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Nucleic acid delivery with microbubbles and ultrasound ☆

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ABSTRACT

Nucleic acid-based therapy is a growing field of drug delivery research. Although ultrasound has been suggested to enhance transfection decades ago, it took a combination of ultrasound with nucleic acid carrier systems (microbubbles, liposomes, polyplexes, and viral carriers) to achieve reasonable nucleic acid delivery efficacy. Microbubbles serve as foci for local deposition of ultrasound energy near the target cell, and greatly enhance sonoporation. The major advantage of this approach is in the minimal transfection in the non-insonated non-target tissues. Microbubbles can be simply co-administered with the nucleic acid carrier or can be modified to carry nucleic acid themselves. Liposomes with embedded gas or gas precursor particles can also be used to carry nucleic acid, release and deliver it by the ultrasound trigger. Successful testing in a wide variety of animal models (myocardium, solid tumors, skeletal muscle, and pancreas) proves the potential usefulness of this technique for nucleic acid drug delivery.

Contents

Abbreviations: AAV, adeno-associated virus; Ang1, angiopoietin 1; DSPC, distearoyl phosphatidylcholine; DSTAP, distearoyl trimethylammonium propane; EGF, epidermal growth factor; eGFP, enhanced GFP; EGFR, EGF receptor; GFP, green fluorescent protein; GLP/GMP, good laboratory practice/good manufacturing practice; JNK, c-Jun N-terminal kinase; MAdCAM-1, mucosal vascular addressin cell adhesion molecule 1; neuroD1, neurogenic differentiation factor 1; Nkx2.2, NK-type homeodomain transcription factor; PDX-1, pancreatic duodenal homeobox-1 transcription factor; PEG, poly(ethylene glycol); PiggyBac, piggyBac transposon; RBC, red blood cell; SDF-1, stromal cell-derived factor 1; siRNA, small interfering RNA; TB4, thymosin beta-4; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelium growth factor; VEGFR2, VEGF receptor 2.

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1. Introduction

The use of ultrasound energy to enhance nucleic acid delivery into the target cells has been considered since the 1980s [1]. While initial efforts were limited to the *in vitro* cell culture testing, ultrasound-assisted transfection studies eventually moved to the *in vivo* setting, with the ultimate aim to apply ultrasound-assisted transfection to clinical practice. The latter goal has not been achieved yet, even for a clinical trial stage. Certain attractive features of ultrasound transfection maintain the interest in this approach; research in this area continues to advance and is rapidly accelerating lately (e.g., [2–5]. In this review we provide examples of the approaches to ultrasound-enhanced nucleic acid delivery. At the initial part of the review (Section 2), we focus on the historical development of ultrasound utilization for transfection and general design logic of the sonosensitive nucleic acid delivery particles. We then look at the characteristic examples of the potential biomedical applications of ultrasound-assisted nucleic acid delivery in the specific animal models (Section 3).

2. Ultrasound delivery: history and nucleic acid carrier design

2.1. Ultrasound and plasmid co-application: the first acoustic transfection tool

As the early discovery step, ultrasound-assisted plasmid delivery to cultured mammalian cells was investigated as a purely mechanical method, *in vitro*, in a cell culture setting, using a laboratory 20 kHz probe-type sonicator unit [1]. Transfection efficacy was reasonable for its time (when calcium phosphate coprecipitate of the plasmid was a common practice, and microinjection was state of the art), and comparable with mechanical scraping of the cells from the dish to achieve delivery of plasmid inside the cells. Co-loading of FITC-dextran in the ultrasound-treated cells was noted in that study. Interestingly, lipofection was developed at the same time [6], and the latter became a standard nucleic acid delivery tool in almost every *in vitro* biomedical lab setting, and reached multiple clinical trials [7]. Ultrasound-assisted delivery required a combination skill set, with a strong knowledge of medical physics, cell and molecular biology; thus, development proceeded much more slowly. Only a decade later medical ultrasound (MHz frequency, as used for physical therapy or medical imaging) was successfully used to enhance cell transfection in culture *in vitro* [8]. Acoustic pressure necessary to achieve transfection during short (20 s) insonation was at ~300–400 kPa level. Use of cavitation nuclei (i.e., microbubbles) helped to reduce the amount of transmitted acoustic energy required for successful transfection, achieved with even shorter (1 s) ultrasound pulses [9]. A combination of ultrasound, dispersed microbubbles and an aqueous solution of a hydrophilic plasmid DNA encoding green fluorescent protein in the cell culture medium led to a successful intracellular delivery of plasmid and expression of the encoded protein. With that study, the interest in ultrasound-assisted transfection started to increase and is reflected in hundreds of published manuscripts at this point.

2.2. Ultrasound, microbubbles and plasmid co-administration: enhancement of intracellular delivery in vitro and in vivo

While microbubbles made their way into clinical practice as ultrasound contrast blood pool agents, they were also investigated as energy deposition foci to enhance drug delivery in general and nucleic acid in particular [10].

The advantage of this combination is in its simplicity and its being an easier path of translation towards clinical practice (Fig. 1): a plasmid (or a shorter nucleic acid fragment), either unprotected, or complexed to a lipofection gene delivery system, is added to cultured cells [9], injected intravenously, intraarterially or locally [11], along with microbubbles (those already approved for human use as ultrasound contrast agents), and ultrasound is applied in the area where the transfection is desired, using a clinical ultrasound imaging apparatus, or a wider insonation field ultrasound equipment as used in physical therapy, or even a recently approved MRI-guided focused ultrasound system. All types of ultrasound systems can provide efficient microbubble cavitation (stable cavitation, where bubbles vibrate continuously, or inertial cavitation, where bubbles rapidly expand, compress and collapse). The gas inside a microbubble is orders of magnitude more compressible than the surrounding biological tissue or blood. Passage of ultrasound waves through the media results in the rapid pressure variations within the tissue, leading to the rapid cyclic compression and expansion of the bubbles, with focal deposition of energy at the bubble surface, leading to microstreaming, cavitation and jetting. If this happens in close proximity to a target cell, formation of transient pores in the cell plasma membrane in response to such treatment takes place [12]. Pores, which cells can seal by energy- and calcium-dependent repair, are relatively small (from tens to hundreds of nanometers) and most of them do not stay open for a much longer than a minute or two [13–15], so most efficient transfer may happen during insonation: plasma membrane needs to seal rapidly to maintain cell viability. Despite this established fact, intracellular delivery of small molecules (e.g., fluorescent dyes) may be efficient hours after ultrasound treatment [16,17]. It is generally assumed

