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SYNTHESIS, CHARACTERIZATION AND APPLICATION OF MICROBUBBLES: A REVIEW

J.V. Prajapati and Y.K. Agrawal

Institute of Research & Development, Gujarat Forensic Sciences University, Sec-18/A, DFS Headquarters, Gandhinagar- 382 007, Gujarat, India

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Correspondence to Author:

Jinal Prajapati

M.S. Forensic Pharmacy, Institute of Research & Development, Gujarat Forensic Sciences University, Sec-18/A, DFS Headquarters, Gandhinagar- 382 007, Gujarat, India

ABSTRACT

Microbubbles are bubbles smaller than one millimeter in diameter, but larger than one micrometer. Microbubbles designate air or gas filled microspheres suspended in a liquid carrier phase which generally results from the introduction of air or gas. The liquid phase contains surfactants to control the surface properties as well as stability of the bubble. Microbubbles have an average size less than that of RBC's i.e., they are capable of penetrating even into the smallest blood capillaries & releasing drugs or genes, incorporated on their surface, under the action of ultrasound. Microbubbles in general have a wide variety of applications. However in the biomedical field these are primarily used as diagnostic agents in combination with ultrasound for molecular imaging of various organs and even tumours. These are also proposed for drug and gene delivery to targeted regions in combination with various ligands. Most of the physicians today prefer imaging with ultrasound in combination with microbubbles compared to other diagnostic techniques for low cost and rapidity. They are used in medical diagnostics as a contrast agent for ultrasound imaging. The gas-filled, e.g., air or perfluorocarbon, microbubbles oscillate and vibrate when a sonic energy field is applied and may reflect ultrasound waves. This distinguishes the microbubbles from surrounding tissues. The unique ability of microbubbles to respond to ultrasound makes them useful agents for contrast ultrasound imaging, molecular imaging, and targeted drug and gene delivery. In practice, because gas bubbles in liquid lack stability and would therefore quickly dissolve; microbubbles must be encapsulated with a solid shell. The shell is made from either a lipid or a protein such as Optison-microbubbles which consist of perfluoropropane gas encapsulated by a serum albumin shell. Microbubbles may also be used for drug delivery and water/waste water treatment purposes.

INTRODUCTION: Microbubbles are also small spherical bubbles comprising of gas, they remain distinct from each other or separate from each other i.e. do not agglomerate, also they have their size range in micrometers usually 1-100 µm. There has been a lot of research on micro bubbles in recent years.

Micro bubbles are miniature gas bubbles of less than 50 microns diameter in water. The micro bubbles, which mostly contain oxygen or air, can remain suspended in the water for an extended period. Gradually, the gas within the micro bubbles dissolves into the water and the bubbles disappear.

In the medical field, microbubbles have been used as diagnostic aids to scan the various organs of body and recently they are being proposed to be used as drug or gene carriers and also for treatment in cancer therapy. Microbubbles have been used in a variety of fields , these have been used to improve the fermentation of soil, used to increase the hydroponic plant growth, have been to used to increase the aquaculture productivity, these have been also used to improve the quality of water, used in sewage treatment . Biomedically microbubbles are defined as small spherical gas bubbles made up of phospholipids or biodegradable polymers , that are approximately the size of RBC's and are used as diagnostic aids, as drug and gene carriers in combination with ultrasound.

Properties of Microbubbles: The ideal properties of microbubbles can be divided into two classes [1];

Functional Properties: The functional properties are those which render them useful for performing their various functions these include,

a) **Injectability**: Since these microbubbles are to be injected into the body so as to exert their various actions they should be injectable.

b) **Ultrasound Scattering Efficiency**: As these microbubbles act in combination with ultrasound they should have ultrasound scattering efficiency. Ultrasound-mediated microbubbles destruction has been proposed as an innovative method for non-invasive delivering of drugs and genes to different tissues. Microbubbles are used to carry a drug or gene until a specific area of interest is reached, and then ultrasound is used to burst the microbubbles, causing site-specific delivery of the bioactive materials.

c) **Biocompatibility**: Microbubbles interact with the vital organs of the body at cellular levels they should be biocompatible.

