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*'Study the past – with a critical eye – if you would define the future.' Confucius (**modified**).*

The role of Ca^{2+} in ultrasound-elicited bioeffects: progress, perspectives and prospects

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Intracellular calcium (Ca^{2+}) transients have been observed in association with exposure to therapeutic ultrasound and correlated to both early- and late-onset bioeffects. For example, it has been suggested that early 'ultra-short' Ca^{2+} transients recorded during sonoporation can mediate Ca^{2+}-dependent exocytosis and endocytosis processes as complementary mechanisms for membrane self-sealing. Moreover, apoptosis induction has been reported to occur through a partial mediation of a Ca^{2+}-dependent pathway. In this review, we attempt to assemble the salient facts into a cogent whole, with special attention given to the relationships arising through altered Ca^{2+} levels, which underscore its crucial role during ultrasonic interactions with biological systems and its consequent implications in the context of therapeutics.

Since the first report on the biological effects of ultrasound in 1927 [1], the concept of therapeutic ultrasound (TUS) has been consolidated considerably and extended in versatility across a wide range of applications. Recent exciting demonstrations – for example, in accelerated bone fracture healing and wound healing – underscore this [2–4]. Moreover, the facility for direct lysing of cells and induction of apoptosis [5,6], together with adjuvant qualities gained by the combination with chemotherapeutic [7,8] and thrombolytic [9,10] agents, also serve to highlight the promise of this approach once its full potential is reached. Such successes paint TUS in a positive light; however, this belies the fact that our understanding of the fundamental processes giving rise to both real-time and downstream effects is somewhat incomplete and, in some instances, rather confused. That the interaction between TUS and biological systems has been shown to produce a wide range of often contrary effects is perhaps not surprising given the complexity and the multitude of parallel processes involved [11,12]. The dominance of any individual effect in relation to another depends on many variables, both acoustic and non-acoustic, which makes inter-comparisons difficult and might actually stymie translational research [13]. Therefore, to

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TABLE 1

Summary of TUS setups used in major studies included in the review

Cell line	Exposure system	Transducer	Transducer position in relation to cells	Central frequency (MHz)	Acoustic pressure (MPa)	Sonication period(s)	Contrast agent	Refs
***Xenopus* oocyte**	Special chamber	Planar	Beneath (2 cm away)	0.96	0.24–1.2	0.1–1.0	Optison	[20][a]
U937	Rotating tube (30 rpm)	Planar	Aside (20 cm away)	1.0	0.6	60	Levovist	[21,22]
CHO	Culture dish	Planar	Above (4 mm away)	1.0	0.30–0.45	0.2	Optison	[25][a]
DU 145	Special chamber	Cylindrical planar	Beneath and sides (the axial and radial centre)	24 kHz	0.7	20	_	[26]
MCF-7	Rotating tube (60 rpm)	Focused	Beneath (the focal length)	1.0	0.19–0.48	40	+	[27]
BAEC	OptiCell	Planar	Beneath at an angle of 45°	1.0	0.22	30	SonoVue	[28]
***Xenopus* oocyte**	Culture dish	Planar	Beneath	1.075	0.3	0.2	Definity	[33][a]
MAT B III	Rotating tube (60 rpm)	(a) Air-backed (b) Focused	Beneath (7.6 cm away)	(a) 1.15 (b) 2.25	(a) 402 kPa (b) 570 kPa	10	+	[34]
BHK-21	Culture plates	Planar	Above	1.0	0.045–0.159	10–40 min	Optison	[36]
H9c2	OptiCell	Planar	Beneath at an angle of 45° (7 mm away)	1.0	0.27	10–15 μs	Definity	[42][a]
RPMI 1788	Rotating tube (200 rpm)	Planar	Beneath (7.5 cm away)	1.0	0.5	30	–	[60]
DU 145	Special chamber	Focused	Beneath (the focal length)	500 kHz	0.6, 1.6, 2.4, 3.0	2, 9 and 34	Optison	[61]
HUVEC	Culture dishes	Focused	Above (5 cm away)	1.6	0.6–1.515	30 min	–	[66]

[a] Real-time analysis. Abbreviations: BAEC, bovine aortic endothelial cells; BHK-21, baby hamster kidney cells; CHO, Chinese hamster ovary cells; DU 145, human prostate cancer cells; HUVEC, human umbilical vein endothelial cells; H9c2, rat cardiomyoblast cells; MAT B III, rat mammary carcinoma cells; MCF-7, human breast cancer cells; RPMI 1788, human peripheral lymphocytes; U937, human myelo-monocytic lymphoma cells.

promote TUS and assist progress towards clinical acceptance and application, it has become a necessary priority to understand the detailed interactions at a fundamental level. In addressing this, two distinct routes seem to be favoured: investigating post-sonication effects via a retrospective analysis, usually on a population of cells or extended tissue, or conducting real-time imaging at the single-cell level for recording *in situ* changes, which can then be correlated with physical cause. Studies using the former approach have given rise to the 'sonoporation' hypothesis [14]. The vast technological strides that have been taken in the past few decades, however, have seen the emergence of real-time sensing systems, such as the patch-clamp technique, affordable (ultra) high-speed cameras and other innovations that have facilitated a significantly enhanced capability to probe TUS-driven bioeffects. The main objective of this review is to assemble a cogent and comparable snapshot of the existing hypotheses, especially in relation to their time-dependent transients in intracellular Ca^{2+} concentrations, in an attempt to draw these into a logically consistent and more unified view.

Calcium ions (Ca^{2+}) have pivotal roles in living cells and are key regulators of cell proliferation and cell death [15]. A key requirement for the regulation of cellular functions by cytosolic Ca^{2+} is to maintain a steep concentration gradient between the extracellular and intracellular environments [16]. In addition, within the intracellular space, a further Ca^{2+} gradient is established between the cytosol and other organelles such as the endoplasmic reticulum (ER) and mitochondria [17,18]. Any change in this balance affects Ca^{2+} homeostasis and can ultimately affect the fate of the cell [19].

Table 1 summarizes – and, indeed, underscores – several key studies that highlighted some specific roles for Ca^{2+} dynamics in the context of TUS-induced bioeffects.

Pioneering studies on Ca^{2+}-dependent TUS bioeffects

A seminal early report highlighting the involvement of Ca^{2+} in TUS bioeffects occurred in 2004 when Deng *et al.* achieved the first parallel electrophysiology measurements on a patch-clamped *Xenopus* oocyte during irradiation with tone-burst ultrasound [20]. There, it was observed that a slightly delayed inward electric current developed, which proceeded to increase in magnitude in a step-wise manner during the sonication procedure, whereupon it returned to control levels upon the termination of the ultrasound irradiation (Fig. 1a). This observation was suggestive of an enhanced cell permeability facilitating a transmembrane ion flux. Moreover, the effect was strongly related to bubble activity because it occurred only in the presence of Optison (5%). Whether acoustic streaming – leading to threshold local shear stresses with the potential to disrupt the membrane – or an alternative mechanism was the cause of permeability could not be discriminated with confidence. It was certainly indicated, however, that the increased ionic permeability was due to the opening of non-specific pores rather than endogenous voltage- or ligand-gated ion channels. Interestingly, the sonoporation process, as inferred from the enhanced electrical current, was irreversible if Ca^{2+}-free buffer was used (Fig. 1b) or if the direction of current was reversed so that little Ca^{2+} could enter the cell from outside (Fig. 1c). This lack

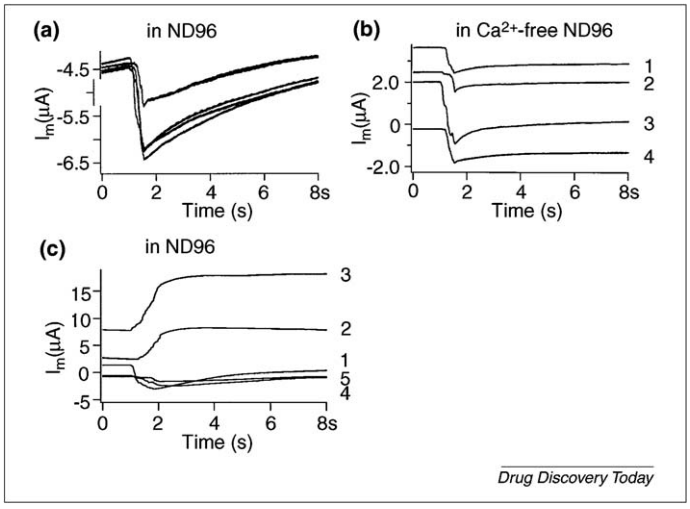

FIGURE 1

Ionic currents occurring across the membrane of a single *Xenopus* oocyte during and after ultrasound exposure (tone burst, 0.29 MPa, 0.5 s duration) in the presence of 5% Optison ($\sim 10^9$ microbubbles/ml). The oocyte bathed in **(a)** ND96 solution (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.6) and **(b)** Ca^{2+}-free ND96 (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 EGTA, 5 HEPES, pH 7.6) and the membrane potential was clamped at −50 mV. Repetitive lines indicate current traces recorded from the same oocyte under repeated exposures. The numbers from 1 to 4 in (b) give the sequence of recordings. **(c)** Ionic currents when the membrane potential was clamped at either −50 or +50 mV (tone burst, 0.29 MPa, 1 s duration). The numbers 1–5 indicate the sequence of recordings. The membrane potential at each recording was: 1, 50 mV; 2 and 3, consecutive recordings at +50 mV; and 4 and 5, consecutive recordings at −50 mV. Inward current decay was only observed in the presence of extracellular Ca^{2+}, whereas the current decay, and hence membrane sealing, failed when the oocyte was bathed in Ca^{2+}-free ND96 or when the membrane potential was clamped at +50 mV to reverse current direction and prevent Ca^{2+} entry to the cell through pores. Reproduced, with permission, from Ref. [20].