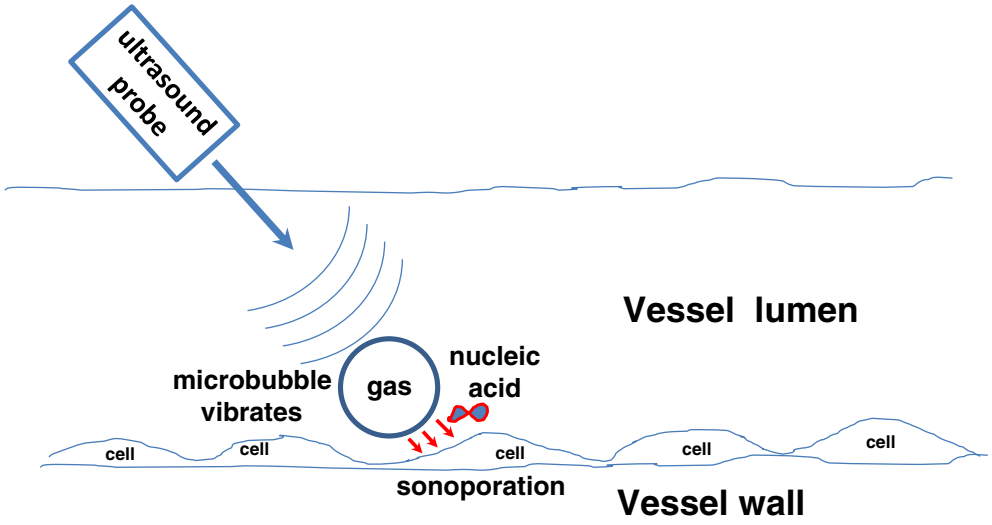

Fig. 1. Microbubble vibration in the ultrasound field near the vessel wall results in sonoporation and intracellular delivery of co-injected nucleic acid.

that nucleic acid can enter cells by diffusion through the transient submicron pores; transfection efficacy is thus expected to be lower, when compared with viral vectors. Yet up to 70% cells were transfected by sonoporation recently in an *in vitro* study in a glioblastoma model [18], which may point at some other mechanisms of nucleic acid delivery inside the target cells. An alternative mechanism to pore formation, a caveolin- and clathrin- dependent increase of cellular endocytosis in response to microbubble insonation by cell surface was suggested [19]. *In vivo*, following intravascular administration of the microbubbles and nucleic acid, the initial target of transfection is endothelium. However, endothelium barrier function may also be altered during microbubble insonation, and nucleic acid may get delivered beyond endothelial lining, either via fenestrations/pores or by transcytosis. Efficacy of all these processes needs to be taken into account.

With a wide availability of microbubbles for clinical use, and a developed manufacturing capability for sterile pyrogen-free nucleic acids, it is possible that the modest transfection efficacy might be the reason that clinical trials with this approach have not yet taken place. Ultrasound-based plasmid transfection cannot yet achieve the same nucleic acid transfer efficacy *in vivo* when compared with viral delivery systems, such as adenovirus, most of the time, but ultrasound can help with selectivity: transfection of non-insonated non-target tissues can be orders of magnitude lower than in the insonated target tissues. Co-administration of microbubbles and nucleic acid can be localized, e.g., intramuscular [20] or intraarterial [12] or systemic/intravenous [11,12]. Targeting tumor nodes, skeletal muscles, kidney, pancreas and myocardium may be most appropriate for ultrasound-assisted transfection applications. In Section 3 of this review we address these specific targets in more detail.

2.3. Ultrasound, microbubbles and separate nucleic-acid carrier complex: transfection enhancement opportunities

Unprotected nucleic acid, such as a plasmid or an oligonucleotide, is rapidly degraded in biological milieu [21]; therefore, delivery efficacy can be improved by complexing nucleic acid with a protective delivery vehicle, and co-injecting this material with microbubbles. Since the initial lipofection studies [6], there has been a whole industry created for the delivery of nucleic acid materials [22], first with plasmids, and, lately, with shorter oligonucleotide, such as antisense oligonucleotides,

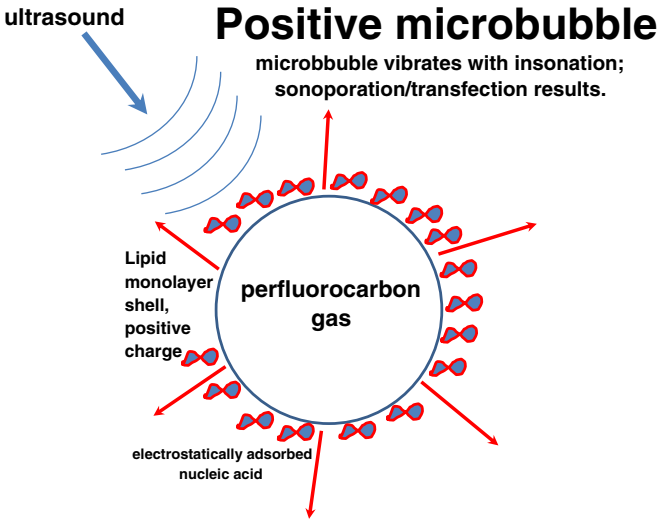

Fig. 2. Nucleic acid (negative electrical charge) is adhered on the microbubble shell (positive charge) via an electrostatic interaction. In response to ultrasound, microbubble vibration releases the nucleic acid, possibly associated with attached shell fragments, and drives it into the cell.

interfering RNA, microRNA, mRNA [23] and minicircles [24]. These particles can be positively charged fusogenic liposomes, smaller-size lipoplexes, polyplexes, or polyethyleneimine derivatives [25], so taking advantage of these nucleic acid carriers is a logical step in improving sonoporation transfection.

Initial combination "ultrasound and microbubbles" studies [26] have confirmed the improved efficacy of this approach over simply co-injecting microbubbles with the unprotected plasmid. As expected, complexing and stabilizing the plasmid with a polymer (PEG-polyethyleneimine with a high degree of substitution) resulted in a very significant enhancement of transfection in a skeletal muscle in a rodent model, with intravenous co-injection of plasmid–polymer construct with microbubbles, followed by targeted ultrasound treatment [27]. One possible undesired feature of using transfection-enhancing particles as nucleic acid carriers could be a potential risk of nonspecific transfection in the non-insonated tissues, but the latter study did not report this problem: PEG-PEI/plasmid complex administration did not result in a significant transfection in the absence of microbubbles or insonation.

2.4. Microbubble nucleic acid carrier constructs

Performing sonoporation directly with a nucleic acid carrier microbubble offers a significant advantage: the material to be delivered to the cell is located in an immediate proximity to the surface of the target cell. Transient pores offer limited time for intracellular transfer [13]; thus, proximity should improve delivery efficiency. So, the plasmid or oligonucleotide should be placed on the microbubble surface, which is easily accomplished by electrostatic binding. Indeed, a direct comparison study has determined that microbubble–plasmid complexes provided significantly better transfection than co-administered plasmid and microbubbles incapable of complex formation [5,28].

Placing positive charge on the surface of microbubbles is simple from the preparative standpoint: a lipid with a net positive charge is simply added to the lipid mixture composition prior to bubble formulation (Fig. 2). It was first suggested by Unger et al. [29], who have successfully modified preparation of MRX-115 microbubbles (lipid shell based on zwitterionic dipalmitoyl phosphatidylcholine), to include a mixture of positively charged dipalmitoylethylphosphocholine and fusogenic dioleoyl phosphatidylethanolamine. The resulting microbubbles were capable of tightly binding plasmid DNA encoding chloramphenicol acetyl transferase; upon ultrasound treatment transfection was observed in cell culture [29] and in the insonated dog heart [30]. Some nonspecific transfection in the lung, liver and other tissues that were not treated with ultrasound has also taken place [31]. MRX-115 (now named Definity, a clinical ultrasound diagnostic imaging contrast) microbubble manufacturing is performed by the bedside, by rapid vibration of a sealed vial with the aqueous media containing lipids and perfluoropropane headspace, in a desktop amalgamator. So, adding a positively charged lipid to the aqueous phase and making it uniformly mixed with other lipid components do not add a significant technical complexity to the formulation.