Structural Properties: These refer to the structure or the physical properties of the microbubbles, these are as follows,

a) Should have an average external diameter between the ranges of 1-10 µm, narrow size distribution so as to avoid complications when injected into the body

b) Density & compressibility difference between themselves & the surrounding body tissues to create an acoustic impedance & to scatter ultrasound at a much higher intensity than the body tissues so as to be used as contrast agents

c) Sufficient surface chemical properties to be modified for the attachment of various ligands to target them to specific tissues or organs

d) Uniformity of shell thickness

Components of Microbubbles (Fig. 1):

Microbubbles basically comprise of three phases [1, 2, 3, 4]:

1. Innermost Gas Phase
2. Shell Material Enclosing the Gas Phase
3. Outermost Liquid or Aqueous Phase

In addition to this, the formulation may also comprise of;

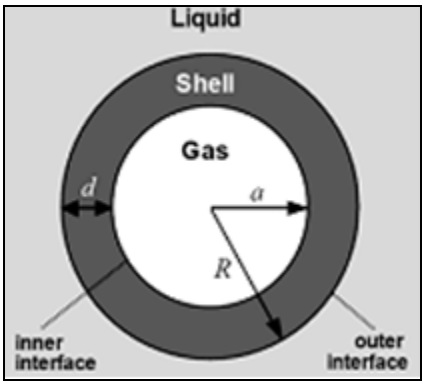

FIG. 1: COMPONENTS OF MICROBUBBLES

1. **Gas Phase:** The gas phase can be a single gas or a combination of gases can be used. Combination gases are used to cause differentials in partial pressure & to generate gas osmotic pressures which stabilize the bubbles. When a combination of gases is used two types of gases are involved one is the Primary Modifier Gas also known as first gas. Air is preferably used as primary modifier gas, sometimes nitrogen is also used as first gas. The vapor pressure of first gas is (760 - x) mm of Hg , where x is the vapor pressure of the second gas.

The other gas is Gas Osmotic Agent also known as second gas; it is preferably a gas that is less permeable through the bubble surface than the modifier gas. It is also preferable that the gas osmotic agent is less soluble in blood & serum. Gas osmotic agent is normally a gas at room temperature or liquid so long as it has a sufficient partial or vapor pressure at the temperature of use to provide the desired osmotic effect. Some examples of second gas are per fluorocarbons or sulfur hexafluoride.

The key to success for microbubbles as drug delivery vehicles is their extreme activity when exposed to ultrasonic waves. The gas core expands during the rarefaction phase of the pressure wave and contracts during the compression phase. Depending on the ultrasound parameters, various phenomena may occur that facilitate ultrasound backscatter and/or the release and local delivery of drugs from the microbubble shell. These phenomena are summarized below and range from subtle effects such as acoustic radiation force, to highly energetic events such as inertial cavitation. Combinations of these phenomena allow for imaging, targeting, controlled release and vascular permeability enhancement.

2. **Shell Material**: The shell material encapsulates the gas phase. It plays a major role in the mechanical properties of microbubble as well as diffusion of the gas out of the microbubble.The shell also acts a region for encapsulation of drug molecules also ligands can be attached to the shell membrane so as to achieve targeting of these microbubbles to the various other components. organs or tissues. It accounts for the elasticity or compressibility of microbubbles.

   More elastic the shell material is more acoustic energy it can withstand before bursting or breaking up, this increases the residence time of these bubbles in body. More hydrophilic the shell material, more easily it is taken up by the body this decreases the residence time of these bubbles in the body eg., the various types of shell materials that can be used are;

   a. Proteins like albumin

   b. Phospholipids like phosphotidylcholine, phospho tidylethanolamine etc.

   c. Biodegradable polymers like polyvinyl alcohol, polycaprolactone etc.

   d. Surfactant Shells

   e. Polyelectrolyte Multilayer Shells

   a) **Protein Shells:** Albumin shelled microbubbles were a pioneering formulation used in contrast ultrasound imaging. They paved the way for several subsequent formulations that could pass the lung capillaries and provide contrast in the left ventricle of the heart. The first albumin microbubbles formulation to be approved by the US Food and Drug Administration (FDA) was Albunex (GE Healthcare). An Albunex suspension consists of roughly $7x10^8$ microbubbles/mL with a size range from 1 to 15 µm diameter [5]. Albunex is stable upon refrigeration for at least two years. Albumin-coated microbubbles are formed by sonication of a heated solution of 5% (w/v) human serum albumin in the presence of air. During sonication, microbubbles of air are formed which become encapsulated within a 15-nm thick shell of aggregated albumin.

      Heating is necessary to denature the albumin prior to sonication and facilitate encapsulation. Biochemical analysis suggested that the shell is a monomolecular layer of native and denatured albumin in multiple orientations [6]. The albumin shell is held together through disulfide bonds between cystein residues formed during cavitation [7]. Covalent cross-linking may explain the relative rigidity of albumin shells observed during ultrasonic insonification [8]. Following Albunex, an albumin formulation was developed encapsulating a perfluorocarbon gas core, named Optison™ (GE Healthcare). The low solubility of the perfluorocarbon gas gave these microbubbles much longer circulation persistence *in vivo* [9]. Optison™ is currently approved by the US FDA for contrast echocardiography.

b) **Lipid Shells**: Lipid-coated microbubbles are one of the most interesting and useful formulations used for biomedical imaging and drug delivery. The lipid shell is inspired by nature, as stable microbubbles found ubiquitously in the oceans and fresh waters of Earth are known to be stabilized by acyl lipids and glycoproteins [10]. The lipid shell of a microbubbles is also bio-inspired, as it mimics the remarkable stability and compliance of lung surfactant [11]. Indeed, lung surfactant was discovered based on the observation of stable microbubbles formed from lung lavage [12]. Moreover, the ability of lung derived fluid to form stable microbubbles is being pursued as a clinical means of assessing lung surfactant viability in neonates [13].

