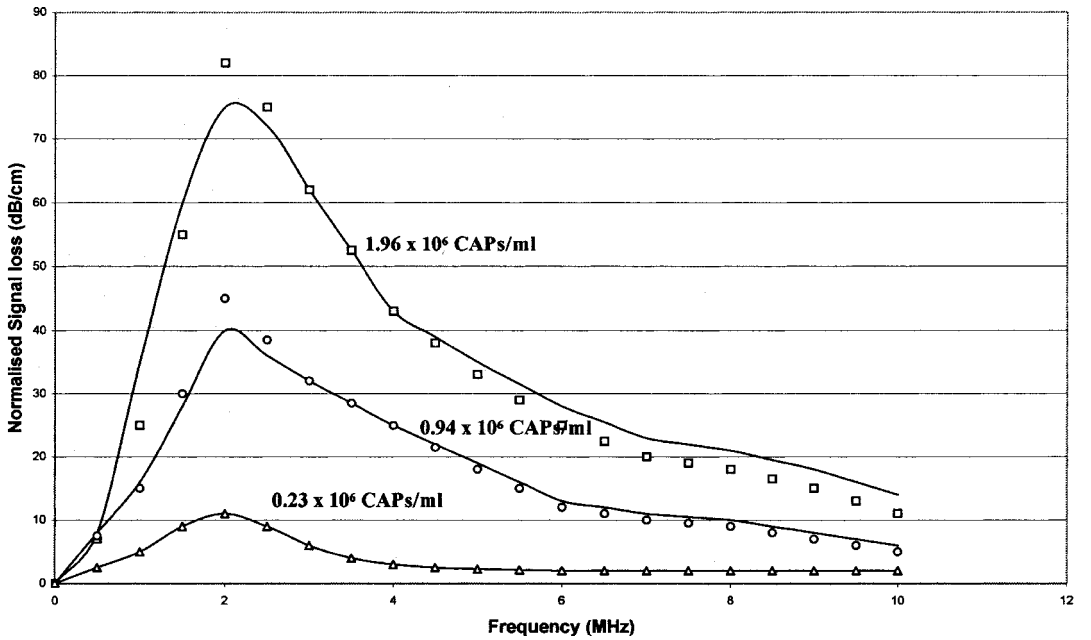


Fig. 6 The effect of increasing CAP concentration upon attenuation measurements from a suspension of Albunex®. Scatter points represent experimental measurements. Solid lines represent fitted theoretical curves. Reproduced with permission from Hughes *et al.* [**80**] (© 1997 The Acoustical Society of America)

out differ considerably from those relevant to ultrasound radiography. The discussion will therefore be restricted to CAPs and the most recent studies of free bubbles of equivalent size.

Hughes *et al.* [**80**] carried out attenuation and phase velocity measurements on suspensions of Albunex® to determine the concentration at which the assumption of negligible multiple scattering was no longer valid. At concentrations of Albunex® exceeding 10^7 particles/ml they found that the peak attenuation shifted towards higher frequencies (Fig. 6). This was not predicted by the de Jong *et al.* and the Church models, i.e. from the summed responses of single CAPs*. Similarly, Zhang *et al.* [**81**] observed an increase in non-linearity parameter with increasing CAP concentration, which they attributed to multiple-scattering effects. As mentioned above, Dayton *et al.* [**15**] also observed a distinct variation in the acoustic response of a CAP cluster compared with that of a low-concentration suspension. This took the form of a reduction in the harmonic content of the scattered signal, i.e. the spectrum decreased in bandwidth, implying that some form of interaction was taking place between the CAPs and encouraging uniform behaviour.