In an effort to improve stability of the microbubble formulation, a switch to longer-chain lipids with higher phase transition temperature was suggested: microbubble shells were made of distearoyl phosphatidylcholine co-mixed with positive distearoyl trimetylammonium propane and a neutral PEG stearate [12]. Poorly water-soluble perfluorobutane gas improved microbubble stability and circulation time; DSTAP/DSPC/PEG-stearate microbubbles were pre-manufactured by sonication dispersion of decafluorobutane gas in the aqueous micellar lipid mixture. These microbubbles were stored refrigerated for many months in sealed vials under perfluorobutane atmosphere. They provided efficient transfection in the insonated skeletal muscle or myocardium in animal models, with minimal non-target expression of the reporter genes [12].

DSPC/DSTAP combination has become applicable generally, with a number of published studies from several laboratories [2,5,32–34]. In

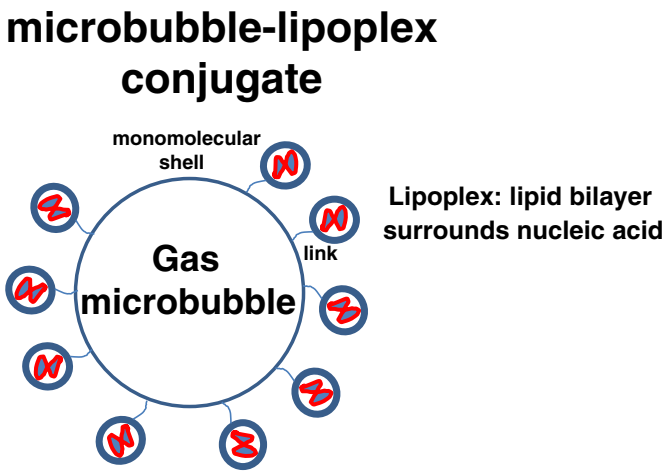

Fig. 3. Micrometer-size bubble is decorated on the outside with a coat of nucleic acid carrier nanostructures, such as lipoplexes (lipid bilayer nanospheres with condensed nucleic acid as the inner cores).

this formulation, microbubbles have to be purified from unincorporated lipid micelles by a short centrifugal flotation in a cell culture centrifuge (~100–200 g, for several minutes); plasmid can be then added directly to the aqueous microbubble dispersion for electrostatic binding. Typically, several thousand plasmid molecules can be placed on each microbubble [12] (this is calculated from 4 μg plasmid bound to 10^8 microbubbles). For the small oligonucleotide fragments, the number of molecules bound per single bubble will be several orders of magnitude higher [35] due to the much smaller molecular mass of an oligonucleotide. Electrostatic interaction between the bubbles and nucleic acid can be controlled by adjusting the ionic strength of the incubation media, selection of the appropriate concentrations of the reactants and their order of mixing, to avoid formation of large aggregates comprising a multiplicity of microbubbles glued by plasmid.

Instead of DSTAP, other positively charged lipids, such as a fully biocompatible phosphate ethyl ester of phosphatidylcholine [3] or lipofection liposomes [36] can be added to the lipid mixture in the aqueous media prior to bubble manufacturing. It has been confirmed that plasmid attached onto DSTAP/DSPC microbubbles that carry a grafted PEG brush is partially protected from nuclease degradation [5].

Some reports of oligonucleotide attachment to protein (human albumin) microbubbles have appeared, but the mechanism of the attachment of the bubble shell and nucleic acid is still not completely understood: albumin bubble shell surface should carry a net negative surface charge, and nucleic acid is highly negatively charged, so these materials should be electrostatically repelled from each other. Nucleic acid is also extremely hydrophilic, so hydrophobic interaction with denatured albumin is not likely. Yet microcalorimetry studies confirm binding of oligonucleotide to microbubbles, with a significant energy and affinity [35].

Additional protection of nucleic acid on the surface of the bubble can be achieved by proper formulation. Positively charged liposomes, lipoplexes or polyplexes, nanostructures that entrap and/or stabilize nucleic acid, can be placed on the surface of the microbubbles (Fig. 3), either via streptavidin–biotin bonding [37,38], or by covalent coupling [39], as has been done earlier for liposome attachment to microbubbles [40]; this approach may significantly enhance delivery efficacy.

Improvement of the nucleic acid load per microbubble can be also achieved by surface engineering: layer-by-layer (LBL) deposition of the opposite-charge polyelectrolytes on surfaces [41] can create multiple-layer sandwiches. This is a perfect tool to increase the plasmid or oligonucleotide load on the bubble surface: layered polyelectrolytes form extremely stable microcapsules, so nucleic acid layers can be embedded between the layers of a biocompatible polymer, e.g., polylysine [42]. Care should be taken to make a proper selection of polyelectrolyte to complex to nucleic acid, though: if not optimal, the complex might be too stable to accomplish effective release of nucleic acid in the cell cytoplasm and/or delivery into the nucleus, if necessary.

Viral particles placed on the microbubble shell may provide the desired transfection efficacy, combined with acoustic activation specificity, if a virus cannot enter the cells by itself. One example of this approach was a study by Taylor et al., who took a deficient form of a retroviral vector incapable of self-replication and entry in the target cells, and placed it on the DSTAP/DSPC microbubbles by electrostatic coupling. Transfection with this system could only take place following ultrasound treatment [43]. Likewise, for cationic AAV virus attached to anionic microbubble shell, AAV6-microbubble constructs achieved an excellent transfection in insonated rat heart, an order of magnitude higher than plain virus—but in this case, the viral particle was fully functional and liver transfection was significant. Interestingly, when the same experiment was performed with AAV9-microbubbles, liver transfection was minimal, perhaps due to a difference in the binding affinity of the virus particle to certain cell types [44]. AAV9 serotype seems to be potentially important for ultrasound–microbubble gene delivery: recently, targeted transfection of brain in a murine model was accomplished by an MR-guided focused ultrasound with a simple intravenous administration of Definity microbubbles, where ultrasound and microbubbles provided blood–brain barrier permeability enhancement, 5 min later followed by AAV9 administration, so that the virus could enter the brain from the vasculature and provide transfection of neurons, astrocytes and oligodendrocytes with long-term expression of GFP [45]—we should note that in this case AAV was not attached to the microbubble surface.

2.5. Targeted microbubbles as nucleic acid delivery vehicles

Combination of positive electrostatic surface charge and negatively charged plasmid on the surface of the microbubbles does not preclude the use of targeting ligands for selective delivery of the particles to the tissue of interest. Phillips [46] tested this hypothesis *in vitro* and achieved selective targeting of activated smooth muscle cells by positively charged microbubbles carrying an eGFP-encoding plasmid, and decorated with anti-VCAM-1 antibodies; as compared with non-targeted bubbles, transfection efficacy increased by 5.5-fold, with just 200 kPa peak negative acoustic pressure. Xie and colleagues [2] compared transfection efficiencies between selectin-targeted and non-targeted positively charged microbubbles carrying a reporter transgene plasmid in a mouse model of hindlimb ischemia. It was found that targeting resulted in significantly higher transfection when sonoporation was performed at 0.6 MPa, although this difference was abrogated at higher acoustic pressures. Tlaxca and colleagues [47] examined inflammation marker-targeted plasmid-carrying microbubbles in a mouse model of inflammatory bowel disease. In this experiment, sonoporation of the abdomen was performed 10 min after anti-MAdCAM-1 or anti-VCAM-1 antibody microbubble administration in order to allow circulating microbubbles to clear the vasculature (Fig. 4). A potentially useful scenario justifying the use of these particles is the ability to minimize non-specific transfection, because targeted bubbles would preferably adhere to the target cells that express disease marker, e.g., VCAM-1. Even more important, there may be no need to use focused ultrasound to perform selective sonoporation of the tissue of interest under imaging guidance: wide unfocused "painting" with ultrasound probe will still allow specific transfection of the target, because circulating (or adherent) bubbles would not be present in other areas.