There are several commercially available lipid-coated microbubble formulations approved for clinical use in the US and abroad, including Definity (Lantheus Medical Imaging) and Sonovue® (Bracco Diagnostics). Lipid shells have several advantages. Phospholipids spontaneously self-assemble into a highly oriented monolayer at the air-water interface, such that their hydrophobic acyl chains face the gas and their hydrophilic head groups face the water. Thus, the lipid monolayer will form spontaneously around a newly entrained gas bubble, just as for surfactants and proteins.

c) **Polymer Shells**: The term "polymer microbubble" typically refers to a special class of microbubbles that are stabilized by a thick shell comprising cross-linked or entangled polymeric species. The bulk nature of the polymer shell makes it more resistant to area compression and expansion than its lipid and albumin counterparts, which reduces the echogenicity and drug delivery activity.

For example, polymer microbubbles have been observed to fracture during insonification, thereby releasing their gas core via extrusion through the shell defect [14]. The resulting gas bubble was unstable and rapidly dissolved according to the classical Epstein and Plesset equation [15].

The shell, on the other hand, remained intact and often propelled away from the gas core; this ballistic effect may be useful for drug delivery. The polymer microbubbles had a broad size distribution ranging from 1–20 µm diameter. Optical microscopy and cryogenic transmission electron microscopy (cryo-TEM) showed that the microbubbles had elongated, crumpled shapes. The polymer shell was typically 150–200 nm thick.

Acoustic tests showed a dose-dependent increase in acoustic attenuation. In 1999, Nayaran and Wheatley reported on microbubbles formed by the biodegradable copolymer poly (D, L-lactide-co-glycolide) (PLGA). Also in 2005,Cavalieri *et al* [16]., described a method to create microbubbles coated with poly (vinyl alcohol) (PVA). PVA microbubbles were created by chemical cross-linking at the air/water interface during high-speed stirring (8000 RPM) of an acidic solution of telechelic PVA. The mean diameter was approximately 6±1µm.

The shell thickness could be decreased from 0.9 to 0.7µm by decreasing the operating temperature from room conditions to 4°C. PVA microbubbles had a shelf life of several months and are capable of carrying hydrophobic drugs, charged polymers (e.g., DNA) and targeting ligands.

d) **Surfactant Shell**: Microbubbles stabilized by mixtures of the synthetic surfactants SPAN-40 and TWEEN-40 were formulated by Wheatley *et al* [17, 18]. The SPAN/TWEEN solution was sonicated in the presence of air to form stable microbubbles. Using a Langmuir trough, they were able to establish the correct ratio of SPAN to TWEEN (roughly 1:1) to use for maximum film stability. Interestingly, they showed that surfactant derived from sonicated micro-bubbles was more stable (i.e., was capable of reaching higher collapse pressures on the Langmuir trough) than that used in the precursor solution, indicating that the sonication process modified the surfactant to form a more stable film [18].

e) **Polyelectrolyte Multilayer Shells**: A new class of polymer-surfactant shell hybrids was recently introduced that involves polyelectrolyte multilayer (**PEM**) shells on preformed microbubbles. The preformed microbubbles are coated with a charged surfactant or protein layer, which serves as a substrate for PEM deposition. The layer-by-layer assembly technique is used to sequentially adsorb oppositely charged polyions to the microbubble shell. Shchukin et al [19]., were the first to report PEM deposition onto microbubbles.They used the polymers poly(allylamine hydrochloride) (PAH) and poly(styrene sulfonate) (PSS) for the polyion pair. This system gave a relatively uniform PEM coating that provided the microbubbles with remarkable stability.

Borden *et al* [20]., developed a PEM microbubble with phospholipid containing the cationic head group tri methyl ammonium propane (TAP) as the underlying shell and DNA and poly (L-lysine) (PLL) as the poly ion pair. Interestingly, the PEMs formed as islands owing to phase separation of the phospholipid species in the shell. The formation of such islands may be useful for surface compartmentalization for multi-functional microbubbles that require both ligand-receptor mediated adhesion and drug release through ultrasound-triggered fragmentation. Lentacker *et al* [21]., Described a multilayer microbubble in which albumin microbubbles were coated with DNA and PAH, where the latter layer served to bind and protect the DNA from enzymatic degradation.