of current decay was suggestive of a role for Ca^{2+} in membrane resealing immediately after TUS exposure, a very early bioeffect. Cases in which the pressure amplitude was higher (>1 MPa) or the insonation period was longer (>0.5 s) also led to prolonged currents, and this was assumed to be due to irreversible damage to the cell membrane leading to cell death even in the presence of Ca^{2+}.

Around this time, Honda *et al.* [21] published a study showing that TUS was able to induce apoptosis in myelo-monocytic lymphoma U937 cells. Apoptosis induction was shown to be strongly related to inertial cavitation and synergistically increased in the presence of microbubbles; however, there seemed to be no correlation between apoptosis and the amount of free radicals generated extracellularly during sonication [22]. Here, a transient increase in intracellular Ca^{2+} was observed during four hours of immediate post-exposure monitoring, after which the Ca^{2+} levels returned to normal levels. In experiments conducted without extracellular Ca^{2+}, no such increase was observed. Based upon these findings, the authors concluded that the TUS-induced apoptosis was regulated, in part, through a Ca^{2+}-dependent pathway and the transient increase in intracellular Ca^{2+} was caused by an extracellular Ca^{2+} influx occurring again through non-specific membrane pores created during sonication. This was further supported by the inability of Verapamil, a well-known Ca^{2+} channel blocker, to suppress increases in intracellular Ca^{2+} concentrations when added before TUS exposure.

After these two studies, a flurry of further reports were published supporting the occurrence of TUS-associated Ca^{2+} changes [23–25]; however, these particular studies offered little conceptual insight into the fundamental interactions arising between TUS and living cells in terms of the consequent biological processes that were triggered and/or controlled by Ca^{2+} levels. The first study embracing this objective was conducted by Schlicher *et al.* [26] using a combination of real-time imaging and advanced microscopies. The group reported that TUS induces 'micron-scale wounds in plasma membrane that reseal using intracellular vesicles by an energy-intensive process requiring Ca^{2+}' [26].

Subsequently, in late 2008 and early 2009, two very complementary studies appeared, which provided a clearer perspective about the Ca^{2+}-mediated responses occurring in the earliest stages of insonation and their implication in molecular delivery [27,28]. Before discussing these important contributions, however, it is prudent to consider the state of salient knowledge in the context of mammalian cells.

What happens when cells are exposed to TUS?

Ultrasound is a mechanical wave that manifests as a series of pressure fluctuations that transmit through a body under free-field conditions or can simply establish a spatial standing wave pattern if reflecting constraints are present [29]. The effects of ultrasound are well known to be amplified when microbubbles are present in solution, either in the form of shelled micrometer-scale contrast agents (as is often the case with modern sonoporation studies) or through the formation of natural bubbles (cavitation). Any bioeffects arising, therefore, will have a direct correlation to the ultrasound parameters used, the location of the affected cells relative to the energy source (transducer) or to any nodal structures (when standing waves are present), and the proximity to acoustically active bubbles, amongst other possible contributants. The possibility of membrane disruptions occurring during insonation has been inferred from many observations of cellular deformation, which also correlate with the uptake of otherwise impermeable dyes within the deformed cells [30], and through measured changes in membrane electrophysiology [20,31–33]. The occurrence of actual physical pores has been confirmed in several other studies that used scanning electron microscopy and atomic force microscopy [34–37]. Pores are thought to arise via the occurrence of excessive shear stresses on the membrane caused by acoustic streaming. Microjetting and other more exotic events can also contribute to the overall state of permeabilization [38]. Perhaps the most important aspect of these pores is their ability to pass, non-specifically, extracellular molecules that would be otherwise impermeable to the cell under normal conditions. Logically, larger pores would be expected to lead to a more ready passage of species across the membrane compared with smaller counterparts; however, this figure of merit (from a drug delivery standpoint) would become moot if the cell were not able to repair itself within some finite timescale whereupon damage were seen as lethal. Interestingly, mammalian cells are known to tolerate pores of the order of 1000 μm^2 [39]. If we consider that gross cellular 'wear and tear' – for example, on load-bearing tissue structures – is a daily occurrence in humans, it might be anticipated that specialized pathways

for the repair of damaged cells would exist and the mechanisms would operate without prejudice or consideration of the actual physical cause of the damage as (fast) repair becomes a biological imperative for cell survival. This hypothesis was first proven by Saito *et al.* [40] and studied further by Schlicher *et al.* [26], who showed that after TUS exposure, 'cells actively reseal these holes using a native healing response' within a short period 'similar to the kinetics of membrane repair after mechanical wounding' with indication to the role of Ca^{2+}.

In membrane repair processes, the pore size dictates the mechanism of resealing [41]. Thus, small pores might be sealed passively, an inherent quality of lipid bilayers that is favoured energetically, whereas the sealing of larger pores requires more complex processes, provided that the extracellular fluid contains Ca^{2+} at near-physiological levels. In such conditions, Ca^{2+} is driven into the cell – amongst other molecules – by the concentration gradient [42]. Proximal bubble activity, stimulated by ultrasound, has been implicated in this process. For instance, the intracellular Ca^{2+} increase has been shown to start at the cell side closer to a bubble located just before irradiation and was presumed either to dislocate or 'to collapse' after exposure [25,42] (Fig. 2a). The influx of Ca^{2+} would be expected to trigger Ca^{2+}-responsive proteins within the cell and to react with the underlying cytoskeleton, perhaps causing depolymerization of the filaments. This depolymerization reaction would be important to free the way for internal vesicles (lysosomes, which are normally subcortical organelles) to approach the disruption site. Lysosomes can be stimulated by a high Ca^{2+} concentration to fuse with the membrane [43], as well as with each other (homotypic fusion) [44] so they can adequately patch the site of disruption (the 'patch hypothesis'). This Ca^{2+}-regulated exocytosis mediated by lysosomes was shown to be prevalent in many cell types [45]. Furthermore, its role was clearly evidenced through immunostaining with antibodies against the luminal domain of the lysosome-specific protein, LAMP-1, in mechanically injured cells [43], as well as TUS-treated cells [27] (Fig. 2b,c).

Alternative pathways

For the sake of completeness, it is worth mentioning the alternative scenarios for membrane self-sealing. It has been suggested that the cytoplasm can build immediate barriers by itself in the presence of elevated Ca^{2+} concentrations. These barriers act to hinder diffusion of extracellular fluid and/or to guard against the loss of cytoplasm through the disrupted membrane, and they are mostly regulated by non-exocytotic Ca^{2+}-dependent pathways [46]. Moreover, it has been hypothesized that elevated Ca^{2+} concentrations can activate a family of cytoplasmic enzymes known as tissue transglutaminases (TGase) that have the capability to cross-link proteins – especially those of the extracellular matrix, a pathway implicated in wound healing [47–50] – thus forming intracellular 'clots'. However, whether these pathways are implicated in TUS has not yet been investigated.

Types of Ca^{2+} transients

Once the membrane disruption is patched, it is expected that Ca^{2+} can no longer enter the cell non-specifically. Researchers have been able to show that the Ca^{2+} transients observed in cells exposed to mechanical injury or TUS application are terminated within approximately 3 min [25,51–53]. This time period might be sufficient to kill the cell if the increased permeability persisted throughout [41]. Rather, the cell could patch the membrane disruptions in much shorter periods (5–20 s is the reported time range taken for the transmembrane potential to return to its pre-exposure value [33], and 3–8 s is the interval during which the Ca^{2+} influx peaks before recovery starts [25]). To be clear, the figure of 3 min represents the time required for the re-establishment of Ca^{2+} homeostasis, and this Ca^{2+} influx defines the first 'ultra-short' Ca^{2+} transient experienced by cells exposed to TUS, which is different from the 'short' transient noticed by Honda *et al.* [21] during the first six hours post-sonication.