2.6. Liposome constructs that entrap fluorocarbon nanoparticles, for ultrasound-assisted nucleic acid delivery

A lipid nanoparticle termed "bubble-liposome" was developed by Maruyama, Suzuki and colleagues at Teikyo University in Japan

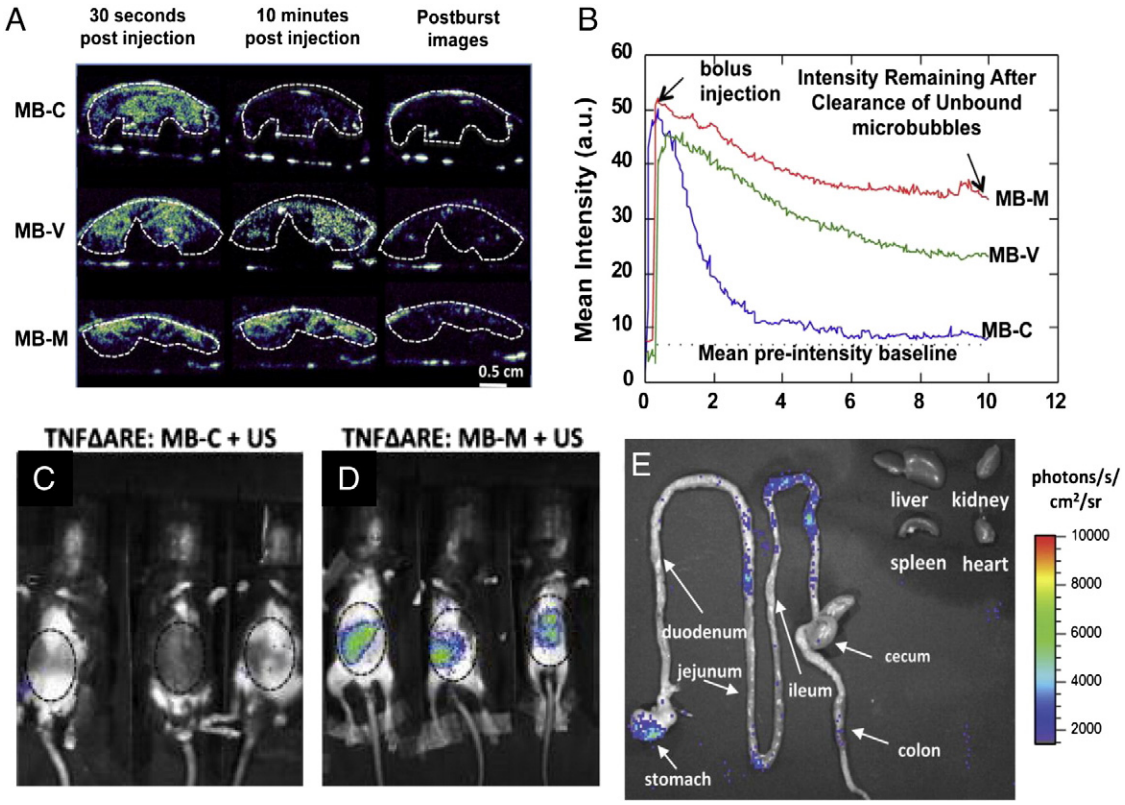

Fig. 4. Delivery of plasmid to inflamed small bowel in the mouse model of Crohn's disease. A) contrast ultrasound images showing the behavior of plasmid-bearing microbubbles targeted with a control antibody (top), or with antibodies to VCAM-1 (mid) or MAdCAM-1 (bottom). B) Time–intensity curves corresponding to images in panel A. Sustained retention of MAdCAM-1 or VCAM-1 targeted agents is appreciated, while the control agents wash out of the bowel within 5 min. 24 h after treatment, *in vivo* bioluminescence imaging shows that C) minimal luciferase is detectible in mice treated with control targeted microbubbles, but (D) significant expression in mice receiving MAdCAM-1 targeted agent. E) *Ex vivo* bioluminescence imaging shows luciferase expression confined to small bowel, with undetectible off-target signal on heart, liver, kidney, and spleen. Reprinted with permission from [47] with permission, Copyright, 2013, Elsevier B.V.

[48,49]. This nanoparticle is a liposome which in addition to a payload encapsulates a perfluoropropane nanoparticle inside it (Fig. 5). Initially tested for drug delivery, this particle is now widely investigated in animal models for nucleic acid delivery: a plasmid or an oligonucleotide can be entrapped inside the aqueous core of the liposome [50]. Alternatively, nucleic acid can be attached to the outside of the nanoparticles, with a standard positive lipid electrostatic interaction—in this case, positively charged lipid component is used for the liposome formulation [51,52]. The advantages of the latter approach are excellent yield of nucleic acid coupling to the particle and ease of use and the disadvantage is the potential nonspecific adhesion in non-target areas due to electrostatic interaction.

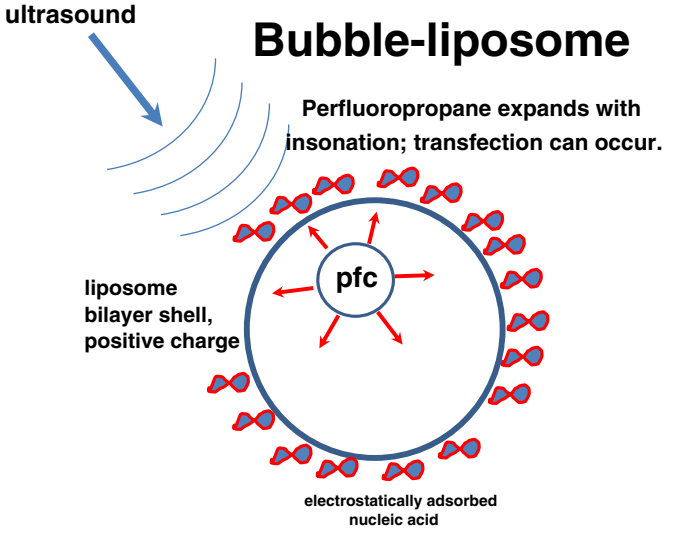

Fig. 5. Bubble-liposome formulation [50]: a liposome nanoparticle, surrounded by a lipid bilayer that entraps even a smaller perfluoropropane particle which can expand in response to ultrasound. Nucleic acid can be electrostatically attached to the outside of the liposome (as in traditional lipofection) or entrapped in the inner aqueous core.