Aqueous or Liquid Phase: The external, continuous liquid phase in which the bubble resides typically includes a surfactant or foaming agent.Surfactants suitable for use include any compound or composition that aids in the formation & maintenance of the bubble membrane by forming a layer at the interphase. The foaming agent or surfactant may comprise a single component or any combination of compounds, such as in the case of co surfactants. Also the persistence of microbubble in body is inversely proportional to La Place pressure which in turn is directly proportional to surface tension of bubble. In other words decrease in the surface tension acting on the bubble increases the persistence time of the bubble in the body eg., copolymers of polyoxy-propylene, polyoxyethylene, sugar esters, fatty alcohols, aliphatic amine oxides, hyaluronic acid esters & their salts, dodecyl poly (ethyleneoxy) ethanol, etc.

- **Nonionic Surfactants**: Polyoxyehylene polyoxy-propylene copolymers eg., Pluronic F-68, polyoxy-ethylene stearates, polyoxyethylene fatty alcohol ethers, polyoxyethylated sorbitan fatty acid esters, glycerol polyethylene glycol oxystearates, glycerol polyethylene glycol ricinoleate etc.

- **Anionic Surfactants**: Fatty acids having 12 -24 carbon atoms Eg. Sodium Oleate.

Other Components: The various other components that may be incorporated in the formulation include osmotic agents, stabilizers, chelators, buffers, viscosity modulators, air solubility modifiers, salts & sugars can be added to fine tune the microbubble suspensions for maximum shelf life & contrast enhancement effectiveness. Such considerations as sterility, isotonicity & biocompatibility may govern the use of such conventional additives to injectable compositions.

Methods to prepare Microbubbles: The various methods that can be used for the preparation of these microbubbles include:

1) Cross Linking Polymerization
2) Emulsion Solvent Evaporation
3) Atomization & Reconstitution
4) Sonication.

1. **Cross Linking Polymerisation**: In this a polymeric solution is vigorously stirred, which results in the formation of a fine foam of the polymer which acts as a colloidal stabilizer as well as a bubble coating agent. The polymer is then cross linked, after cross linking microbubbles float on the surface of the mixture. Floating microbubbles are separated & extensively dialyzed against Milli Q water eg., 2% aqueous solution of telechelic PVA is vigorously stirred at room temperature for 3 hrs at a pH of 2.5 by an Ultra Turrax T-25 at 8000 rpm equipped with

a Teflon coated tip, fine foam of PVA is formed. The PVA is then cross linked at room temperature and at 5˚C by adding HCl or H2SO4 as a catalyst, the cross linking reaction is stopped by neutralization of the mixture and microbubbles are then separated.

2. **Emulsion Solvent Evaporation**: In this method, two solutions are prepared, one is an aqueous solution containing an appropriate surfactant material which may be amphilic biopolymer such as gelatin, collagen, albumin or globulins. This becomes the outer continuous phase of the emulsion system. The second is made from the dissolution of a wall forming polymer in a mixture of two water immiscible organic liquids. One of the organic liquids is a relatively volatile solvent for the polymer & the other is relatively nonvolatile nonsolvent for the polymer. The polymer solution is added to the aqueous solution with agitation to form an emulsion. The emulsification step is carried out until the inner phase droplets are in the desired size spectrum. It is the droplet size that will determine the size of the microbubble.

   As solvents volatilizes, polymer conc. in the droplet increases to a point where it precipitates in the presence of the less volatile nonsolvent. This process forms a film of polymer at the surface of the emulsion droplet. As the process continues, an outer shell wall is formed which encapsulates an inner core of nonsolvent liquid. Once complete, the resulting microcapsules can then be retrieved, washed & formulated in a buffer system. Subsequent drying, preferably by freeze-drying, removes both the nonsolvent organic liquid core & the water to yield air filled hollow microbubbles.

3. **Atomisation & Reconstitution**: A spray dried surfactant solution is formulated by atomizing a surfactant solution into a heated gas this results in formation of porous spheres of the surfactant solution with the primary modifier gas enclosed in it. These porous spheres are then packaged into a vial, the headspace of the vial is then filled with the second gas or gas osmotic agent. The vial is then sealed, at the time of use it is reconstituted with a sterile saline solution. Upon reconstitution the primary modifier gas diffuses out & the secondary gas diffuses in, resulting in size reduction. The microbubbles so formed remain suspended in the saline solution & are then administered to the patient.

4. **Sonication:** Sonication is preferred for formation of microbubbles, i.e., through an ultrasound transmitting septum or by penetrating a septum with an ultrasound probe including an ultrasonically vibrating hypodermic needle. Sonication can be accomplished in a number of ways, for eg., a vial containing a surfactant solution & gas in headspace of the vial can be sonicated through a thin membrane. Sonication can be done by contacting or even depressing the membrane with an ultrasonic probe or with a focused ultrasound “beam”.

