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Microbubble Therapies

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1. Introduction

Microbubble technologies have recently gained acceptance as radiological molecular imaging or perfusion agents. These intravenous ultrasound contrast agents consist of gas-filled microspheres stabilized by a 'shell' layer which interfaces with a surrounding solvent pool. Development in the form of encapsulation with complex biocompatible polymer shell consisting of proteins, lipids and surfactants has improved their stability and half-life significantly. These shell modifications have optimised the mechanical index of microbubbles, making them more resistant to compression rather than expansion.

The relatively inert nature of microbubbles contributes to a favourable safety profile and 'stealth' delivery profile. These indolent properties of microspheres are fundamental to their ability to function as targeted delivery vehicles for potential therapeutic modalities. Their small size (1-8 micrometre) enables unobstructed diffusion between the vascular and capillary compartments. Complete isolation of the active therapeutic components from normal native tissues and liberal exposure to the target disease tissue is the panacea of drug delivery.

Incorporation of lipopolysaccharide ligands adhering to selective cell surface epitopes, to the microbubble surface interface, is the principal method by which these vehicles can be precisely targeted. Selective bioactive material delivery into the target cell significantly improves therapeutic efficacy and can completely negate the adverse side-effects.

Externally applied focused ultrasound directed at the ligand bound microbubbles and target cells complex is the crucial final step ensuring optimal delivery. Application of sonication results in the oscillation followed by destruction of the bubbles and liberation of the bioactive compound; the high intensity focused acoustic energy disrupts the microbubbles. Passage of the active product into the intracellular space is facilitated through cellular sonoporation, at least in part as a result of acoustic cavitation of the target cell's membrane.

It has been demonstrated that microbubble technology can accommodation active constituents, hence be 'drug loaded'. Microbubble delivery technology has been utilised as a feasible drug delivery modality for gene therapies, cytotoxics, drugs and dyes. Co-administration of the microbubbles with the active product, without drug loading, has also been utilised to improve targeted drug delivery.

The premise of the clinically applicable microbubble delivered therapeutic agent delivery is an attractive proposition, but the clinical experience is still limited. Establishing the clinical benefits of microbubble technologies in the field of diagnosis has opened the door to a precise and accurate drug delivery vector. The proliferation in interest surrounding microbubbles has yet to meet its climax; the scientific, translational and clinical challenges of this exciting technology are still to be fully elucidated.

In this chapter we will consider the current understanding of the elucidated theory underlying this technology, and the potential clinical applications with drug and gene delivery. We also endeavour to summaries uncertainties and controversies.

2. The evolution of microbubble

High-intensity focused ultrasound is used routinely for the lithotripsy of the renal calculi. The ablation of the solid tumours and regions of tissue with high-intensity focused ultrasound is a less proven application. Since the inception of the microbubbles their application has grown exponentially with incremental advances in their formulations (Tinkov et al., 2009).

The dawn of microbubble technologies began with an innocuous *in vitro* observation that simple agitated saline enhanced ultrasound signal. This observation was demonstrated in cardiological and aortic ultrasound echocardiogram signal within the aortic root and chambers of the heart (Gramiak & Shah, 1968). The first generation microbubble contrast ultrasound agents were largely only applicable to diagnostic situations where additional opacification of the myocardium and coronary vessels is was necessary. The limited spectrum of clinical application was the result of the inherent characteristics of these first generation microbubbles agents (Kaul. 1997).

The fragile *in vitro* composition of these microbubbles is the result of unstable gas fluid interface and large bubble size. First generation microbubble agents consist of simple air bubbles dispersed in aqueous solution with an absence of a stabilising shell. The large bubble diameter ensured these agents do not to pass through the small capillaries, including the intrapulmonary circulation. This with combined with the exceptionally short life-span beds means that these first generation agents will never become detectable in within the left ventricle after intravenous injection (Kaul. 1997).

Later generations of microbubble agents have increasingly sophisticated colloidal gaseous/aqueous shells, which results in enhanced stability characteristics. The improved modalities of the later generations of microbubbles contrast dyes are now are recognised part imaging adjunct. The smaller size distribution of second generation enabled transpulmonary passage. Following intravenous administration of this generation of microbubble agents with stabilised solvent-gaseous interface they can become detectable within the left heart chambers. This generation is limited by unstable gaseous cores. Despite the composite shells the air cores have a tendency to dissolve in the blood within minutes. Subsequent generations replaced these highly soluble gaseous air nucleuses to overcome this inherent characteristic which limited their half-life (Voci et al, 1994).