At this juncture, it is natural to ask why the increase in intracellular Ca^{2+} noticed by Kumon *et al.* [25], despite being localized in the beginning, later transpired to be in a diffuse state within the cytoplasm (Fig. 2a). Outwardly, logic might suggest that the existing large Ca^{2+} concentration gradient between the extracellular and intracellular environments will facilitate an appreciable ion flux as long as the disruption is sustained during and immediately after TUS exposure, a unique feature of ultrasound treatment. Acoustic streaming, either owing to the acoustic beam or from cavitational activity, could also augment the ion flux through convection. In the present context – that is, with direct comparison to the paper by Deng's group [20] – this seems not to be the case because the acoustic pulse took only 0.2 s, after which the localized transient could still be discernible. It was not until 1.8 s post-sonication that intracellular homogenous fluorescence was observed. For this particular cell experiencing an immediate transient (i.e. immediate membrane disruption), this phenomenon might be justified by the persistence of an open pore for a short period until complete patching was achieved [26,30,54]; however, this justification cannot work for the surrounding cells, which also encountered delayed Ca^{2+} transients after TUS termination. According to the authors, the transmission of an inter- and intracellular messenger in response to TUS exposure and/or the ultra-short Ca^{2+} transient might offer a more plausible explanation. In such a case, the persistence of localization for finite time periods might be in support of the formation of a cytoplasmic barrier [46]. Interestingly, cells after TUS irradiation experienced two modes of ultra-short Ca^{2+} transients: a Ca^{2+} transient followed by a monotonous recovery and Ca^{2+} oscillations [24,25,42]. Ca^{2+} oscillations – which are responsible, in part, for Ca^{2+} signalling and the subsequent release of ER Ca^{2+} stores – are usually produced by the generation of inositol phosphate (IP_3) [19], reflecting that the observed Ca^{2+} transients might be, in part, of an intracellular origin (as discussed below). Because the authors in this study did not identify the fate of these cells in the longer term, the justification of these different transient patterns and their real correlation to TUS-induced effects, such as apoptosis, still warrants dedicated attention. On one hand, there is evidence that TUS-induced Ca^{2+} oscillations are not essentially indicative of cell death, owing to the diversity of Ca^{2+} oscillations and the complex processes associated with their encoding [55]. On the other hand, these Ca^{2+} oscillations could simply reflect sub-threshold responses to trigger apoptosis [56]. In all cases, the presence of intra- and intercellular Ca^{2+} waves indicates that sealing is necessary but not sufficient to retain viability and that Ca^{2+} will be a mediator and/or stimulator in the end-stage TUS-induced ultimate responses.

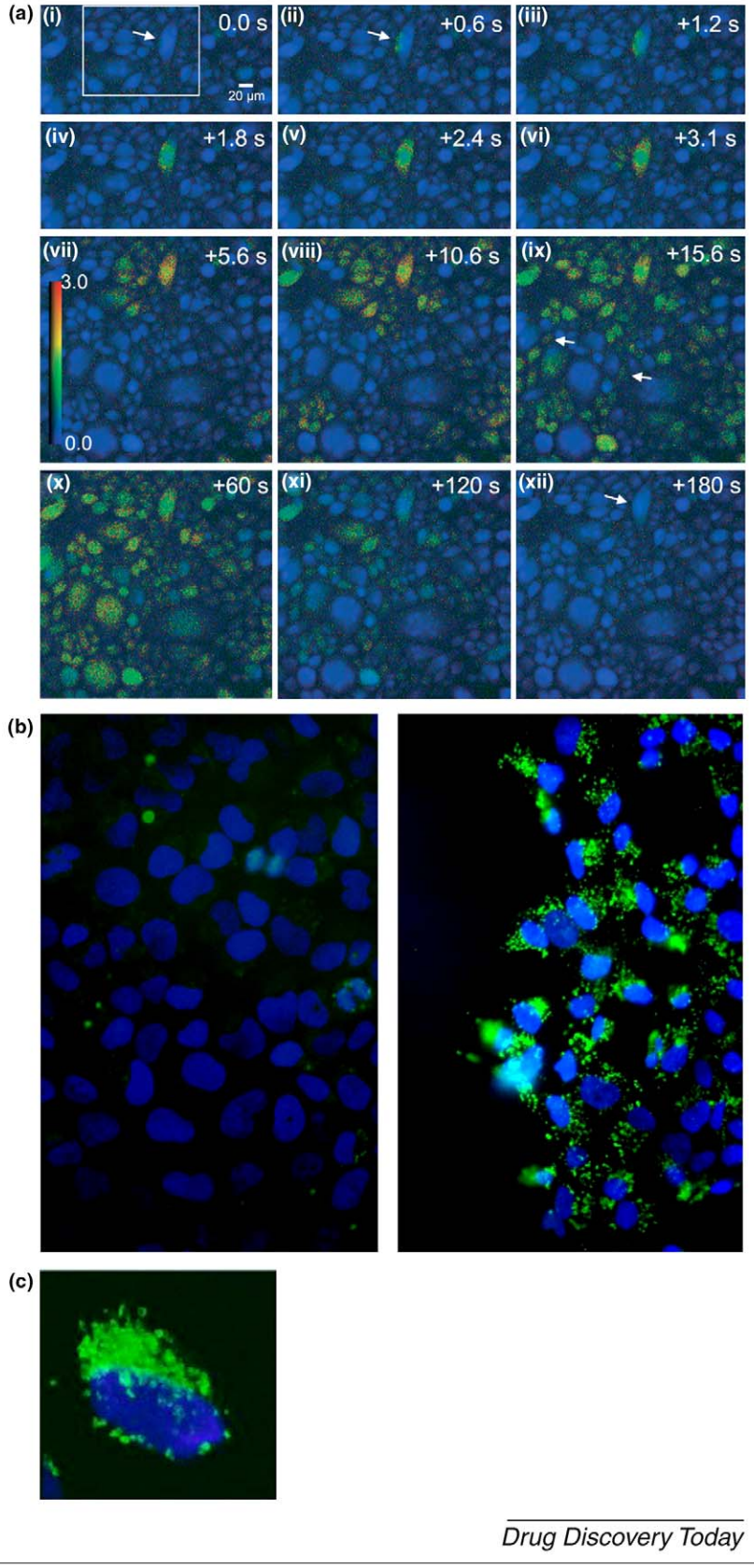

FIGURE 2

(a) Time-lapse ratio images showing calcium waves induced by ultrasound pulse for Chinese hamster ovary (CHO) cells in phosphate buffer saline (PBS) containing Ca^{2+} ions at a concentration of 0.9 mM. The colour bar indicates the local fura-2 fluorescence ratio $R = F_{340}/F_{380}$. The labels in the upper right corner of each image list the time since the first image. The images before ultrasound application show variation in baseline fluorescence intensity within different cells. (i), ratio image before ultrasound pulse. The arrow points to the cell that will be immediately affected by the ultrasound pulse. (ii), ratio image at first frame after ultrasound pulse

TUS-induced endocytosis

If clear evidence on the involvement of lysosomes in pore self-sealing after TUS exists, an obvious question at this point is: 'where does the lytic cargo of lysosomes go?' In fact, the lytic enzymes enclosed within the lysosomes are released to the external environment upon the fusion of the vesicle with the membrane. Momentarily, these enzymes are suggested to play a part in facilitating the fusion process. For secretory lysosomes, the release of their luminal contents could participate in several effector functions [57]; however, the unfavourable external pH (~7.4) strongly curtails their activity. Moreover, Ca^{2+}-stimulated endocytosis after lysosomal exocytosis helps clear the area from residuals [58,59]. The stimulation of endocytosis in membrane-repair mechanism is better described by Idone *et al.* [58] as that 'the discovery of injury-induced endocytosis provides a new framework for understanding plasma-membrane-repair pathways in mammalian cells' and that 'it is no longer clear whether new membrane addition by exocytosis directly mediates plasma membrane resealing, or whether it functions indirectly, by triggering a subsequent endocytotic response that represents the true resealing event'.