   Once sonication is accomplished, the microbubble solution can be withdrawn from the vial & delivered to the patient. Sonication can also be done within a syringe with a low power ultrasonically vibrated aspirating assembly on the syringe.

5. **Characterisation of Microbubbles:** Once prepared these microbubbles are characterized as per the following parameters;

   A. Microbubble Diameter & Size Distribution: The average diameter as well as size distribution of these microbubbles can be determined by Laser light Scattering, Scanning Electron Microscopy (SEM), Transmission Electron Microscopy.

   B. Shell Thickness: Shell thickness is determined by coating the shell with a fluorescent dye like Red Nile, this is then determined by Fluorescent Microscopy against a dark background.

   C. Microbubble Concentration: The microbubble concentration is determined by counting the no. of microbubbles per ml by using the Coulter Counter Machine.

   D. Air Content by densitometry: The content of air encapsulated within the microbubbles in the suspension samples is measured by oscillation U-tube densitometry with a DMA-58. The instrument is calibrated with air and purified

water prior to use. The density of the suspension is measured before and after elimination of encapsulated air. The complete removal of encapsulated air is achieved by 5 min high powered sonication in a sonicator. The air content is calculated as,

$$C_{air} = \rho 1 - \rho 2 / \rho 2 * 100$$

Where, C_{air} is air content (%v/v); ρ1 (g/ml) density before elimination of encapsulated air; ρ2 (g/ml) density after elimination of encapsulated air

E. **Ultrasound Reflectance Measurement**: Experimental set up consists of transducer, microbubble contained in a vessel consisting of metallic reflector and cellophane membrane, this vessel is in turn kept in another vessel containing water. The signals which are reflected are evaluated for the ultrasound reflecting capacity of these microbubbles.

F. **Biomedical Applications** [22, 23, 24, 25]**:**

1. **Diagnostic Aids**: Microbubbles are elastic and compressible, these undergo compression and rarefaction thereby creating an acoustic impedance mismatch between biological tissues and fluids as these are efficient reflectors of ultrasound, hence used as contrast agents. These are used as diagnostic aids for:

    a. Organ Edge Delineation

    b. Blood Volume and Perfusion

    c. Inflammation

    d. Cancer

    e. Liver

    f. Also used to scan the tumors arising in the body.

    g. Used for imaging the gall bladder stone.

2. **Gene Delivery**: The next most promising application of these microbubbles is these can be used as tools for gene delivery. The salient features of these microbubbles which make them useful for gene delivery are as follows:

    1) Microbubbles are metabolically inert

    2) When injected into the body they do not produce any immune response

    3) Also the gene encapsulated or attached to the microbubble is carried to its target without getting digested by the various enzymes.

Charged drugs can be stabilized in or onto the surfaces of microbubbles by virtue of electrostatic interactions lipid-coated microbubbles to bind DNA. DNA, because of the sugar phosphate groups in the molecule, is a polyanion (i.e. negatively charged). DNA is avidly bound to cationic (positively charged) microbubbles. The gene is released when ultrasound energy cavitates the microbubble (**Fig. 2**).

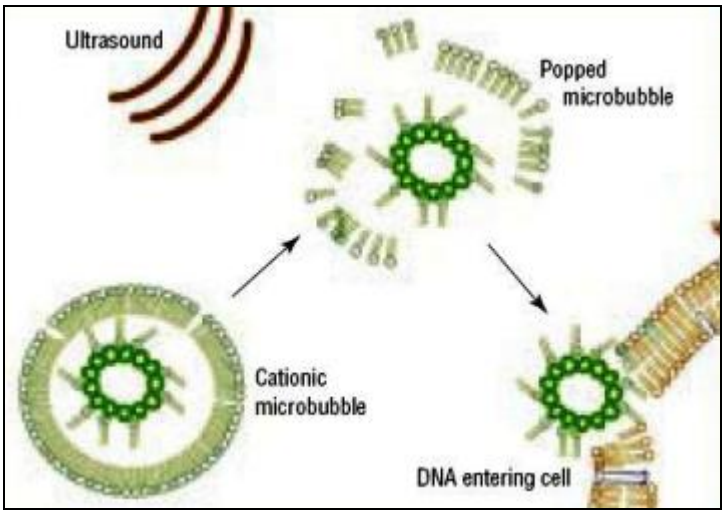

FIG. 2: ULTRASOUND SCAN OF LIVER USING

Levovist Microbubbles: The clinical use of viral vectors for gene therapy is limited because viral proteins elicit an immune response within the target tissue [23] and have been shown to cause an intense inflammatory activation of endothelial cells [24]. On the other hand, the nonviral delivery of vehicles, such as plasmids and antisense oligonucleotides, has been associated with a lower transfection efficiency and transient expression of the gene product [25]. The first published report of targeted DNA delivery was performed in 1996, using surface ultrasound and intravenously delivered microbubbles carrying antisense oligonucleotides [29].