The experimental evidence would thus seem to indicate that multiple-scattering effects cannot be neglected at CAP concentrations similar to those recommended for use in clinical examinations (see, for example, reference [**82**]). It is possible of course that the suspension of CAPs is sufficiently diluted in the blood stream after injection for multiple scattering to be negligible, especially if the pulse repetition rate is too low for secondary radiation forces to be significant. Until this is confirmed, however, it must be assumed that multiple-scattering effects cannot be ignored, and to date there has been little agreement between experimental results and the theoretical treatments of CAP populations described earlier. In recent work on free bubbles, Kapodistrias and Dahl [**83**] measured the acoustic response from two identical air bubbles (600 μm diameter) attached to a fine thread and found that multiple-scattering effects were detectable. Their results were in good agreement with predictions obtained from an expansion of the multiple-scattering series. Accurate treatments of an entire population have yet to be developed, however, although some interesting results have been obtained from investigations of chaotic behaviour as discussed below. Moreover, care must be taken in extrapolating from results for relatively large free bubbles to contrast agents whose size and encapsulation has a significant effect upon their behaviour [**35**].

3.5 Interaction with boundaries

Zhong *et al.* [**50**] performed experiments to assess how the presence of a vessel wall (simulated by silicone and cellulose tubes) affected the oscillation and inertial

* These workers did point out that their results were themselves subject to error, however, since linear behaviour was assumed in the calculation of the attenuation coefficient and the apparatus was extremely sensitive to temperature changes.

collapse of a free bubble induced by lithotripter shock waves. They found that smaller vessels gave rise to a greater constraining effect upon bubble oscillation and hence less violent bubble collapse. However, vessel dilation and tendency to rupture also increased with decreasing vessel diameter and larger numbers of bubbles. The mathematical model employed in the work did not take account of the vessel.

Lindner *et al.* [**84**] found that phagocytosis of CAPs by leukocytes could be used to gauge the extent and severity of tissue inflammation because the leukocytes adhere to cells in the inflamed region. Following up on this research, Dayton *et al.* [**55**] found by optical and acoustic observation that phagocytosis reduced the amplitude of CAP oscillation, increased damping, increased mean frequency of response to a single rarefaction first pulse (but not compression first) and increased the frequency shift observed. They used a modified RPNNP equation [**85**] to predict radius–time curves for the CAPs and hence the echoes and frequency shifts which would be detected. They found good agreement with their optical results for the radius–time curves but only qualitative agreement for the frequency shifts. They attributed the discrepancy in the latter to the low-pass filtering effect of the surrounding water bath upon the propagating wave; this was neglected in the model. They did not address the question of the survival time for phagocytosed CAPs.

3.6 Chaos

As mentioned above, Lauterborn and his co-workers successfully confirmed their predictions of chaotic behaviour (i.e. highly irregular, apparently random motion) with experimental observations of collections of free bubbles. Their findings have been reviewed by Lauterborn and Parlitz [**86**]. Briefly, however, their results indicate that the interactions between bubbles in a cloud gives rise to strong uniformity, with the surprising result that the response of the cloud as a whole is qualitatively similar to that of a single bubble (as opposed to a summation of single bubbles of different sizes). This seems, outwardly at least, to concur with the change in acoustic response observed by Dayton *et al.* [**15**] and may be of assistance in developing an effective multiple-scattering model.

As regards the chaotic behaviour of CAPs, Morgan *et al.* [**85**] suggested that the agreement between their theoretical and experimental results shows that CAP oscillations are deterministic, i.e. capable of being predicted by equations, rather than chaotic. However, agreement was only obtained for certain types of CAP [MP1950 (see Table 1)] and for relatively small amplitude oscillations.

4 SAFETY OF CONTRAST AGENTS: DAMAGE MECHANISMS

4.1 Toxicity

Clearly the shell material and filling gas should be selected so as to satisfy standard pharmacological/toxicological tests. References to relevant studies in this area have been given in the review by Mornstein [**87**] The risks arising from acoustically induced chemical changes leading to the formation of toxic products are less easily assessed, however, as discussed below.

4.2 Microembolism

Blockage of any blood vessel supplying a major organ could have extremely serious, even fatal results. The risks associated with introducing any particle, especially one having dimensions similar to those of a capillary (1–10 μm), into the bloodstream must therefore be considered carefully. According to Nyborg [**88**], single microbubble CAPs do not present a significant risk and Uhlendorf [**89**] stated that albumin-shelled CAPs do not coalesce to form larger particles. This may not be the case with surfactant-coated CAPs, however, and there is some evidence that coalescence does take place between Albunex® particles [**90**]. More fluidic shells would be more likely to allow coalescence (as evidenced by the coating of CAP fragments after rupture described above), and thus a stabilized particle could be produced, of sufficient size to block a capillary.