Following on from this statement, and setting in the context of TUS, the first experiments to prove the concept were conducted by Brayman *et al.* [60] who noticed the removal of CD19 receptors from the cell surface after sonication. Although the authors did not refer to this phenomenon as endocytosis-mediated, a similar removal of the stable pores formed by the bacterial protein streptolysin O (SLO) from the cell surface was shown to be mediated through endocytosis [58]. Recently, Meijering *et al.* [28] reported the occurrence of endocytosis post-TUS exposure in endothelial cells. The group found that low molecular weight species (4.4 and 70 kDa fluorescein isothiocyanate [FITC]-dextran) could be pushed into the cell through pores, thus appearing diffuse in the cytoplasm, whereas high molecular weight species (155 and 500 kDa FITC-dextran) were able to traverse the cell membrane only through endocytosis (Fig. 3a). Upon inhibition of the endocytotic pathways, they found that endocytosis played a part, even in the traversal of low molecular weight dextrans. The latter finding is consistent with the sealing scenario because the stimulation of endocytosis during the operation of the sealing machinery will lead to the engulfment of part of the extracellular solution, including all its constituents, regardless of their molecular weights. In fact, inspection of the literature with respect to the dependence of delivery on molecular size reveals that several other reports have found enhanced TUS uptake for species as large as 2000 kDa. The internal dispersion manifested size dependence in terms of the number of molecules taken up [34], the number of cells internalizing the molecules [7,61] and the intracellular distribution [26,61]. It is notable that these studies used different acoustic arrangements; thus, exposure to focused beams [34,61] could not eliminate the presence of different pore sizes, including those larger than the diameters of these molecules. Yet according to Guzman *et al.* [61], the lack of intermediate pore sizes internalizing intermediate molecules, such as bovine serum albumin (66 kDa), suggests that the uptake of larger sizes (probably >60 kDa) is accomplished by a mechanism other than sonoporation that could be a form of post-insonation-induced endocytosis. Careful examination of the confocal fluorescence micrographs (Fig. 3b) reveals spots of increased fluorescence that could represent localization. In addition, the still obvious diffuse pattern seen in the images and the statement by the authors that 'among HUP (high uptake subpopulation) cells, intracellular macromolecule uptake appears to have reached thermodynamic equilibrium with the extracellular solution for all the molecules studied' indicates that the internalization in this subpopulation is due to extensive damage of the membrane that, despite being fixed in the period immediately after sonication and therefore endowing the cell with an apparent short-term viability, leads, in the longer term, to cell death, possibly caused by a lack of full re-establishment of homeostasis [62] or perhaps in response to the detection of DNA damage. In conclusion, these recent studies imply that the two hypotheses employed in the interpretation of TUS-enhanced delivery (i.e. sonoporation and endocytosis-mediated delivery) might be occurring as sequential processes at least in some cases. The generalization of this concept still requires further attention.

Is post-insonation endocytosis a reality?

In 2010, Cheri Deng and co-workers discovered that not all Ca^{2+} ultra-short transients were associated with uptake of extracellular matter [42]. Only those that occurred immediately during TUS exposure showed concomitant delivery of the extracellular dye (propidium iodide, or PI), whereas the delayed transients did not (Fig. 4a). They also found that these delivery-associated transients were only correlated to bubble activity in close proximity to respective cells, despite the exposure of the whole field of view to acoustic irradiation. They further negated the presence of any localization of the dye in the permealized cells and thus concluded that endocytotic delivery post-TUS was an invalid route. We believe that although the authors succeeded in proving that Ca^{2+} transient measurements are not reliable indicators for delivery (i.e. transient permealization) – a noteworthy finding (see later) – their argument about endocytosis seems incomplete. This is because PI cannot be considered as a high or as an intermediate molecular weight species as previously classified [61] (molecular weight 668.4 Da) and because PI is not excluded by viable cells owing to its size but because it is being actively pumped out of the cells with intact membranes [63]. Again, the more proximal any bubble activity, the

(duration 0.2 s). The first significant change is seen in only one cell (arrow). Subsequent ratio images show (iii–v) propagation of an intracellular wave and (vi–x) propagation of intercellular calcium waves originating from this immediately affected cell and locations likely from outside the field of view. Arrows in (ix) indicate examples of locations showing cell-to-cell propagation of the calcium wave, connecting the two calcium transient regions in the image. (xi), dissipation of the calcium waves; (xii), full recovery of all the cells in the field of view. Note that the immediately affected cell also recovers (arrow). Reproduced, with permission, from Ref. [25]. Sections (b) and (c) show immunostaining with antibodies against the luminal domain of lysosome-specific protein, LAMP-1. **(b)** Control monolayer of human osteosarcoma (U2OS) cells that were not insonated (left) and monolayer subjected to ultrasound (1000 × 1 ms pulses at 3.2 MPa) (right). Insonated cells show a clearance zone (CZ; dark area to the left): cells near the periphery of the CZ exhibit LAMP-1 staining, whereas those further to the right are much less affected, suggesting that the occurrence of a jetting event that flowed over the cells with sufficient shear force to permealize the cells. **(c)** Confocal fluorescent imaging of human breast cancer (MCF-7) cells fixed within 60 s of insonation (1000 × 1 ms pulses at 3.2 MPa) showing the anti-LAMP-1 stain extending within the interior of the cell. Photomicrographs courtesy of Paul Campbell, University of Dundee.

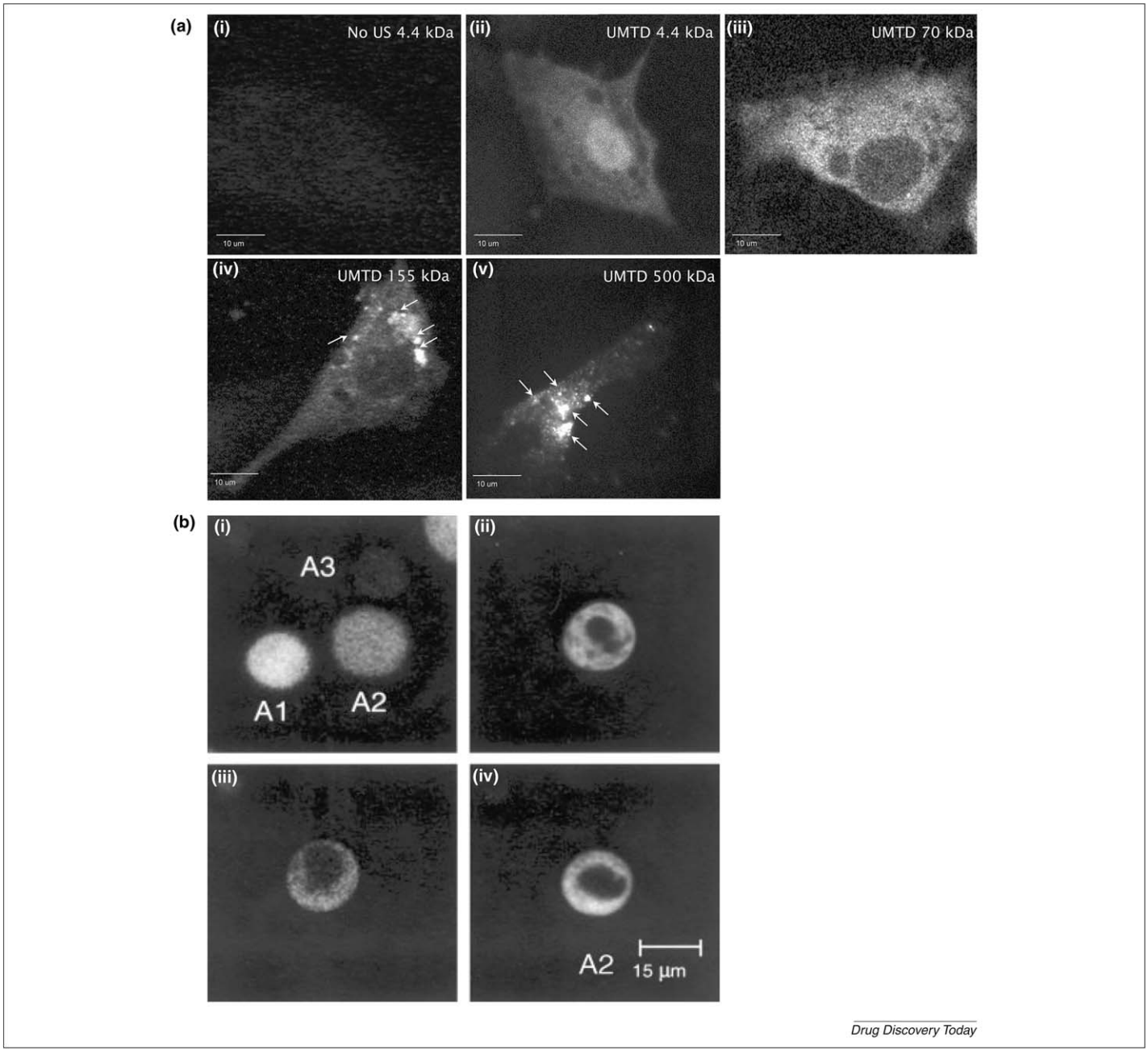

FIGURE 3

Cellular distribution of different fluorescent markers with different molecular weights after ultrasound microbubbles targeted delivery (UTMD) exposure. **(a)** Confocal laser microscopy images showing the uptake of fluorescein isothiocyanate labelled (FITC)-dextrans (m.wt. 4.4 kDa (ii), 70 kDa (iii), 155 kDa (iv) and 500 kDa (v)) in bovine aortic endothelial cells immediately post-sonication. Homogenous distribution in the cytosol and nucleus can be seen with 4.4 kDa dextran, whereas the 70 kDa dextran was excluded from the nucleus. Larger dextrans were localized in vesicle like structures (arrows) in the cytosol only. Reproduced, with permission, from Ref. [28]. **(b)** Confocal fluorescence micrographs showing intracellular uptake of calcein (m.wt. 623 Da, i), bovine serum albumin (BSA, m.wt. 66 kDa, ii), FITC-dextran 42 kDa (iii) and FITC-dextran 464 kDa (iv) in human prostate cancer (DU145) cells immediately after TUS exposure. Calcein is distributed throughout the whole cell, whereas BSA and dextrans molecules are excluded from the nucleus. Hoechst nuclear stain (not shown) was used to identify cell nuclei. Figure A1–A3 show the simultaneous presence of three cells having different levels of calcein uptake. A1, the brightly fluorescent cell is indicative of cells in the high uptake subpopulation (HUP); A2, the dimmer fluorescent cell is indicative of low uptake (LUP); and A3, the dimmest cell is indicative of nominal uptake (NUP). Reproduced, with permission, from Ref. [61].