In 1997, Bao *et al* [26]., described the use of ultrasound and albumin-coated microbubbles to enhance the transfection of luciferase reporter plasmid in cultured hamster cells. Since then, many studies have

confirmed the efficacy of ultrasound-mediated microbubble destruction for drug and gene delivery, both in vitro and in vivo [29, 30-31].

Shohet *et al*., demonstrated for the first time with an adenovirus vector that the ultrasound-mediated disruption of gas-filled microbubbles could be used to direct transgene expression to the heart *in vivo* [31]. They showed that intravenously injected recombinant adenovirus vectors encoding a beta-galactosidase reporter gene were successfully delivered to normal rat myocardium using microbubbles and transthoracic 1.3 MHz diagnostic ultrasound, at a mechanical index of 1.5, delivered at a burst of 3 frames of ultrasound every 4 to 6 cardiac cycles.

Of note, transfection was not observed if the adenovirus was administered in the same dose without microbubbles, or if the adenovirus was administered with microbubbles but in the absence of ultrasound. Importantly, using the same model the authors confirmed that plasmid transgene expression can be directed to the heart, with an even higher specificity than viral vectors, and that this expression can be regulated by repeated treatments [27]. Taniyama *et al*., have also shown effective transfection of a plasmid DNA to endothelial and vascular smooth muscle cells with albumin-coated microbubbles (Optison) and ultrasound [30].

In vivo studies demonstrated that transfection of wild-type p53 plasmid DNA into balloon-injured blood vessels was effective and resulted in significant inhibition of the ratio of neointimal-to-medial area, as compared with transfection of control vector. In contrast, transfection of p53 plasmid DNA by means of ultrasound without microbubbles failed to inhibit neointimal formation in the rat carotid [30]. In a recent study, Teupe *et al*., have documented efficient transfer of plasmids encoding either beta-galactosidase or endothelial nitric oxide synthase to the endothelial cells of conductance arteries with preservation of the functional integrity of the transfected endothelial cell layer after ultrasound treatment [28].

3. **Drug Delivery**: Microbubbles have also been demonstrated an effective technique for targeted delivery of drugs and genes [32-41]. Drugs can be incorporated into the microbubbles in a number of different ways, including binding of the drug to the microbubble shell and attachment of site-specific ligands.

   As perfluorocarbon-filled microbubbles are sufficiently stable for circulating in the vasculature as blood pool agents, they act as carriers of these agents until the site of interest is reached. Ultrasound applied over the skin surface can then be used to burst the microbubbles at this site, causing localized release of the drug [42-45]. This technique then permits using lower concentrations of drugs systemically, and concentration of the drug only where it is needed. This improved therapeutic index may be extremely advantageous in cases of drugs with hazardous systemic side effects, like cytotoxic agents. Albumin-encapsulated microbubbles have also demonstrated to adhere to the vessel walls in the setting of endothelial dysfunction [46]. This also may be a method of targeting delivery with microbubbles but without the application of ultrasound.

   On application of low frequency ultrasound, these microbubbles start oscillating & undergo a process of cavitation resulting in bursting or break up of the bubble, drug molecules if incorporated within the bubble are released by this process & these are useful in drug delivery.

- Two factors which are taken into account for drug delivery are:

1) **Incorporation of drug into Microbubbles *(*Fig. 3*)***: Drug molecules can be incorporated in a variety of ways within the microbubble as follows;

a) Drug molecules can be incorporated within the bubble.

b) Drug molecules can also be incorporated within the bubble membrane or shell material of the microbubble.

c) Drugs can also be attached to the shell of the microbubble (for eg. by noncovalent bonds)

d) These can also be attached to the microbubble surface via a ligand (for eg. avidin-biotin complex).

e) Also if the microbubble is made up of multiple layers it can also be incorporated within the various layers of these microbubble.

4. These microbubbles are proposed to cross the blood brain barrier.

Targeted microbubbles are created by attaching a targeting ligand (such as a monoclonal antibody or a peptide) specific for the desired (endothelial) marker onto the shell of the microbubbles. Targeted ultrasound contrast agents have been used to assess vascular pathology associated with several intravascular markers, including P-selectin, ICAM-1, GpIIb/IIIa, the αv integrins and other markers of tumor angiogenesis.