Free bubbles, released after some form of shell disruption, would certainly be able to coalesce and thus pose a risk, particularly if a low-solubility gas were used. Conversely CAPs having thick shells to increase their longevity would also pose a risk, since their inability to deform would increase the chance that they could cause a blockage. Furthermore, as mentioned above, clusters of CAPs held together by secondary radiation forces were found to be highly resistant to separation. Thus some care would be needed in selecting the scanning parameters, i.e. the insonation pressure, frequency and pulse repetition rate to avoid clustering.

4.3 Heating

Absorption of energy as ultrasound passes through any medium causes a heating effect. The higher the frequency used, the greater is the absorption and hence the risk of thermal damage. Thus the generation of harmonic components in the CAP echoes, while desirable for image enhancement, might lead to some undesirable heating effects. Heat will also be dissipated during CAP pulsation due to viscous effects and conduction during compression of the filling gas. Hilgenfeldt *et al.* [**91**] used a simplified Keller equation to simulate the effects from

single free bubbles (such as would be produced after shell rupture) and used the results to predict subsequent heating of the surrounding tissue. They concluded that the risk of damage was minimal for normal clinical conditions but that this was not necessarily the case at higher insonation pressures. The heating effects associated with inertial cavitation are discussed below.

Absorptive heating will also arise as a result of the attenuating nature of the CAP suspension itself [**92**, **93**], and from non-linear propagation of the incident and scattered waves, which will result in the development of harmonics [**94**]. The latter effect is not directly linked to the presence of CAPs but the modification of the incident wave will affect CAP response to some extent. According to the theoretical study by Hilgenfeldt *et al.* [**91**], the effect should be small. Chomas *et al.* [**33**] on the other hand identified the shape of the incident wave as an important determinant of CAP behaviour experimentally. Also, as mentioned above, Frinking *et al.* [**34**] found that frequency content of the insonating pulse affected the size of the free bubbles produced upon CAP rupture.

4.4 Radiation force

One aspect of non-linear propagation of the incident wave, which is significant for CAP behaviour, is the primary radiation force. This arises as a result of the pressure gradient produced by the passing of the wave and can lead to rapid translation of free bubbles in liquids. So-called 'bubble bullets' have been observed to travel at 5–10 cm/s [**95**] and have been shown to destroy living cells [**96**] *in vitro*. *In vivo*, however, these harmful effects appear to be somewhat mitigated. Later experiments by Miller *et al.* [**65**] showed that a very high concentration of bubbles was needed to cause damage to cell concentrations similar to those found in blood/tissue. According to Mornstein [**87**], this may be because the higher levels of carbon dioxide in the blood associated with high cell concentrations exert an additional damping effect upon bubble activity. Alternatively the 'mirror' effect, as observed by Miller *et al.* [**97**] (see below) may cause bubbles to become attached to nearby cells, thus preventing their being accelerated.

In addition, it may be that bubble translation is negligible at the pulse repetition rates used clinically. It was shown by Tortoli *et al.* [**98**] that bubble displacement was not sufficient to interfere with Doppler measurements and investigations by Dayton *et al.* [**77**], discussed in the next section, recorded maximum speeds less than 0.001 m/s.

4.5 Mirror effect

Just as the secondary radiation forces between two neighbouring bubbles may cause them to move towards each other, so a bubble may become attached to a nearby surface that is reflecting energy emitted from the bubble [**47**]. This may have a direct effect if the pulsation of the bubble is of sufficient amplitude to damage the cell (although attachment may affect the bubble's ability to pulsate). The increased proximity may also contribute to the risk of damage due to streaming and microjetting described below.