more prone the cells become to poration of the membrane and, thus, the more probable the above-mentioned scenario will be. Similarly, the opposition to the TUS-induced endocytosis-mediated delivery by Schlicher *et al.* [26] can be argued by the smaller molecular weight of calcein molecules (623 Da) used in the experiments compared to the 150 and 500 kDa dextrans observed in endocytotic vesicles [28], as well as the reliability of the FM1-43 staining test in acoustic treatments, which requires further confirmation [64,65]. It could be, however, that the endocytotic role in the uptake of low molecular weight species was overestimated upon inhibiting the endocytotic activity by Flipin and chlorpromazine [28] owing to their effects on membrane structure, the factor that

was eliminated when the inhibition was carried out by potassium depletion and FM1-43 stain [26].

Which endocytotic pathway is involved?

Whereas Meijering *et al.* [28] revealed that the TUS-enhanced delivery for FITC-dextrans stimulated clathrin-mediated endocytosis more than caveolae-mediated endocytosis and macropinocytosis in primary bovine aortic endothelial cells (BAEC), Lionetti *et al.* [66] found that the exposure to diagnostic-level ultrasound enhanced the uptake of the fluorescent probe for caveolae-mediated endocytosis more than that for the clathrin-mediated endocytosis in human umbilical vein endothelial cells (HUVEC). It is still unknown whether this discrepancy is due to the dependence on the delivered substance [67] or due to the duration of sonication, which was 30 s in the former and 30 min in the latter. Furthermore, Basta *et al.* [68], who conducted their work in the same laboratory under similar conditions, showed that the same later cell line (namely, HUVEC cells) exhibited a DNA ladder after exposure for 15 and 30 min. Do these findings reflect a dependence of the type of endocytosis involved on the cellular response or vice versa, or is it merely the effect of incubation time before ultrasound application? Basta *et al.* [68] performed TUS immediately after the addition of the fluorescent markers, whereas Lionetti *et al.* [66] allowed the cells to incubate for 90 min before different protocols of exposure were applied. These questions still require rigorous investigation to achieve a complete clarification.

Waypoints along the route to self-sealing

As mentioned earlier, the cell membrane returns to its pre-exposure transmembrane potential in less than 30 s after the insonation burst. In addition, the time in which the Ca^{2+} influx continues to increase is of a comparable period, after which a recovery process starts to occur; thus, this period is the first waypoint that the cell reaches on the way to resealing. The second point represents the establishment of Ca^{2+} homeostasis. This step might comprise different processes including Ca^{2+} efflux, Ca^{2+} uptake by intracellular stores and so on. This step has been shown to be accomplished within 3 min.

Studies using scanning electron microscopy and atomic force microscopy showed that altered membrane roughness and pit-like structures were observed in the cell membrane post-TUS exposure and, interestingly, did not recover until 24 hours later [35,36]. What is notable about these studies is that both of them used Optison microbubbles (heat-denatured human albumin shell with perfluorocarbon [PFC] gas core). In a recent study, another 24-hour recovery was reported with PFC-filled phospholipid-shelled microbubbles [37]. Whether these long-term membrane changes are a part of the normal sealing procedure after the patching of the membrane disruption (in which case, there should be discrimination between membrane 'patching (sealing)', which can be defined as the closure of membrane disruptions, and membrane 'recovery', which indicates the return to a pre-disruption condition; Fig. 6) or a specific response to TUS exposure in the presence of PFC-filled microbubbles is unknown at present [69]. It should be noted, however, that some controversy still pervades the use of perflutren-based contrast agent microbubbles with the FDA raising concerns over the safety of such products since 2007, whereas others have argued strongly for their efficacy [70,71].

Cell morphology after TUS exposure

Cells exposed to TUS were observed to shrink in size and acquire a smoother surface [34]. This phenomenon, termed the 'shaving effect', might be due to the mechanical stripping of microvilli, for example, by radial flow from collapsing bubbles in the vicinity of cells (Fig. 5). Other studies have shown sonicated cells with smaller cross-section diameters but with irregular surfaces and villiform structures [37]. In a recent study of immediate morphological changes occurring post-sonication, cells were found to exhibit 'balloon'-like membrane blebs consisting of intracellular lipids sprouting outward and finally shedding off into the extracellular environment and 'blisters' that can eventually re-integrate with the plasma membrane [72]. These membrane blebs were observed in cells recovering from membrane wounds caused by acoustic exposure, as well as laser ablation, mechanical shear and pore-forming polypeptides (SLO) [73], reflecting their role in membrane sealing. How these altered surface topographies are correlated, what the exact mechanisms behind them are and the role of membrane blebbings in self-sealing (Is it mere occlusion of the 'hot spots of Ca^{2+} entry' or intended shedding of plasma membranes?) all require further investigation to clarify.

Having sealed: will cells always survive?

Cells that fail to seal their membranes because of deficiency in Ca^{2+} in the surrounding environment or because of extensive membrane trauma seem to die immediately. However, those that have their membranes sealed still retain a facility to die in a selective manner mediated via other pathways [50,74]. For instance, apoptosis was shown to be induced under certain acoustic conditions [22,69,75–77]. The evidence for the integrity of membranes at this point is the externalization of phosphatidylserine, a marker of early apoptosis, on the outer leaflet of the cell membrane with a simultaneous exclusion of dyes indicative of disrupted cell membrane permeability, such as PI, as detected by double staining technique performed by flow cytometry [78]. Thus, 'sealing' is not simply sufficient for 'survival', but rather a necessary step for cells to regain decision-making ability. Fig. 6 summarizes the possible survival–death pathways in the post-insonation period.

What are the stimuli that trigger the apoptotic cell death after TUS?

It is doubtless that Ca^{2+} contributes to the apoptosis signalling in mammalian cells [19,79,80]. In TUS-induced apoptosis, there is evidence that the molecular pathway of apoptosis proceeds via a Ca^{2+}-dependent pathway together with the intrinsic mitochondrial pathway [21], during which an increase in intracellular Ca^{2+} was noticed over a period of four hours and then returned to the normal levels, probably as a result of impaired membrane permeability and the consequent leakage of Ca^{2+} at later stages of apoptosis. This inference is supported by the occurrence of larger percentages of secondary necrotic cells after six hours incubation compared with early apoptotic cells. Although the authors of this study concluded that the rise was, in part, due to the extracellular Ca^{2+} influx through non-specific pores because no increase was observed under sonication in Ca^{2+}-free buffer, it is now clear that this conclusion cannot be drawn from such an experimental protocol because the lack of extracellular Ca^{2+} prevents the resealing of the membrane and in such a case, the cells die immediately,