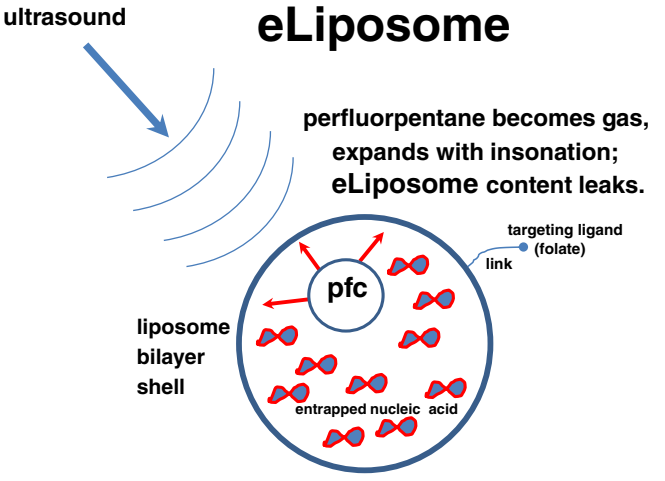

Fig. 6. Eliposome formulation [54]: a liposome nanoparticle, consisting of a lipid bilayer that entraps the inner aqueous core with perfluoropentane superheated droplets which can quickly expand to become gas at physiological temperatures in response to ultrasound. Triggered liposome content release can thus take place. Nucleic acid can be entrapped and sequestered inside the liposome. For molecular targeting, selective ligands (e.g., folate) can be attached to the liposome surface.

Preparation of bubble-liposomes is quite simple: pre-formulated liposomes as an aqueous dispersion were placed in a vial with perfluoropropane atmosphere, further pressurized with perfluoropropane gas via a syringe and sealed, and subjected to bath sonication at 42 kHz for several minutes. Electron microscopy confirmed the presence of nanoparticle "droplets" inside the liposomes. For nucleic acid delivery, it was added to pre-formulated positively charged bubble-liposome preparations; successful *in vitro* and *in vivo* delivery [51,52] and application of this approach for cancer vaccine have been reported [53]. It was noted by other investigators [54] that a perfluoropropane nanoparticle inside a liposome, due to its small size, under 60 nm, might be subjected to excessive Laplace pressure, calculated to reach tens of atmospheres. In their opinion, such gas particle would be unstable. Alternatively, one might hypothesize that perfluoropropane inside bubble-liposome may be in a liquid form. In that case, liquefied superheated perfluoropropane droplet, sequestered inside a liposome, would be triggered by ultrasound to become a much larger gas bubble, quite efficiently, despite the small initial size of the nanodroplet [55,56]. It will expand and trigger the release of the liposome content in response to ultrasound treatment, which might explain the stability and functionality of the "bubble liposome" formulation.

A formulation with a similar structure, termed "eliposome", was manufactured by a different protocol and entraps not perfluoropropane, but liquid perfluoropentane gas precursor (Fig. 6), which would expand to become gas at physiological temperature during ultrasound treatment [57]. A method of preparation of eliposomes is more complicated than for bubble liposomes: a lipid-stabilized liquid perfluoropentane nanoemulsion is prepared first, followed by co-entrapment of plasmid and nanoemulsion particles inside the carrier liposomes. The advantage of this formulation is in the lack of positive electrostatic charge on the external surface (thus, nonspecific adhesion to non-target cells could be reduced). To achieve selective adhesion, targeting ligands directed at the target cell receptors (e.g., folate) can be placed on the outer layer of eliposome [57].

A significant advantage of these nanoparticles when compared with gas-filled microbubbles comes from their smaller size: if liposomes are <250 nm in diameter, with proper PEG coating they can generally circulate in the bloodstream for hours [58]. Presumably, encapsulated liquid fluorocarbon nanoparticle will stay inside the liposomes during recirculation, providing enough time to perform ultrasound treatment and improve delivery efficacy to the target by extending the time of ultrasound treatment. Smaller liposomes can also extravasate in the areas of disease, such as tumor (leaky neovasculature) or ischemic cardiac or skeletal muscle, where endothelial inflammation leads to permeabilization of the vessel lining [59]. Typical microbubble formulations used for gene delivery have the same size parameters as typical microbubbles approved for diagnostic ultrasound imaging, i.e., diameter range is ~1–3 μm. Microbubbles will circulate only for minutes, with relatively rapid gas loss by exhalation via a lung route. Microbubbles stay intravascular, unless intense ultrasound treatment would result in the extravascular delivery of microbubble-associated plasmid, as was indeed detected by fluorescence microscopy of plasmid-associated dye in the interstitial space following plasmid–microbubble insonation in the cremaster muscle vasculature [12]. As a result of intense insonation, some RBC extravasation was also observed, which might not always be a desirable outcome. Thus, moderation of applied acoustic energy may result in directing transfection mostly to the endothelial lining of the vessel wall [2,60], which could be beneficial for certain research and clinical scenarios.

A multilamellar formulation, termed "echogenic liposome", proposed almost two decades ago [61], was manufactured by hydration of the dry lyophilized precursor matrix. These particles have been tested for ultrasound-assisted plasmid delivery [62] and oligonucleotide delivery [63]. Particle size here is significantly larger than for eliposomes or bubble-liposomes. Acoustic response of these particles was found to be dependent on the pockets of entrapped air [64]: air core is postulated to be placed within the lipid bilayer of the liposome membrane [65]. Choice of air as an entrapped gas may reduce the lifetime in the bloodstream and in response to ultrasound treatment even if the gas pocket is inside the liposome; it is generally believed that low-solubility fluorocarbons, such as perfluorobutane or perfluoropropane, with water solubility orders of magnitude less than of air, provide much longer lifetime and slower gas exchange. Thus, further improvement of echogenic liposome formulations might be feasible.

3. *In vivo* nucleic acid delivery with sonoporation: tissues and diseases

Small animal studies have evaluated sonoporation based delivery using microbubbles in nearly every organ system, including heart [66], pancreas [36], liver [67], kidney [68], bowel [47], skin [24], eye [69], solid tumors [70,71], joint synovium [72], dental pulp [73] and tendons [74]. While not sonoporation *per se*, opening of the blood–brain barrier with microbubbles and focused ultrasound has also been demonstrated [75]. Although there are numerous applications for *in vitro* and *ex vivo* use in life science research and approaches towards clinical use, we will confine our discussion to key *in vivo* applications.

3.1. Myocardial ischemia

Early *in vivo* work in the field was concentrated on the heart, although this has shifted somewhat in recent years. Numerous studies have demonstrated delivery of reporters to myocardium. In general, most studies have confirmed that the ultrasound energy must be applied to the microbubble for payload delivery [76–78]; that is, delivery outside the ultrasound beam or mediated by ultrasound alone (without microbubbles) occurs at a very low rate. However, early work by [30] and [79] found transgene expression in distant (non-sonoporated organs), although this may be due to the use of a viral payload in the case of the latter. Delivery of functional luciferase-encoding plasmid to the myocardium in rats was reported [80], with little gene expression in the non-target organs, when microbubble composition was selected appropriately. Microbubble-assisted siRNA delivery to coronary endothelium was demonstrated [77].

Induction of therapeutic angiogenesis as a treatment for ischemic heart disease has been a significant focus in the cardiac sonoporation field. In one of the earliest studies, Mukherjee et al. [81] demonstrated the delivery of recombinant VEGF protein directly (not via gene delivery) and resultant endothelial proliferation in hypertensive rats. Similar findings of increased vascular density were reported by [78] and [82] following treatment with VEGF165 plasmid. Delivery of plasmid coding for stem cell factor (SCF) was explored by Fujii and colleagues [82,83]. As was found with VEGF treatment, SCF delivery improved vascular density, cardiac function, and progenitor cell recruitment in a mouse model of myocardial ischemia. Repeated SCF treatment (up to 6 times over ten days) resulted in increased vascular density, improved cardiac function, and reduced scar size relative to a single treatment [83]. Delivery of hepatocyte growth factor to rodents, either in the form of recombinant protein [84] or (more appropriate for the topic of this review) a plasmid [66] also resulted in proliferation and improvement in cardiac function. These results demonstrate the feasibility of sonoporation to deliver functionally significant quantities of plasmid, siRNA, and even protein to the myocardium.