4.6 Acoustic microstreaming

The oscillation of a bubble or CAP may cause steady 'streaming' flows to be set up in the surrounding fluid, due to the friction between the fluid and the wall of the bubble/CAP when they move relative to one another. These flows may in turn impose shear stresses on nearby surfaces, such as the walls of cells, and thus damage them. Experimental evidence for cell damage by microstreaming around free bubbles was obtained, *in vitro*, by Clarke and Hill [**99**] and Rooney [**100**]. More recently similar results were obtained with CAPs by Ward *et al.* [**101**].

From studies of the streaming around a free bubble attached to the base of a tank, Elder [**102**] identified four distinct streaming patterns corresponding to different combinations of liquid viscosity and acoustic pressure. Gormley and Wu [**90**] performed a similar experiment using latex particles to trace the streaming patterns around Albunex® CAPs in a 160 kHz standing-wave field for various insonation pressures. At low pressures the patterns were similar to those observed by Lee and Wang [**103**] around solid spheres. As the pressure was increased, a translational motion was superimposed upon CAP pulsation (giving rise to a dipole response acoustically). In addition, jets were observed to emanate from the CAP, although it was not clear whether these were purely fluid motions or due to escaping gas. The streaming speed was estimated to be between 50 and 100 μm/s.

The majority of the theoretical studies of streaming around microbubble CAPs have been based on the analysis by Nyborg [**104**], e.g. that by Wu [**105**]. In the latter study the streaming velocities and associated stresses were calculated using CAP wall velocities obtained from the model developed by de Jong *et al.* [**13**]. They indicated that cell damage due to streaming would be expected at insonation pressures as low as 0.12 MPa. It should be noted, however, that the analysis was derived for a hemispherical free bubble attached to a plane surface and therefore has limited applicability to the case of a spherical shelled CAP.

4.7 Inertial cavitation

As discussed above, there is some controversy regarding the relationship between CAP destruction and inertial

cavitation. This is to some extent irrelevant in considering contrast agent safety, however, for as long as there is a possibility of free bubbles being released the risks associated with cavitation cannot be ignored. These are considered separately as follows.

4.7.1 Heating

The inertial collapse of a free bubble is accompanied by an extremely large and rapid reduction in volume and consequently by a rise in pressure and temperature to several thousand kelvins [**106**]. Clearly, direct damage to cells would be more or less inevitable if the temperature of the surroundings reached a fraction of this level. As noted by Crum *et al.* [**107**], however, these extreme conditions will be highly localized, the centre of the bubble being very small. According to the analysis by Hilgenfeldt *et al.* [**91**], the temperature rise in the fluid just a few micrometres from the centre will be relatively negligible ($\ll 1$ K) and persist for no more than a few microseconds.

Similarly, Hilgenfeldt *et al.* also showed that, while the generation of higher harmonics and viscous effects would be more significant at high insonation pressures, the associated temperature rise would still be relatively small. Moreover the actual temperature rise achieved in practice would almost certainly be lower than predicted, as a result of asymmetrical bubble collapse and cooling due to circulation of the surrounding fluid. If it is the case therefore that cell damage is proportional to the total amount of energy absorbed, the risk from thermal effects would seem to be quite low. This relationship has not been conclusively proven, however, and clearly, if the number of bubbles collapsing were very high relative to the number of cells, the overall temperature rise could be significant.

4.7.2 Fragmentation

At high acoustic pressures, collapsing bubbles may become unstable, causing them to fragment into a mass of smaller bubbles. This is especially likely to occur if the symmetry of their surroundings is interrupted for example by the presence of another bubble. The energy released in this process may cause direct damage to cells and 'bubble nuclei', which may subsequently undergo inertial cavitation, will also be produced. The same will be true for fragmentation of CAPs regardless of whether or not the process is genuinely cavitation. The importance of this mechanism will depend upon the number of bubbles/CAPs destroyed during insonation and, as noted above, this has been the subject of some controversy.

4.7.3 Microjetting

The application of a large acoustic pressure may induce involution of a bubble attached to a surface such as a cell wall, leading to the formation of a high-speed 'microjet' which can damage the surface. If the bubble fragments during the process, bubble nuclei will also be formed. Direct observation of this effect, and the resulting damage, has been made *in vitro* [**49**] but its occurrence *in vivo* (or at least in a tissue mimicking environment) has not apparently been verified.