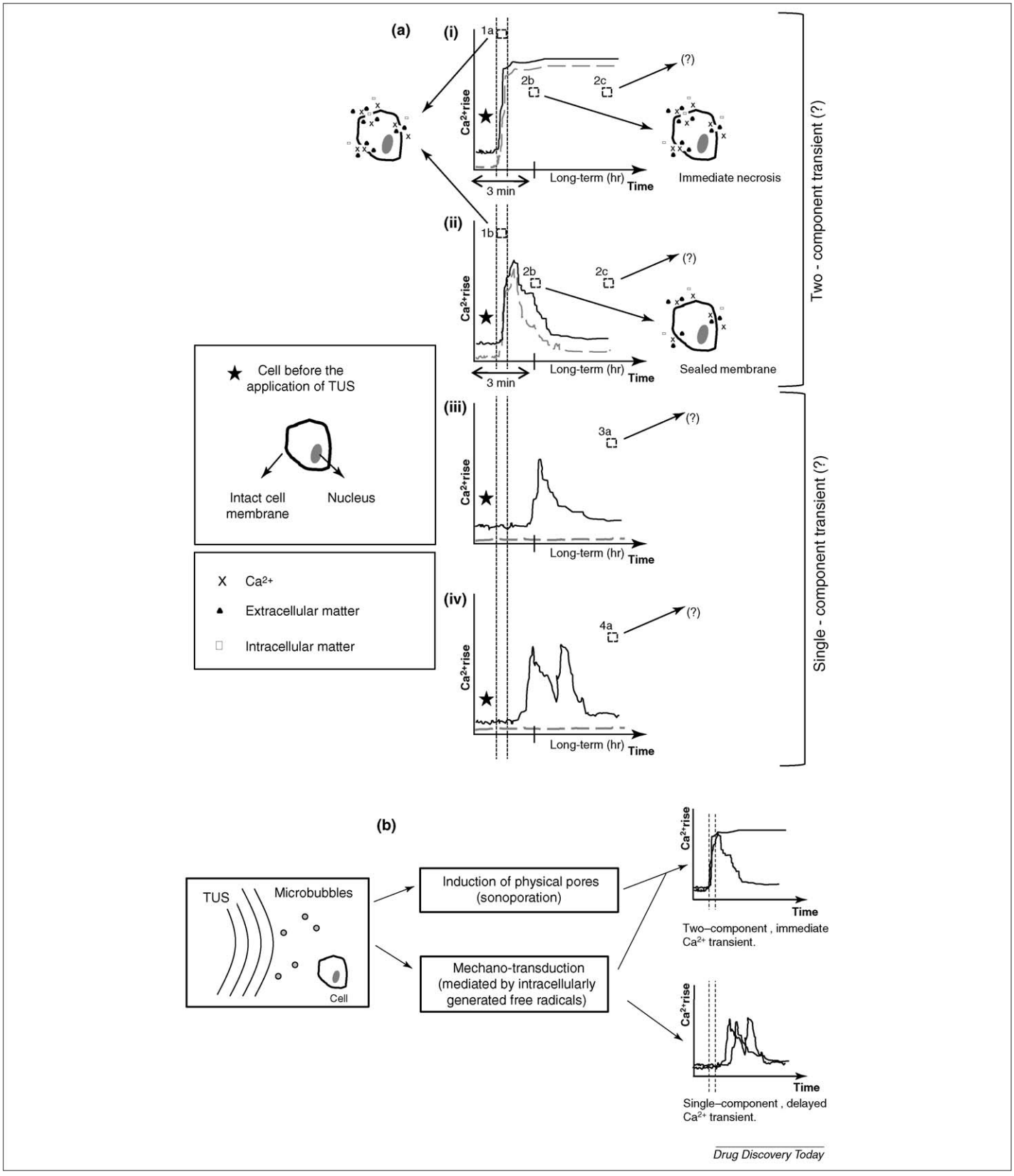

FIGURE 4

(a) A representation of the different types of Ca^{2+} 'ultra-short' transients in response to TUS exposure in relation to increased permeability. (i) and (ii) represent the immediate transients, and (iii) and (iv) represent the delayed transients. The vertical dashed lines indicate the sonication period; the solid black lines indicate the Ca^{2+} transients, whereas the grey dashed lines indicate the uptake of extracellular matter (implication in delivery). Dashed squares refer to the temporal condition of the exposed cells. (i) Extensive cell disruption occurs, resulting in immediate and sustained influx of Ca^{2+} and extracellular matter simultaneous with efflux of intracellular matter (necrosis). (ii) The cell succeeds in patching the disrupted membrane, yet, its long-term fate is unknown (indicated by a question mark). Delayed transients, which can be monotonous (iii) or in the form of oscillations of different frequencies (iv), possess lower amplitude than immediate transients.

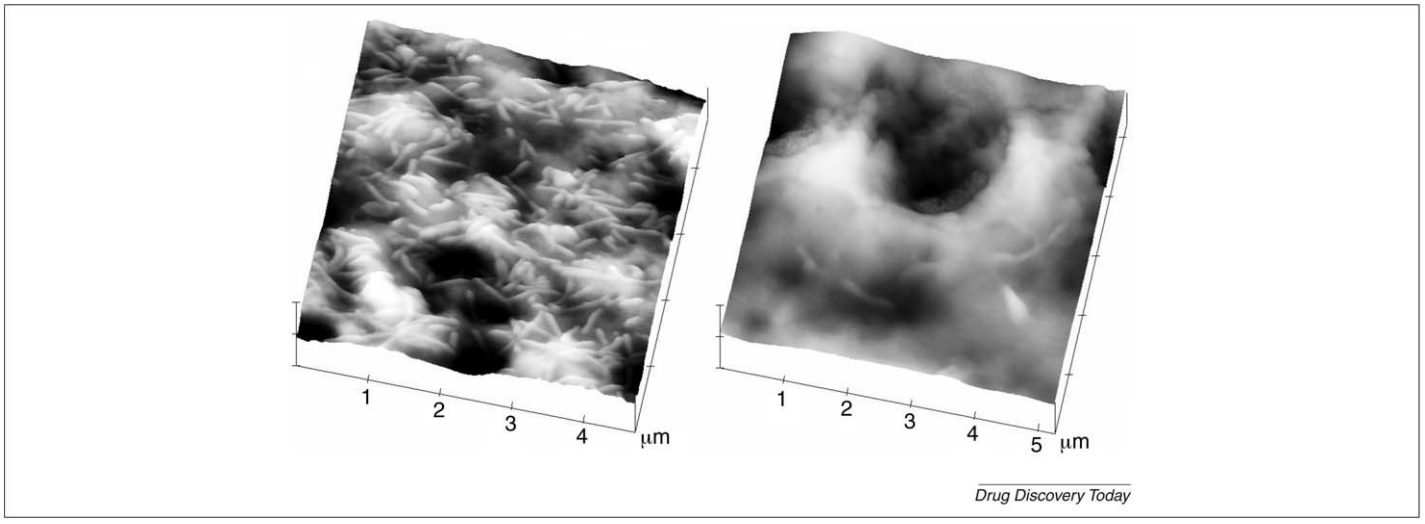

FIGURE 5

Scanning probe microscopy representative image of an unexposed human prostate cancer (DU145) cell cultured as a monolayer. The cell surface is dominated by microvilli and shallow (circa 20–30 nm) depressions (left). Exposure to 2 × 60 ms ultrasound bursts at peak negative pressure of 2.1 MPa in the presence of Optison (1.7%) resulted in microvilli clearance (shaving effect) with the appearance of a micron-radius 'sonopore' extending 450 nm into the cell (right). Notably, the inner leaflet of the cell membrane seems to have flapped out over the cell surface, suggestive of a 'bursting' response entrance wound. Courtesy of Paul Campbell, University of Dundee.

as was discussed earlier. The observed increase in the intracellular Ca^{2+} could, rather, be due to the mobilization of intracellular Ca^{2+} stores and a possibly delayed influx [19]. Whether the initial ultra-short Ca^{2+} transient is a stimulus, partially or wholly, to these events is still unknown. Thus, in all cases, a role for the extracellular Ca^{2+} cannot be excluded in apoptosis induction [81].

Ca^{2+} has been shown to activate a diverse range of Ca^{2+}-sensitive factors that are compartmentalized in cellular organelles such as the ER and mitochondria and even in the cytoplasm [82,83]. Several hypothetical possibilities can operate. If Ca^{2+} entering the cell during sonication succeeds in accessing the mitochondrial matrix, activation of the mitochondrial efflux mechanisms and matrix-Ca^{2+} buffering occurs. One might expect that success in these mechanisms could lead to recovery of homeostasis and that failure would result in Ca^{2+} overload in mitochondria, leading to a decrease in its membrane potential and, thus, opening the permeability transition pores, releasing cytochrome *C*, which, in turn, regulates the mitochondrial downstream events for apoptosis. Although this sequence might hold true for sonically disrupted cells, it cannot work for those cells showing post-irradiation delayed ultra-short transients. These cells experience different 'frequency-modulated oscillations', which are believed to be a cellular language to affect Ca^{2+}-sensitive targets and, thus, the encoding of these oscillations (and waves) might contribute to the modulation of cellular responses to TUS [84].