Cell-based therapy strategies have been examined in the context of ischemic cardiac disease, generally for stimulation/recruitment of progenitor cells. Delivery of plasmid coding for SDF-1 and SCF [83] or VEGF [82] was shown to increase the number of cardiac progenitor cells in the treated region. Sustained expression of TB4 over 12 weeks was shown to induce proliferation and differentiation of cardiac progenitor cells [70]. The use of sonoporation-based delivery strategies to induce the post-ischemic heart to create or recruit endogenous cell

factors, either alone or with concomitant revascularization therapy, is a rapidly developing research topic.

3.2. Skeletal muscle and peripheral arterial disease

Skeletal muscle is a versatile tissue for sonoporation-based treatment, due both to its ease of access (typically hindlimb in experimental animals) and relevance to cardiovascular disease. Numerous studies have demonstrated transfection of muscle cells in mice, rats, and rabbits following intramuscular administration of microbubbles and reporter plasmids [20,85–88]. It should be noted that skeletal muscle represents one of the few tissues in which contact-based delivery techniques (such as electroporation) can be readily performed without surgical access. However, the ability of intravascularly administered microbubbles to direct delivery specifically to the skeletal muscle vasculature represents a key advantage over competing systems.

Work by Christiansen [12] using cationic microbubbles showed successful transfection following either intra-arterial or intravenous administration. Intravital microscopy suggested that the vascular endothelium and associated cells appeared to be the dominant site of delivery, a finding supported by later studies [33]. Kobulnik [32] subsequently compared the efficacy of intravenously administered microbubble–nucleic acid complex with sonoporation to direct intramuscular injection of the same mass of transgene. It was found that direct intramuscular administration resulted in a significantly higher transfection, although this was confined to cells adjacent to the injection site. In contrast, sonoporation with intravenously administered microbubbles resulted in a more uniform treatment pattern. Interestingly, both methods resulted in a similar therapeutic efficacy in a rat model of hindlimb ischemia.

Compelling work has been done using cationic microbubbles bearing pro-angiogenic transgenes in the context of chronic ischemic disease. Leong-Poi [33] demonstrated up-regulation of VEGF and other pro-angiogenic factors for up to two weeks following a single sonoporation treatment with a VEGF plasmid. Functional improvement in blood flow was observed at two weeks post treatment and persisted, although at a reduced level, for up to 6 weeks. Taniyama [88] used a protein shell microbubble and co-injected hepatocyte growth factor plasmid in a rabbit model of hind limb ischemia. Increased capillary density and arterial flow were demonstrated at 5 weeks after sonoporation treatment.

A recent study [34] explored treatment with an Ang-1 coding plasmid two weeks following VEGF-coding plasmid treatment, and demonstrated that this two-phase protocol produced a sustained improvement in the functional blood flow over 6 weeks (Fig. 7). This result was likely due to the formation of functionally mature vessels in response to the temporally separated delivery of two distinct pro-angiogenic therapies. The authors reported the predominant site of transfection to be the arteriolar endothelium, with minimal capillary involvement.

3.3. Nucleic acid delivery in pancreas and in diabetes treatment

Targeted gene delivery and interference strategies hold particular attraction for diabetes and other chronic metabolic disease, due to high incidence of the disease and difficulty in finding long-term treatment. A

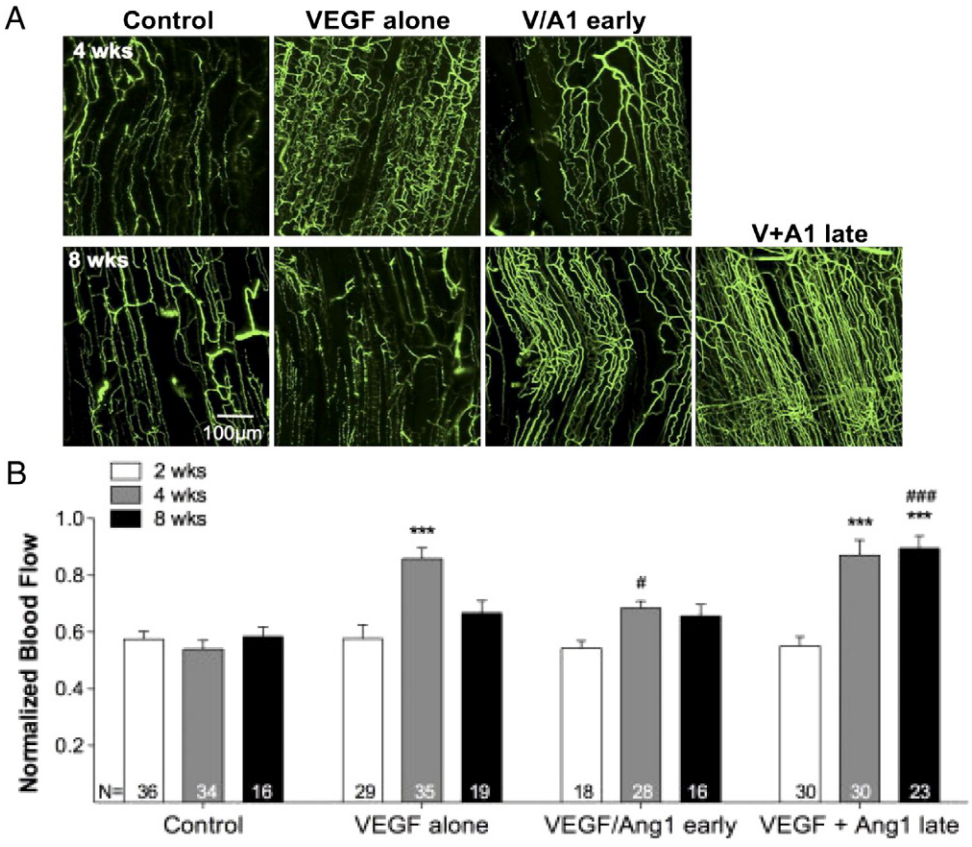

Fig. 7. Sequential delivery of VEGF and Ang1 plasmids improves formation of functional vasculature in a rat model of ischemic injury. A) Fluorescence microangiography images showing vascularity in injured tissue at 4 (top row) and 8 (bottom row) weeks after treatment. Untreated (control) animals exhibit low vessel density. Treatment with VEGF alone results in formation of immature vessels that largely regress within 8 weeks. Delivery of both VEGF and Ang1 together (V/A1 early) results in improved vessel density, while delivering Ang1 2 weeks after VEGF treatment (V + A1 late) yields sustained and functional vasculature. B) Non-invasive imaging of blood flow revealed that simultaneous or sequential treatment with VEGF and Ang1 results in a sustained improvement to vascular function relative to treatment with VEGF alone. Reprinted with permission from [34], Copyright, 2012 by the American College of Cardiology Foundation.