4.7.4 Chemicals

It has also been shown that the high temperatures and pressures associated with inertial cavitation may lead to the formation of free radicals and toxic chemicals such as hydrogen peroxide (H_2O_2). Evidence of their ability to cause damage has been provided by the results of tests in which cell death has been observed outside the area exposed to ultrasound [**99**]. Riesz and Kondo [**108**] found that the concentrations of these chemicals/species produced under normal clinical conditions were sufficient to cause cell damage. However, a study of the relative production rates *in vivo* of H_2O_2/free radical and chemicals such as cysteine (which is naturally present in the blood and has a scavenging effect upon free radicals) does not appear to have been made.

In theory, if the insonation pressure is lower than the threshold level for the smallest bubble released, inertial cavitation should not occur. There is, however, some uncertainty regarding the thresholds defined by Holland and Apfel [**32**], since their analysis did not take into account blood compressibility or viscoelasticity [**109**]. This should in fact mean that the existing thresholds are conservative but, until this and the limits of the range of bubble sizes produced have been confirmed, inertial cavitation must still be regarded as a potential source of harm.

5 EVIDENCE FOR BIO-EFFECTS

Dalecki *et al.* [**110**] observed an enhancement in cavitation induced haemolysis *in vivo* upon the introduction of a CAP suspension, but they concluded that the risk of cell damage associated with the size of dose normally used in clinical practice was negligible. More recently Carstensen *et al.* [**111**] reached the same conclusion. Coleman and Saunders [**112**] and Delius [**113**] found evidence of damage to DNA which they attributed to cavitation. However, these studies were concerned with the much higher acoustic pressures used in lithotripsy, and previously Miller *et al.* [**96**] found that cavitation damage only affected non-viable cells and hence that there would be no lasting deleterious effects.

The optical and acoustic observations by Klibanov *et al.* [**59**] and Chomas *et al.* [**33**] indicated that the main mechanism for CAP destruction (of those types tested) is gradual deflation rather than violent collapse, and that the risks associated with their use are therefore small.

Similarly, Uhlendorf *et al.* [**2**] stated that extensive studies by Causemann [**114**] have indicated there to be no bio-effects associated with PES or contrast agents in general. This study is apparently based on experimental evidence only, however, and is therefore relevant only for the particular agents and scanning conditions investigated.

The current view of contrast agent safety given in the reviews by Mornstein [**87**] and Nyborg [**88**] seems to be that there is no immediate cause for concern, provided that regulations (see, for example, reference [**115**]) are adhered to and contrast-assisted scans are not closely followed by treatments such as lithotripsy. However, this view seems to be based on the fact that no definite evidence for adverse effects has been reported over the 20 years that ultrasound contrast agents have been in use, rather than the results of extensive theoretical and experimental study.

6 RESEARCH AND DEVELOPMENT

Their potential for improving both diagnostic capability and targeting of various treatments has made ultrasound contrast agents the subject of a wide and rapidly developing field of research. What follows here is an overview, albeit inevitably selective, of recent and projected future advances.

6.1 New imaging techniques

The second-harmonic component of CAP response has already been exploited quite extensively, but there are other parts of the scattered signal which are now receiving attention. Under the right conditions of pressure and frequency, bubbles/CAPs will scatter at subharmonic frequencies, usually half the insonation frequency [**26**]. While certain tissues may scatter quite strongly at the second harmonic, the subharmonic component is usually negligible [**116**]. Thus distinguishing between CAP and tissue echoes is made easier. Spatial resolution in subharmonic imaging is currently limited by the requirement for narrow-band insonation signals, but it seems likely that such problems will be overcome as the field develops [**117**]. In addition to subharmonics, the CAP response may also contain ultra-harmonic components, i.e. at non-integer multiples of the insonation frequency. These also offer the possibility of improved CAP detection [**118**].