Generally, the release of cytochrome *C* is known to activate the IP_3 receptor leading to Ca^{2+} efflux from ER [85]. Recently, the existence of cross-talk between ER and mitochondria during apoptosis signalling and regulation has been reported [80]. Moreover, ER has been shown to be able to initiate the apoptotic signalling before mitochondrial involvement through caspase 4. Other reports have shown that the delocalization of Bak and Bax on the ER because of ER stress resulted in Ca^{2+} release to the cytosol, which can then be taken up by juxtaposed mitochondria. The involvement of ER in TUS-induced apoptosis is also to be expected, especially when heme oxygenase-1 increased expression after TUS application is considered [86]. Although heme oxygenase-1 overexpression is anti-apoptotic, it has been found to be induced by ER stress, which simultaneously initiates apoptosis [87,88]. The involvement of ER stress was further evidenced to contribute to TUS-induced apoptosis through the twofold increase in the expression of GRP78/Bip protein protein after TUS application [89]. The study by Honda *et al.* [21] stated that the mitochondrial pathway is only partially rather than wholly affected by the intracellular Ca^{2+} increase, which indicates the presence of other initiators, at least in leukaemia cells [90]. In support of this study, the occurrence of delayed apoptotic effects with increasing post-sonication incubation time augments the presence of another initiator stimuli [68,69,83,89]. Nevertheless, the exact interplay between organelles mediating apoptosis and whether the extracellular Ca^{2+} influx is an initiator or a delayed consequence of TUS-induced apoptosis remain to be explored. In addition, apoptosis might not be the sole programmed response to TUS exposure. Future research might expose alternative processes, especially when TUS-induced DNA damage [91–93] is considered (under study).

Is inertial cavitation important for TUS-mediated bioeffects?

Much of the recent research ascribes sonoporation to the presence of artificial microbubbles during sonication. In such cases, two possibilities can operate: that these microbubbles collapse (inertial cavitation) or that they merely oscillate upon exposure to ultrasound. Some researchers not only claim that the bubble collapse is a prerequisite for enhanced delivery but also suggest a role for the

The extracellular matter uptake is solely controlled by normal transport mechanisms that are molecule dependent. **(b)** A hypothetical diagram of the possible origins of the observed ultra-short Ca^{2+} transients.

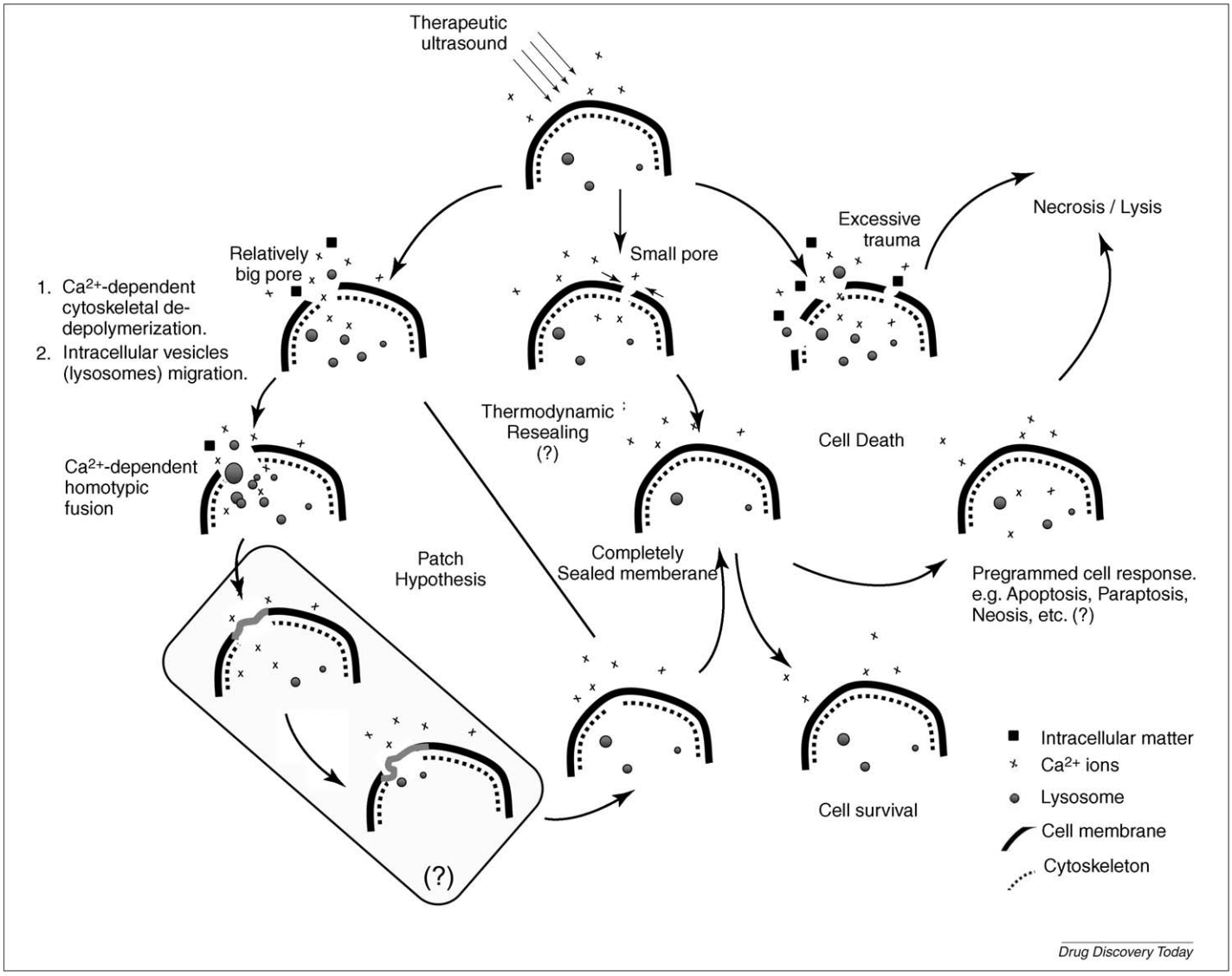

FIGURE 6

A schematic representation of the evolutionary possibilities post-insonation in relation to the extent of cell membrane trauma. The validity of thermodynamic resealing in which pores are sealed passively has been argued recently and replaced by the 'patch hypothesis', at least for larger wounds. Endocytosis has been shown to occur in the course of membrane self-sealing, together with exocytosis, yet the exact details are still unresolved (shaded square). It is still unknown whether the pits observed by atomic force microscopy correspond to a sort of endocytotic activity or a specific response to PFC-filled microbubbles (see the section 'Waypoints along the route to self-sealing').

free radicals generated upon the collapse, namely the hydroxyl radicals ($OH^{\bullet}$) [53]. In fact, the notion that inertial cavitation is a prerequisite for induction of pores might not be true [94]. van Wamel *et al.* have shown that vibrating bubbles are also capable of internalizing normally impermeable molecules and that internalization was accompanied by membrane deformation and poration that was followed by self-sealing within a few minutes [30]. Another report by Mortimer and Dyson [95] showed that Ca^{2+} uptake was increased in fibroblasts exposed to TUS in the absence of transient cavitation. Moreover, the presence of a microbubble near the cell observed by Kumon *et al.* [25] and its disappearance upon ultrasound application is not indicative of inertial cavitation, for several reasons. (i) The authors did not perform a test for free radical formation accompanied by microbubble collapse or other relevant tests. (ii) The disappearance of 98% of the microbubbles after sonication can be justified by their dissolution in the large volume of sonicating medium (7–10 ml). (iii) The 4 mm distance between the emitting transducer surface and the cells might not induce instantaneous *in situ* collapse; rather, it could be the violent dislocation of the bubble that caused shearing of the membrane.

As for the role of free radicals, the literature has conflicting reports as to their involvement in permealization and TUS-induced apoptosis. For example, the presence of free radical scavengers was shown to decrease the selective enhancement of caveolar-dependent permeability and the oxidative stress in endothelial cells treated with ultrasound [66,68]. It has been reported by Burlando and Viarengo [96] that free radicals can stimulate Ca^{2+} increase intracellularly, which is a net of both extra- and intracellular Ca^{2+} contributions. Their observations revealed that this action is selectively carried out by $OH^{\bullet}$ radicals rather than superoxide radicals. Hydroxyl radicals are the major free radicals generated from water sonolysis upon the collapse of