Third generation microbubble compounds contained physiologically inert gas cores propagated their stability almost tripling half-life to at least 15 minutes. This generation has

extended the application of microbubble agents to any ultrasound amenable organs. Further manipulation of the agents will ensure further broadening of their scope of application (Tinkov et al., 2009).

The trends in the developments in later generations of microbubbles agents have moved away from the diagnostic efficacy and towards therapeutic modalities with drug carrying capacities. Precise formulation and engineering is necessary to ensure the efficacious delivery of specific active products (Pitt et al., 2004).

3. Drug targeting

The essential facet of effective targeted drug delivery is the ability to improve the therapeutic efficacy and minimise the quantity of circulating application of unbind active drug. The implications of this include reduction of the adverse effects and a significant minimisation of the effective required dosage necessary. The second property is paradoxically essential for the premise of the drug-loaded microbubbles to be efficacious because of the shell drug-loading capacity is limited. Because of this, only extremely potent agents with a low toxicity threshold would be amenable to this form of delivery. Poly/oligonucleotide gene therapy, cytotoxic, steroid, antimicrobials and active protein compounds may benefit from microbubble compounds.

It is necessary to expose the systemic circulation to the active compound bound to microbubble to ensure close enough proximity to the target organ. It is undeterminable and unpredictable to accurately the proportion of the active product liberated by focused ultrasound destruction at the desired target organ site. Of the remaining non-utilised microbubble complexes some will be eliminated or be dispersed remotely from the target organ. Well perfused organs, such as the renal (Koike et al 2005) and myocardial (Vannan et al 2002; Shohet et al 2000) systems will witness higher microbubble agent concentrations, making an ultrasound liberation of the active products more effective. Organs with inferior blood perfusion levels may not witness the necessary concentrations to make the targeted ultrasound microbubble destruction efficacious. Hence, the extent of blood supply alone may significantly ameliorate any possible projections of the efficacy of targeted therapeutic microbubbles (Tinkov et al., 2009).

The bodies' innate mechanisms can be harnessed to preferentially channel microbubbles to the desire target tissues. This approach of passive targeting relies on the innate non-specific macro- or microcellular characteristic. Reliance on these non-specific in vitro mechanisms means that these agents will be amenable to the influences of pharmacodynamics forces. Two relevant mechanisms include the particle clearance, aspects of the innate and even adaptive immune systems. An example of such a modality includes the effects of immuno-receptive microbubbles typically include those with phospholipid microbubbles containing phosphatidylserine and those which possess polymer-shells. Phagocytic immune white blood cells consume these antigenic microbubbles and convey these microbubbles to sites of active inflammation. Spontaneous non-macrophage mediate passive accumulation of ligand-containing microbubble agents via pathological or physiological enhancement in endothelial permeability and then retention has been found not to be possible (Bloch et al., 2004).

Active molecular targeting is essentially the incorporation of intentionally designed microbubble manipulations to ensure preferential affinity to the desire target tissues. Cell targeting can be achieved with a series of complementary immunoliposome (Siwak et al., 2002) and nanoparticle ligands (Shi et al., 2007) to specific cell surface receptors attached to the microbubble shells. Isolated or preferential expression of particular diseased cell-specific receptors on their surface provides a means by which targeting discrimination can be achieved. Complex formation between the complementary cell surface and attached microbubble shell protein ensure both selectivity and persistence of the vector to the target.

A major area of research has been into the active molecular targeting of microbubble agents are that they wholly reside within the intravascular compartment and will only come into contact with potential target markers situated on the endothelial wall. The shear force of vessel Blood-flow does significantly weaken microbubble ligand complexes-receptor binding complexes. Increasing the ligand density present on the microbubble surface has been found to be a reliable method by which to target binding can be enhanced. No further improvement in receptor binding is exhibited above a certain ligand concentration (Tinkov et al 2009). Supple ligand-microbubble spacer arms are a promising method by which endothelial wall receptor binding can be stabilised against shear blood-vessel forces. It has been theorised that the formation of the ligand endothelial wall receptor complexes induces microbubble core pressurization. The resultant deformation in the shape of the microbubble shell produces limited gaseous leakage and wrinkling microbubbles with outward protrusions, which enhance adhesion characteristics (Kilbanov et al 1999).