Alternative imaging strategies include 'pulse inversion imaging' [**119**] and 'release burst imaging' [**67**]. In the former an initial imaging wave is transmitted into the subject, followed, after a suitable delay, by an inverted copy of itself. If the two waves are scattered linearly, the sum of the resulting echoes should be zero. If, however, they encounter non-linear scatterers such as CAPs, there will be a residual signal after summation, which will be proportional to the degree of non-linearity (Fig. 7). Superior image resolution may be attained because, in contrast with harmonic imaging, the signal bandwidth does not need to be limited. Moreover, relatively low insonation pressures may be used, thus reducing the risk of CAP destruction and damage to tissue. Incorporation of Doppler techniques into the imaging process has enabled tissue motion to be compensated for [**119**].

Release burst imaging utilizes the PES phenomenon. The 'release burst' consists of a high-pressure, relatively low-frequency pulse which causes CAP disruption. In addition to this, a separate set of high-frequency broadband pulses are used for detecting the CAPs/bubbles before and after PES. CAP presence is indicated by the difference between the response and the imaging pulse. Separating the imaging and disruption signals enables both processes to be optimized, thus avoiding loss of either resolution or contrast sensitivity. This technique has also been combined with Doppler imaging, to remove noise due to tissue motion, and with real-time imaging to compensate for the necessarily intermittent nature of the scanning [**63**]. It has also been suggested that CAP destruction could be used for measuring the rate of blood perfusion, by using a high-energy pulse to destroy all CAPs in a given region and then monitoring the rate at which CAPs from further upstream flow [**120**]. However, quite apart from the safety concerns, there is some uncertainty as to the reliability of this method. According to Moran *et al.* [**62**], not all CAPs are necessarily destroyed even at high pressures and reperfusion measurements may therefore be inaccurate.

To accompany the development of new imaging strategies, scanning equipment has also become a focus for improvement and innovation in both academic and commercial groups. Projects are currently under way for example at the Interuniversity Cardiology Institute of The Netherlands [**121**] and the University of California, Davis [**122**]. The latter's aim is to produce a high-frequency (50 MHz) system, able to resolve and measure blood flow in vessels with diameters down to 40 μm. Some recent studies have also drawn attention to the

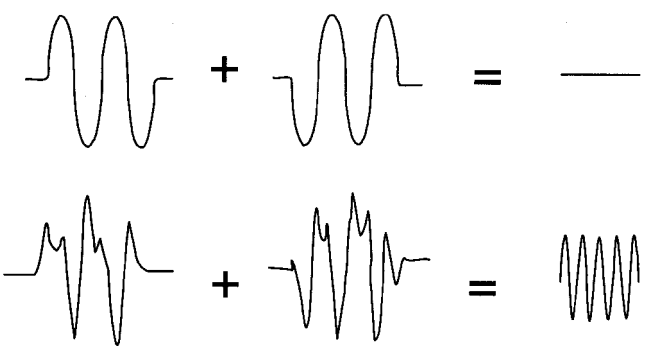


Fig. 7 Schematic illustration of the principle of pulse inversion imaging

possibility of altering the response of the CAP itself, by increasing the shell thickness so that additional modes of oscillation will be excited during insonation [**24**]. The higher-mode signals would offer similar advantages to the subharmonic and ultra-harmonic signals discussed above.

6.2 CAP targeting

The use of microbubble CAPs as vehicles for drug and gene delivery, and even for deliberately blocking capillaries supplying cancer tissues, has attracted considerable interest in recent years (see, for example, references [**123**] and [**124**]). This, in addition to the interest in PES for imaging applications, has strengthened the need for understanding and optimizing CAP destruction processes. The mechanisms of CAP destruction are the subject of ongoing work at the University of Virginia [**125**], in the Netherlands [**121**] and by the present authors at University College London. Clearly, in both imaging and therapeutic applications, accurate targeting of CAPs to particular areas of tissue is highly desirable. It has been suggested that the primary radiation force, described above in the context of 'bubble bullets', could be used for 'steering' a stream of CAPs to a required location [**77**, **78**, **126**]. Further investigations are being made both by the group at the University of Virginia [**122**] and at Virginia Polytechnic Institute [**109**].