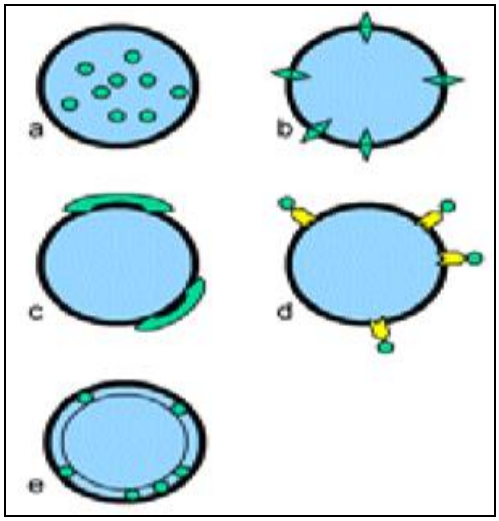

FIG. 3: INCORPORATION OF DRUG INTO MICROBUBBLES

2) **Drug release from Microbubbles (Fig. 4):** Microbubbles are also proposed to be carriers to be used in drug delivery. Microbubbles on application of ultrasound undergo a process known as cavitation which results in bursting or breakup of the microbubble on application of ultrasound. On cavitation the body fluids start insonating creating acoustic cavitation. Further, as the microbubbles oscillate they then give rise to small eddies, these eddies give rise to micro streaming or micro jets resulting in increase in permeability of the cell membrane & facilitating drug transfer across the membrane. Sometimes the microbubbles may also be phagocytosed by the cell membrane resulting in drug release. Another possible mechanism is fusion of the phospholipid microbubble with the phospholipid bilayer of cell membrane resulting in delivery of the drug or genes directly into the cytoplasm of the cell membrane. This is the proposed mechanism for gene delivery as it transfers the gene in close proximity of the nucleus. The following figure shows drug delivery via the microbubbles;

a. Drug delivery by cavitation (**Fig. 5)**

b. Drug release by cavitation as well as increasing the permeability of cell membrane

c. Phagocytosis of the microbubble by cell membrane

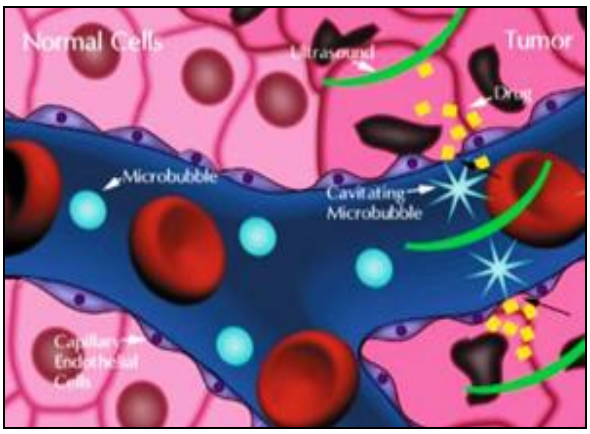

FIG. 4: DRUG RELEASE FROM MICROBUBBLES

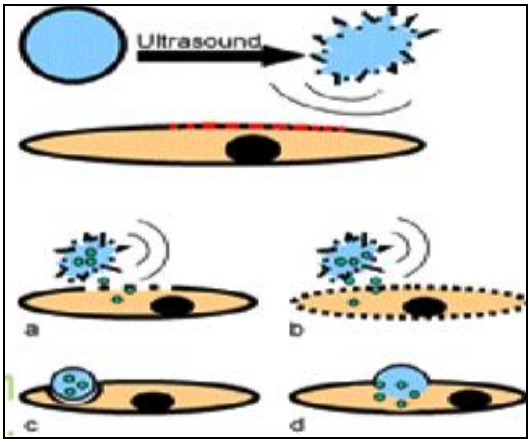

FIG. 5: DRUG DELIVERY VIA MICROBUBBLE BY CAVITATION

Advantages:

1. Since the microbubble delivers the drug in proximity to its target smaller dose of the drug is required as compared to conventional.

2. Also since the drug is released near its target & due to the small dose a decrease in the side effects is noticed especially for antineoplastic drugs.

3. By attaching various ligands these can be used for targeted drug delivery.

Mechanisms for Target Drug Delivery Using Microbubbles: Two possible strategies for delivering drugs and genes with microbubbles are emerging. The first consists on the ultrasound-mediated microbubble destruction, which is based on the cavitation of microbubbles induced by ultrasound application, and the second is the direct delivery of substances bound to microbubbles in the absence of ultrasound. Different drugs and genes can be incorporated into the ultrasound contrast agents. It has already been demonstrated that perfluorocarbon-filled albumin microbubbles avidly bind proteins and synthetic oligonucleotides [47].

In a similar way, microbubbles can directly take up genetic material, such as plasmids and adenovirus and phospholipid-coated microbubbles have a high affinity for chemotherapeutic drugs [47, 48, 49]. Furthermore, specific ligands for endothelial cell adhesion molecules, such as P-selectin and leukocyte intercellular adhesion molecule 1 (ICAM-1), can be attached to both lipid- and albumin-encapsulated microbubbles, which increases their deposition to activated endothelium [50, 51].