The laminar flow patterns present in larger vessels may ameliorate the possibility for significant target binding. The application of ultrasound beams can deflect circulating microbubbles on to the endothelial vessel walls enabling improved contact targeting. This property can help overcome the problem of sclerotic plaque targeting in larger vessels and the microbubble targeting inflammatory deep venous areas (Dayton et al 1999).

4. Therapeutic microbubble vectors

Specificity of drug delivery and targeting is the panacea pharmaceutical technologies. The biological barrier forms a worthy barrier to entry of the therapeutic compounds. These biological barriers include the cell membrane, capillary endothelium, and blood - brain barrier and vessel walls. These barriers can selectively transport molecules of varying molecular sizes. Ultrasound targeted microbubble destruction will enable the transport of 2-3 MDa, 6-8 μm, ~ 9 nm,~100 nm through the cell membrane, capillary endothelium (vessel diameter <7 μm), vessel wall diameter ~55 μm) and blood brain barrier respectively (Schlicher et al., 2006; Skyba et al., 1998).

A dichotomy exists between the desired characteristics necessary for the delivery of compounds to intracellular target sites. A simple innately lipophilic natured compound will preferentially access the intracellular compartment but, lacks the ability to freely transit in the aqueous haematological system to the cellular compartment. Uncomplicated hydrophilic agents can easily around the circulation, but without the benefit of selective specialised transport proteins are unable to enter intracellular compartments. Interiorisation of active compounds may be augmented through the application of ultrasound to cell-membrane barrier. Small molecular compounds, including polynucleotides and proteins can pass into

the intracellular compartment through the process of cavitation when facilitated with focused ultrasound. High energy ultrasound cellular damage is an undesirable effect of focused high-energy ultrasound. The acoustic cellular damage induced at these energies required to induce significant gases cavitation in vitro make this prospect clinically unreasonable (O'Brien 2007).

The application of ultrasound resonance field to microbubbles induces their frequency dependent oscillation. Microbubbles destabilisation and destruction can be induced with targeted high-energy ultrasound. Local delivery can be achieved through the liberation of the active product at the target site with the microbubbles acting cavitation nuclei. Significantly lower levels of the ultrasound energy are necessary when used in combination with microbubble agents.

If insonated microbubbles pass in the near vicinity of cellular membranes, the characteristics of the cell membranes may be altered and demonstrate the presence of sonopores. Their appearance may be the result of the proposed phenomena specific to microbubbles, these include microstream swirling, micro-jetting, the impact of enhanced ion-channel conductance, the formation of hydrodynamic shock waves or controversially free radical formation (12-24)(Miller & Quddus 2000; Miller & Pislaru 2002; Marmottant & Hilgenfeldt 2003; Barnett 1998; Guzman et al., 2003; Wang et al., 1999; Wei et al., 2004; Juffermans et al., 2006; Miller & Thomas 1993). There remain dissenters as to the whole validity of the cavitation theory, whilst others propose alternative ionic channel conductance mechanisms (Lawrie et al., 2003; Bouakaz A et al., 2006).

Bioactive substances may be able to infiltrate into the intracellular compartment through these sonate induced pores. These induced cellular membrane transit pores are transient (seconds to minutes) in their nature because of an endogenous vesicle-based healing responses resulting in these channels resealing. These healing processes are dependent calcium and ATP based processes. Their estimated sizes range from 30-100nm in size up to a maximum of a couple of millimetres (Pan et al., 2005).

It is questionable whether the formation membrane pores results in proportional changes in the nuclear membrane. Some studies have found that the viscous cytoplastic nature meant that the formation of these pores do not affect the nucleus. In contradiction to this one in vivo study has demonstrated that targeted ultrasound microbubble destruction resulted in nuclear uptake of rhodamine-labelled (Duvshani-Eshet et al., 2006).