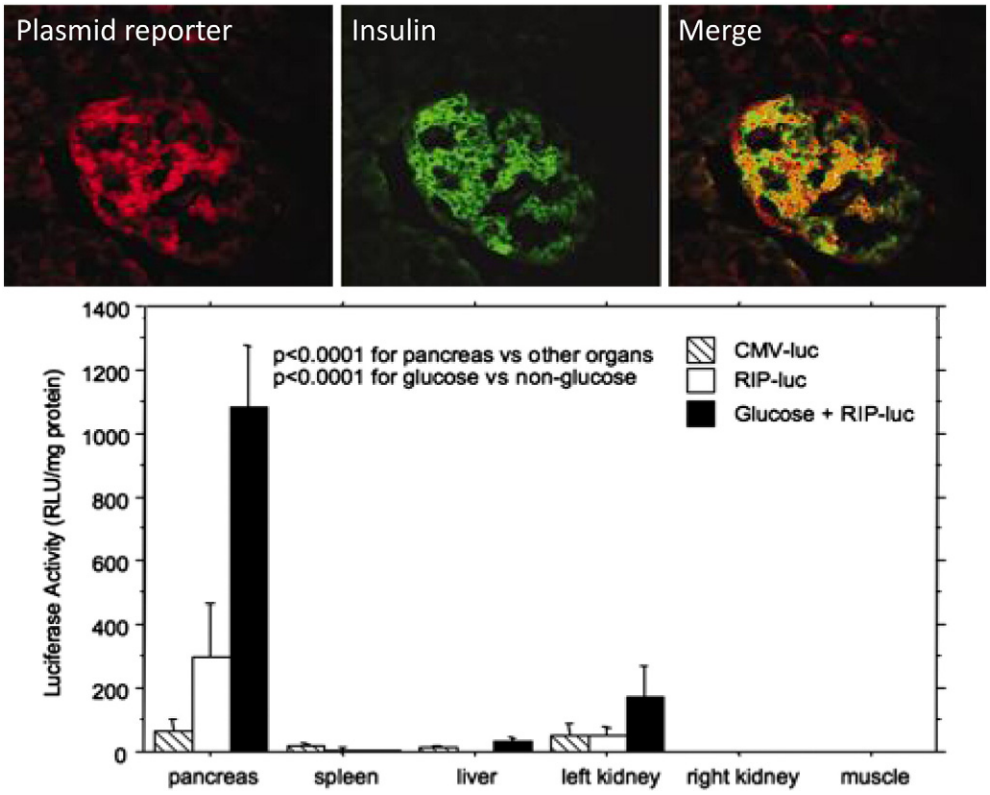

Fig. 8. Delivery of plasmid to islet beta cells in rat. Top row is the confocal micrographs of an islet from an animal treated with DsRed-coding plasmid. The location of the transferred gene (red) co-localizes with insulin-staining beta cells, demonstrating specific delivery. Bottom panel shows minimal payload delivery outside pancreas. Reprinted with permission from [36]. Copyright, 2006, National Academy of Sciences USA.

significant technical challenge here is confining the acoustic beam to the pancreas, owing to the close association of the pancreas to other abdominal organs and its complex anatomy in mice. The use of organ-specific promoters in the context of gene therapy appears to be a potential solution to this, with relatively low off-target transfection reported in the literature [36,89].

In an elegant set of studies, Chen and colleagues [36] demonstrated specific delivery and expression of the plasmids that encoded human insulin and hexokinase to islet beta cells in rats (Fig. 8). Use of a rat insulin promoter [36,89] enabled specific transgene expression in the islets, with low to undetectable expression in the spleen, liver, and kidney. Transgene expression peaked at day 4 and then decayed steadily over 4 weeks following a single treatment, while improvement in functional parameters was demonstrated to persist for at least 10 days. Increased insulin secretion and commensurate reduction in plasma glucose were demonstrated in healthy rats following delivery of a hexokinase plasmid. Subsequent studies have focused on gene therapy approaches for beta cell regeneration.

Sonoporation-based delivery of plasmid coding for both the mitogen betacellulin and pancreatic duodenal homeobox-1 (PDX-1) transcription factor (necessary for pancreatic development and β cell maturation in rodents) was shown to improve insulin production and glucose tolerance in a rat model of chemically induced beta cell destruction [90]. Interestingly, neither mature islets nor true beta cells were observed by traditional histological analyses. Yet a population of insulin producing cells that expressed certain beta cell markers was found in the exocrine pancreas of treated animals. Subsequent studies in which plasmid coding for transcription factors neuroD1 under an optimized [89] insulin promoter [91] or Nkx2.2 using a piggyBac transposon plasmid [92] were used, and initiated proliferation of adult pancreatic progenitor cells within islets to mature islets with β cells and α cells. A single ultrasound–microbubble–plasmid treatment demonstrated islet regeneration. Pretreatment with a JNK protein kinase inhibitor was shown to normalize blood glucose and insulin response for up to 90 days after a single treatment. Long term normalization (up to 6 months) was reported [93] following single treatment with a cocktail of plasmids designed to enable residual beta cell replication. A key aspect of these studies is that sustained transgene expression is not necessarily required to effect a clinically significant improvement in diabetes. Rather, transient expression (generally reported as 2–4 weeks) can be sufficient to initiate a sequence of events that alters the disease phenotype, and provides long term therapeutic effects.

3.4. Nucleic acid delivery to solid tumors

Solid tumors are an attractive target for many targeted delivery technologies, including sonoporation. At the current time, the volume of published work on tumors is significantly greater than that for any other organ system. A systematic assessment of the literature in this field is unfortunately beyond the scope of our current review; rather, we present experimental details and some key findings from the literature in Table 1 and discuss several most interesting examples below. We have concentrated on the studies in which therapeutically meaningful payloads have been reported.

Much of the work in this field has been done in subcutaneous tumor models in mice. Although this minimizes the technical challenges of performing the sonoporation procedure, it should be noted that subcutaneous models may not represent a realistic scenario for the tumors that would be treated in human disease. Several notable studies have utilized orthotopic models, e.g., of prostate cancer [94], providing compelling evidence of efficacy.

Of particular interest for cancer therapy is the ability to either express the genes encoded in plasmids or deliver small molecules such as siRNA with simple positively charged perfluorobutane microbubbles used as a carrier, combined with ultrasound to achieve delivery specificity. This approach was described by Villanueva for targeted ultrasound-

Table 1
Sonoporation for nucleic acid delivery in cancer: animal model studies.