The mechanisms by which ultrasound facilitates the delivery of drugs and genes result from a complex interplay among the therapeutic agent, the microbubble characteristics, the target tissue, and the nature of ultrasound energy. The presence of microbubbles in the insonified field reduces the peak negative pressure needed to enhance drug delivery with ultrasound. This occurs because the microbubbles act as nuclei for cavitation, decreasing the threshold of ultrasound energy necessary to cause this phenomenon. The results of optical and acoustical studies have suggested the following mechanisms for microbubble destruction by ultrasound: 1- gradual diffusion of gas at low acoustic power, 2- formation of a shell defect with diffusion of gas, 3- immediate expulsion of the microbubble shell at high acoustic power, and 4- dispersion of the microbubble into several smaller bubbles. Cavitation of the bubbles is characterized by rapid destruction of contrast agents due to a hydrodynamic instability excited during large amplitude oscillations, and is directly dependent on the transmission pressure [52, 53]. It has been reported that the application of ultrasound to contrast agents creates extravasation points in skeletal muscle capillaries, and this phenomenon is dependent on the applied ultrasound power [37, 54].

High intensity ultrasound (referred to as a high mechanical index) can rupture capillary vessels, resulting in deposit of protein and genetic material into the tissues. Skyba *et al*., demonstrated in an exteriorized spinotrapezius preparation that ultrasonic destruction of gas-filled microbubbles caused rupture of micro vessels with diameter ≤ 7 µm (capillaries), with local extravasations of red blood cells [36].

Price *et al*., have shown that polymer microspheres could be driven as much as 200 µm into the parenchyma with this method [37]. The authors calculated that only a small number of capillary ruptures were required to deliver large quantities of the colloidal particles to the muscle. Using the same model of polymer microspheres bound to microbubbles and ultrasound, it has also been demonstrated that the ultrasound pulse interval and microvascular pressure influence the creation of extravasation points and the transport of microspheres to the tissue. Both were greatest when the pulse interval was around 5 seconds, which allows maximal microbubble replenishment within the micro-circulation after destruction by the ultrasound pulse [54].

The formation of pores in the membranes of cells as a result of ultrasound-induced microbubble cavitation has been proposed as a mechanism for facilitating the drug deposition. Taniyama *et al*., demonstrated the presence of small holes in the surface of endothelial and vascular smooth muscle cells immediately after transfection of a plasmid DNA by ultrasound-mediated microbubble destruction, using electron microscopic scanning [42]. It was then postulated that these transient holes in the cell surface caused by microbubbles and ultrasound resulted in a rapid translocation of plasmid DNA from outside to cytoplasm.

Mukherjee *et al*., demonstrated by electron microscopy of a rat heart performed during application of ultrasound, that disruption or pore formation of the membrane of the endothelial cells occurred with acoustic power of 0.8 to 1.0 W/cm^2 [46]. However, it was a lower intensity of ultrasound (0.6 W/cm^2) that caused more drug delivery with microbubbles. More recently, voltage clamp techniques were used to obtain real-time measurements of membrane sonoporation in the presence of albumin-coated microbubbles (Optison). Ultrasound increased the transmembrane current as a direct result of membrane resistance due to pore formation [55].

Another important therapeutic property of microbubbles is their increased adherence to damaged vascular endothelium. Albumin-coated microbubbles do not adhere to normally functioning endothelium, but their adherence does occur to activated endothelial cells or to extra-cellular matrix of the disrupted vascular wall, and this interaction could be a marker of endothelial integrity [46]**.** Because of this characteristic, the delivery of drugs or genes bound to albumin-coated microbubbles could be selectively concentrated at the site of vascular injury in the presence or absence of ultrasound application [56, 57].

CONCLUSION: Microbubble ultrasound contrast agents offer a wide range of potential benefits for both diagnostic and therapeutic applications. As a result, they have become the subject of a broad and rapidly developing field of research. The application of microbubble with ultrasound which gives a synergistic effect for drug/DNA delivery is currently in its infancy. The use of targeted microbubbles is a great step forward and has created various challenging therapeutic options, in treatment of various diseases.

Microbubbles have rapidly evolved from a diagnostic adjuvant to a possible therapeutic agent. At present, however, their behaviour is by no means fully understood, and consequently their effectiveness has yet to be maximized. Moreover, while no definite evidence of harmful effects has been obtained, there remain some concerns as to their safety. As these questions are resolved by advances in the subject, it is anticipated that more and more of the benefits of contrast agents will become realizable.

The aim of this review has been to examine the existing theoretical and experimental evidence to clarify the extent to which contrast agents are currently understood and to identify the most fruitful areas for future research.

In the coming years, this promising technique needs further development to make it available for clinical applications.

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