An alternative approach has been to incorporate particular species (ligands) into the CAP coating which will cause the CAP to be attracted to specific sites. A number of commercial products have been developed on this basis, e.g. Aerosomes® [**127**], Filmix® [**128**] and Bisphere® [**129**] which consists of capsules of a biodegradable polymer, with a biocompatible outer coating.

The polymer shell provides physical rigidity and may be filled with either a gas or a drug. The outer coating provides the biological interface and a 'scaffold' for targeting species (Fig. 8).

6.3 Modelling

In addition to clinical and *in vitro* experimental studies, further theoretical investigations of CAP behaviour are also being made. The effects of fluid viscoelasticity upon CAP behaviour and cavitation threshold for example, were recently examined by Allen and Roy [**8**, **9**] and are the subject of current studies by Khismatullin [**109**]. The effect of a boundary upon the symmetry of bubble/CAP collapse is also being investigated by the latter and is the subject of further work by Krasovitski and Kimmel [**130**]. Advanced treatments of scattering from single bubbles are being undertaken by Psychoudakis [**131**], in the form of a 'hybrid' Rayleigh–Plesset–scattering model, and by Allen *et al.* [**24**]. The scattering from a bubble/CAP population is under investigation by Ye [**132**] and Chin and Burns [**133**] as discussed above. The longer-term response of the shell in terms of material changes, fatigue processes, etc., is being examined by Deng *et al.* [**66**] and similar issues are the subject of current work at University College London by the present authors.

6.4 Bio-effects

While each new contrast agent must be approved on the basis of standard pharmacological tests, it is impossible to simulate the whole range of conditions under which contrast agents may be employed clinically. The risks associated with, for example, inertial cavitation cannot therefore be eliminated. The concerns regarding the current regulations pertaining to the onset of cavitation have already been mentioned and are the subject of current investigations by Khismatullin [**109**]. The other main focus of bio-effects research is microstreaming, and a number of recent studies have been conducted at the University of Vermont [**132**]. More general studies of bio-effects *in vivo* are planned by Deng *et al.* [**66**], and it is hoped that a clear link between harmful effects and some aspect of the acoustic response, such as

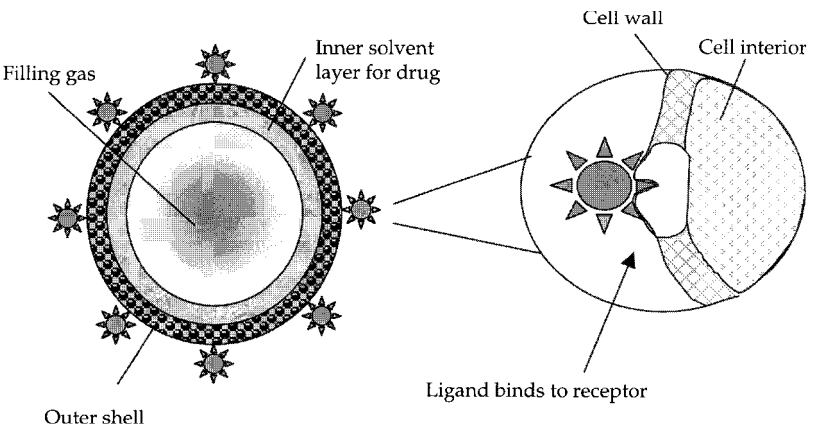


Fig. 8 Design of a CAP for targeted drug delivering

subharmonic emission, may be established to enable reliable and convenient monitoring.

7 SUMMARY

Microbubble ultrasound contrast agents offer a wide range of potential benefits for both diagnostic and therapeutic applications. As a result, they have become the subject of a broad and rapidly developing field of research. At present, however, their behaviour is by no means fully understood, and consequently their effectiveness has yet to be maximized. Moreover, while no definite evidence of harmful effects has been obtained, there remain some concerns as to their safety. As these questions are resolved by advances in the subject, it is anticipated that more and more of the benefits of contrast agents will become realizable. The aim of this review has been to examine the existing theoretical and experimental evidence to clarify the extent to which contrast agents are currently understood and to identify the most fruitful areas for future research.

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