Tumor model	Payload	Method	Findings	Reference
Pancreatic adenocarcinoma, mouse SubQ	p16 (plasmid)	Polymer microbubbles with conjugated payload, single treatment, IV	Reduced tumor growth rate	Hauff et al., 2005 [71]
Hepatic adenocarcinoma, mouse SubQ	IFN-beta (plasmid)	Lipid microbubbles, single treatment, intratumoral	Reduced tumor volume at 6 weeks	Sakakima et al., 2005 [99]
Cervical carcinoma, mouse SubQ	Human survivin (shRNA)	Lipid microbubbles + PEG, single treatment, IV	~4-fold reduction in survivin expression at 3 weeks	Chen et al., 2010 [100]
Colon carcinoma, mouse intra-hepatic	IFN-beta (plasmid)	Cationic "liposome" microbubbles, single treatment, intratumoral	~2 fold increase in survival in microbubble treated animals, slight increase with addition of ultrasound	Hayashi et al., 2009 [101]
Gingival squamous carcinoma, mouse SubQ	Bleomycin (protein) or cytolethal descending toxin B (plasmid)	Albumin microbubbles, 8 treatments, intratumoral	Tumor regression over 28 days, apoptosis	Iwanaga et al., 2007 [102]
Radiation induced fibrosarcoma (RIF-1), mouse SubQ	Luciferase (plasmid)	Lipid microbubble, single treatment, intratumoral	Peak luciferase expression at day 4, sustained expression over 15 days	Li et al., 2009 [103]
Liver (BNL, SubQ or orthotopic), lung (LL/2,orthotopic), glioma (RT-2, orthotopic). All mouse.	Calreticulin or endostatin (plasmid)	Lipid microbubble co-injected with plasmid, 4 treatments, intramuscular.	Plasmid delivery to skeletal muscle can treat distant tumors.	Liao et al., 2012 [104]
Breast carcinoma (MDA-MB-231), mouse orthotopic	Adenovirus coding for secreted tumor marker	Lyophilized lipid microbubble reconstituted with virus, targeting VEGFR2/integrin/P-selectin, single treatment, IV	Expression of reporter for 2 days after treatment.	Warram et al., 2012 [105]
Melanoma (C32), mouse subQ	IFN-beta (plasmid)	Lipid microbubbles co-injected with plasmid, 4 treatments, intratumoral	Reduction in tumor growth rate during treatment	Yamaguchi et al., 2011 [106]
Squamous cell carcinoma, mouse SubQ	HSV thymidine kinase (plasmid) + daily ganciclovir	Cationic lipid microbubble, single treatment, IV	Increased doubling time and apoptosis in treated tumors	Carson et al., 2011 [4]
Squamous cell carcinoma (SCC-VII), mouse SubQ	EGFR (siRNA)	Lipid microbubble manufactured with siRNA, 2 treatments, IV	Decreased EGFR expression and increased doubling time	Carson et al., 2012 [3]
Prostatic carcinoma (DU-145), mouse SubQ	Adenovirus coding for MDA-7 (IL-24)	Lyophilized lipid microbubble reconstituted with virus, 5 treatments, IV	Eradication of both sonoporated and distant tumors	Greco et al., 2010 [107]
Prostatic carcinoma, orthotopic (Hi-Myc transgenic mouse)	Adenovirus coding for MDA-7 (IL-24)	Lyophilized lipid microbubble reconstituted with virus, 9 treatments, IV	Reduction in size of prostate and increased tumor apoptosis in treated animals	Dash et al., 2011 [94]

triggered thymidine kinase gene expression, which resulted in tumor growth inhibition in a murine cancer model when combined with prodrug gancyclovir, activated by the enzyme [4]. Suppression of tumor growth was reported by Leong Poi [95], who also used decafluorobutane microbubbles with DSPC/PEG stearate shell with positively charged lipid for nucleic acid attachment. These microbubbles were carrying a short hairpin RNA-encoding plasmid to inhibit VEGFR2 in rodents. It was noted, that for the most efficient delivery, ultrasound application in a slow tumor vasculature blood flow scenario should be performed intermittently, with long intervals (up to 10–20 s between ultrasound pulses), to allow complete replenishment of the blood vessels with the microbubble–nucleic acid construct, followed by the subsequent ultrasound pulse. Likewise, ultrasound-triggered activation of microbubbles carrying siRNA directed to EGF receptor in a murine squamous cell cancer model allowed to decrease EGFR expression and inhibited tumor growth [3].

One of the interesting strategies in the development of cancer vaccines is to transfect the gene that encodes the desired antigen into dendritic cells for its expression and proper presentation, which would be then applied to obtain antitumor immune response. This transfection can be achieved with ultrasound activation of microbubble–lipoplex complexes, where a plasmid entrapped in lipoplex is encoding the antigen. Cell viability was retained, and transient protein expression was achieved. Authors propose to co-transfect the antigen and dendritic cell maturation signals to improve the efficacy [23].

Use of virus-based systems for tumor therapy should not be overlooked: virus–microbubble mixture can be injected intravenously, followed by focused ultrasound treatment [96], which resulted in the increase of luciferase expression in the insonated tumor up to 50-fold.

Recently, the first clinical trial utilizing sonoporation and microbubbles for local therapeutic delivery was published by Kotopoulis and colleagues [97]. In that study, five patients with inoperable pancreatic cancer were administered gemcitabine followed by sequential doses of microbubble contrast agents and ultrasound within a 30 min interval. Low-intensity ultrasound was administered with a diagnostic imaging ultrasound system, enabling simultaneous visualization and image-guided sonoporation. Compared with a historical control group of 80 patients treated with gemcitabine alone, treatment patients were able to tolerate a greater number of chemotherapy rounds and exhibited a reduction in tumor size and/or growth rate. No adverse effects relating to the sonoporation procedure were reported, although the treatment protocol used somewhat lower intensity than is typically applied in animal studies. This study establishes that a sonoporation protocol can be performed successfully in a clinical setting, and represents an important first step toward clinical development of this promising technique. While there was no nucleic acid delivery reported in this particular study, its general importance and relevance for the nucleic acid delivery field are clear, especially combined with the point that nucleic acid delivery may avoid systemic toxicity associated with regular chemotherapy. An example of such combination gene therapy is an animal study by Machluf et al. [98], where Optison microbubbles were locally administered in the tumor along with a plasmid encoding anti-angiogenic hemopexin-like domain fragment (PEX) in mice bearing subcutaneous prostate cancer, and clinical ultrasound applied with a physical therapy apparatus. Local administration of microbubbles and the plasmid allowed efficient and direct transfection of the cells in the tumor mass. Thus, a single treatment resulted in a significant reduction of the tumor growth rate, for up to 28 days.

4. Conclusions

Application of ultrasound for nucleic acid delivery provides a unique ability to trigger the transfection specifically in the areas of disease. Ultrasound can be applied with the simplest physical therapy apparatus,

or with diagnostic clinical equipment which also provide ultrasound imaging guidance, or even more precise and powerful MRI-guided focused ultrasound systems. While sonoporation transfection efficacy levels are generally not perfect, the ability to achieve nucleic acid delivery specifically in the ultrasound-treated areas, and not in the traditional nonspecific targets (e.g., liver or lung) conveys a significant advantage to this approach. With the existing ultrasound contrast agents (microbubbles) already approved for clinical diagnostic imaging, and a wide array of nucleic acid materials and carriers available as GLP/GMP materials in clinical trials already, a combination approach that takes advantage of the localized energy deposition and nucleic acid carriers should reach clinical trial stage in the near future.

More complicated structures, which combine nucleic acid and ultrasound-triggered carrier into micro- or nanoparticles, realistically, should take a much longer time to bring to clinic (they will be regarded by FDA as drugs, and historically it takes many years). These combined particles might provide more efficient therapy, especially for the delivery of short nucleic acid fragments (antisense, siRNA, microRNA). Use of viral structures, such as adenovirus or AAV, regarded as therapeutic biological products by FDA, in combination with microbubbles and ultrasound, might also reach clinical testing within a few years, especially if viral transfection in the remote organs is negligible, and safety is assured. Overall, with the progress of technology, ultrasound-assisted nucleic acid delivery may provide much needed therapeutic breakthrough, especially for the scenarios where only palliative treatment is currently available.

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