microbubbles. According to the authors, however, the Ca^{2+} increase took more than 8 min incubation with the free-radical-releasing mixture to be discernible; therefore, it is unlikely that the short-lived free radicals formed within the short periods of sonication could contribute directly to the Ca^{2+} transients observed. Yet their being localized by TUS still revives the hypothesis [53], and an indirect role cannot be excluded except by further detailed investigations. For instance, although the cavitation-induced free radical formation occurs only during sonication, which – in most cases – extends to very short periods, it could still be that these radicals can initiate reactions with the medium contents that extend for longer times and thus exert some effect downstream of their initial generation. This conjecture is supported by the observation that transferring the sonicated media to untreated cells resulted in a similar oxidative stress to that found with sonicated cells [68]. In addition, free radicals that could be produced intracellularly might be responsible. If we consider the work done by Juffermans *et al.* [53], we find that the authors, using two different TUS conditions with different mechanical indices, namely 0.1 and 0.5, stated that at these conditions, inertial cavitation was not prone to occur – even at the higher mechanical index (MI), which is already beyond the reported MI threshold for inertial cavitation, the thing that was argued elsewhere [69]. Despite that, they showed a close correlation between $OH^{\bullet}$ formation and transient permealization as indicated by Ca^{2+} transients. Their results suggest that the $OH^{\bullet}$ radicals might be responsible for the Ca^{2+} influx as indicated by the blockade of entry in the presence of catalase. This work demands closer attention because when combined with two other studies [42,52], it might provide new insights into TUS interactions. First, the authors detected hydrogen peroxide (H_2O_2) formation by using a specific intracellular dye; it is thus suggested that the radicals might be produced intracellularly. Second, the authors used an extracellular scavenger, catalase, to check the effect of H_2O_2 on Ca^{2+} transients. The catalase present during sonication blocked the Ca^{2+} transients at MI 0.1 and partially suppressed them at MI 0.5. It is notable that the cells used (H9c2 rat cardiomyoblasts) – being muscle cells – responded with a separate transient at each pulse compared to human umbilical vein endothelial cells under similar sonication conditions [52]. In the presence of catalase, the suppressive effect increased with each pulse, implying that catalase was being internalized with successive pulses, thus further augmenting the possible intracellular origin of H_2O_2. Moreover, if we recall this group's statement: 'It is suggested that superoxide is formed by high shear stress, especially by the vortex like microstreaming around oscillating microbubbles. Superoxide is rapidly dismutated to H_2O_2,' together with the fact that mechanical stress was also shown to result in Ca^{2+} oscillations [23] and the observation that Ca^{2+} transients were not detected except in the presence of microbubbles in most of the respective studies cited here, we can infer that the Ca^{2+} transients detected could be two-component transients with one component related to pore formation from which extracellular Ca^{2+} fluxes into the cell (leading to immediate transients) and the other component related to mechanotransduction that results in a comparatively delayed Ca^{2+} transient initially of intracellular origin. The former component has been shown to be correlated to delivery [42] and could mask the later component. In summary, the ultra-short Ca^{2+} transients might be either immediate two-component or delayed single-component transients (Fig. 4b). It should be noted that these are merely analytical conclusions, however; more directly evident practical data are still required to present a logically consistent overview. Finally, the exact interplay between free radicals, transient permealization and Ca^{2+} transients is not yet clear in terms of which one stimulates the other and what the outcomes are [97].

Can Ca^{2+} contribute to other cellular responses?

Since 2005, interest in the role of TUS-enhanced delivery in nuclear localization has continued apace since it was proved by Duvshani-Eshet *et al.* [36,98] that increased plasmid DNA (pDNA) localization occurs in cell nuclei after acoustic application. What was particular to their studies is that they employed a long sonication schedule, reaching 30 min, whilst retaining high viability in the cell population through sonicating the cells from above, thus minimizing the detachment of cells as when sonicated from beneath, which seems to play a part in cell death [99]. They found that increasing the sonication duration could increase the percentage of cells localizing pDNA in their nuclei. Furthermore, they succeeded in specifying the percentage of cells localizing the pDNA at different cellular sites after different treatment protocols. By contrast, the uptake of high molecular weight species, though enhanced by short sonication periods, the molecules were commonly excluded from the nucleus despite the attainment of a thermodynamic equilibrium at least in the HUP cells as previously mentioned [26,61]. Taken together, it might be concluded that prolonged sonication increased the nuclear access of these molecules, despite their reported poor diffusion through the cytoplasm under normal conditions.

It has been found that the intermediate-sized DNA molecules (10–70 kDa) traverse the nucleus through a nuclear pore complex (NPC) that spans both layers of the nuclear membrane. The regulation of NPC is achieved by the cisternal Ca^{2+} release and storage, where its release causes the NPC to close, and vice versa [100]. The intracellular Ca^{2+} stores depletion through IP_3 mediation results in Ca^{2+} release from the cisternae of the nuclear membrane as well, leading to the closure of the NPC. In such a case, the DNA cannot access the nucleus. In calcium phosphate nanoparticles-mediated transfection, the presence of excessive free intracellular Ca^{2+} in the cytosol inactivates the IP_3 through complexation, preventing the IP_3 receptor-assisted drainage of cisternal Ca^{2+}, and the NPC will be open for pDNA to traverse and transfect the cells successfully. Similarly, if we suppose that prolonged sonication leads to an increase in the intracellular Ca^{2+} owing to the sustained opening of the pores and consequently increasing the cytosolic free Ca^{2+}, the same scenario might operate. Whether this is true is still unknown and requires further efforts to ascertain in a definitive fashion, but the low transfection levels obtained in the U937 cell line despite the successful cytoplasmic delivery produced by TUS might provide a proof of concept if studied carefully. U937 cells have been shown to be a very sensitive cell line in which TUS can induce apoptosis that is accompanied by intracellular Ca^{2+} increase, as mentioned previously. Thus, if this increase in Ca^{2+} is due to intracellular depletion of Ca^{2+} stores, then it is probable that the cisternal Ca^{2+} will also be depleted, with a consequent closure of the NPC. This postulation was implicitly embraced in a recent study conducted by Miller and Dou [11], in which the authors compared leukaemia-

derived HL-60 cells – a cell line with an inherent capacity for apoptosis – and epithelium-like CHO-K1 cells, with 'a lower tendency for apoptosis', with respect to simultaneous apoptosis induction and gene transfection by ultrasound. The study showed that although sonoporation occurred successfully in both cell lines, more cells of the loaded population showed apoptosis in the former cell line, rendering the percentage of the viable gene-expressing cells lower. Interestingly, the authors referred to extracellular Ca^{2+} influx in their discussion, although they did not clarify their views concerning its role. Moreover, the attractiveness of muscle cells as targets for gene delivery [101], compared to tumour cells, might involve a role of Ca^{2+} in muscle physiology that one might expect to be more adapted to intracellular Ca^{2+} transients and to their rapid buffering as a part of their normal function as 'contractile cells' [73]. Again, this postulation requires verification and, at least until now, does not preclude the involvement of other mechanisms. For instance, prolonged sonication might deactivate some degrading enzymes or promote the diffusion of large molecules through the cytoplasm.

Finally, it is worth mentioning that there are other responses to TUS exposure that involve the Ca^{2+} signalling system, including the stimulation of normal physiological processes, such as accelerated healing of bone fractures by TUS [51,102,103] and wound healing [104]; however, a discussion of these responses and their mechanisms is beyond the scope of this review.

Concluding remarks

If intracellular Ca^{2+} is involved in both cell proliferation and cell death, what specific stimulus is there for activating either pathway? The answer to this question might be the key for progressing TUS 'from bench to bedside' and into a fully realized clinical application. Could it be that the sonication conditions and their associated chemical and mechanical effects prevail? Or that the cell type and its inherent response to external stimuli dictate the outcome? Is it the encoding of Ca^{2+} oscillations? Could it be a threshold dictated by the intracellular Ca^{2+} concentration of sonically porated cells? It has been shown, for instance, that membrane self-sealing can operate at elevated intracellular Ca^{2+} concentrations between 5 and 10 μM, above which the cell reaches the point of no return, undergoing immediate lysis [73]. If we can extend these results to sonically porated cells in the light of, for example, the study conducted by Hutcheson *et al.* [62], could it be that the cells previously designated as 'HUP' showing initial short-term viability are the same cells having the maximum tolerated intracellular Ca^{2+} elevation at which sealing can be achieved but meanwhile seen as toxic and apt to trigger apoptosis? If yes, why did only a small percentage of cells in this regime undergo apoptosis? And what was their intracellular Ca^{2+} profile within the period from sonication until apoptosis occurrence?

The success of lithotripsy and more recently, high-intensity focused ultrasound, as interventional tools, underscores the power and versatility of ultrasound as a minimally invasive therapy. However, the full and complete therapeutic potential of ultrasound, especially for the compelling scenarios of molecular delivery, tissue sensitization, and for the subtle control of biochemical pathways, remains untapped at a clinical level. We conclude here that one important route to addressing this lies in developing a concerted effort that is focussed on achieving a more complete understanding of the role of Ca^{2+} transport during insonation. This will consolidate the present knowledge base, and in the longer term, assist in paving the way towards a much broader and powerful implementation of ultrasound for therapeutic purposes. Evidently, the intervention to save cells from TUS-induced apoptosis through post-sonication Ca^{2+} chelation would not have been studied successfully but for the knowledge of the role of Ca^{2+} in membrane resealing. Although this approach is suggested to enhance delivery *in vitro* only, it offers some suggestion as to how the achievement of detailed fundamental knowledge can initiate fast strides likewise *in vivo*.

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