It has been proposed that the ultrasound focused destruction of microbubbles increases the capillary permeability at the cellular microvascular level. The implication is that permeability can be enhanced active compounds can be enhanced. The reciprocal undesirable effect is that the application of focused-ultrasound microbubble destruction can result in local haemorrhage (Tinkov et al., 2009)

5. Drug loading

Manipulation to the microbubble formulation alters their physiochemical characteristics significantly, particularly the shell volumes and widths. There are four classes of microbubble structures with the capacity as gene and drug carriers. Potentially higher drug loads can be loaded into acoustically-active lipospheres (shell thickness [triacetin layer 500-

1000 nm], [soya-bean oil layer 300-700nm]), microcapsules(usually emulsification method, shell thickness 50-200 nm) and protein-shelled microbubbles (HSA-shelled microbubbles, probe-type sonication method, thickness 200-300 nm) possess larger shell volumes to which active products can be embedded. Whereas, Phospholipid-microbubbles (shell thickness 2-3 nm), because they have smaller shell volumes have better acoustic properties (Tinkov et al., 2009).

The most common approach for drug-loading in phospholipid and protein microbubbles is for shell surface loading. The loading process of the active agents within the entire volume of the shell is appropriate for microcapsules and on occasion protein composite microbubbles.

Microbubble drug coupling can be achieved through a number of the modalities. In essence microbubbles are gas-filled colloidal particle, which comprises a surfactant wrapping a flexible protein and polymer shell.

The outer shell surface can be used as a foundation by which active products can be attached. Drug compound attachment of the microbubble composite polymer shell can be utilise van-der-waal, hydrophobic or electrostatic forces. The association of active agents can be achieved through the association of smaller secondary shell surface anchor/carrier particles proteins or complexation to adhesive human serum albumin molecules. The nucleic acids, DNA and RNA can be attached to the outer microbubble shells with charge coupling.

Physical encapsulation in biodegradable polymeric shells offers the advantage of facilitation of the appropriate elimination from the systemic circulation. These biocompatible shells can consist of compounds such as gelatin, and may reduce immunogenicity and unfortunately ligand affinity.

Hydrophobic drugs molecules incorporated within can be submerged within the oily layer of lipospheres or intercalated between the monolayer of phospholipids.

A 'phase-shift' colloid emulsion vehicle is an elaborate system consisting of a perfluoropentane microemulsion system stabilised by biodegradable surfactants. This colloid emulsion is then loaded with the active compound possessing the appropriate physiochemical properties. The droplet-colloid composition is phase-shifted to bubble form through the application of heated sonication (Rapport et al., 2007).

6. Co-administration drug targeting

The drug loading of microbubbles is not essential to glean the benefits of their potential drug targeting properties. Administration of microbubble agents can be accompanied by the active product. Ultrasonic beam application at the target sites may facilitate the entrance of otherwise circulating active product into the interstitial compartment and into the intracellular compartment. Further study is necessary to study the therapeutic modality of this approach (Tinkov et al., 2009).

7. Targeted microbubble production

The process production and formulation begins with the identification of an amenable disease and correlated target disease specific receptor. A therapeutic mechanism and active

compound are prerequisites to ensure the efficacy of any potential enhancement with microbubble targeting. Comprehensive understanding of pharmacological, pharmacodynamics, physiochemical and dosages are essential to ensure realistic and cost-effective production of clinically applicable microbubble compounds. The active compounds must be amenable to the microbubble formulation process, which in many instances may denature and degrade biological compounds (e.g peptide, nucleic acid and monoclonal antibodies) and many synthetic compounds. Highly thermodynamic and immunogenical components be they active compounds or ligands are unsuitable for incorporation into microbubble formulations. For several molecular therapeutic compounds the impact of the formulation and manufacture process makes targeted microbubble modalities non-feasible.

The production process must incorporate uncompromising quality control based on sound reproducible manufacturing practices and in-depth understanding of both components and the required end-product.

Complexation of the active products to the shell utilises the relative physiochemical characteristics of the shell and the active product. Non-covalent bonding to the shell ensures appropriate strength to their loading and for ultrasonic liberation. Electrostatic charged albumin and phospholipids can be coupled to charged active products.

7.1 Phospholipids

Semi-synthetic flexible phospholipid thin monolayer possesses suitable acoustic properties. When present in solution the microbubble the hydrophobic portion orientated internally towards the gaseous centre and the electrostatic hydrophilic elements coming in continuity with the aqueous solvent. The condensation of the saturated fatty acid chains portions of the phospholipids structure imparts integral stability to the gel micelle shells.

There is a wide spectrum of the phospholipid compositions, including unsaturated fatty acid chains. Amalgamation of certain phospholipids and excipients can drastically alter the microbubble stability. An example, includes the integration of unsaturated chained phospholipids into the shell significantly reduces the stability of the microbubble shells. Alternatively, the steric stabilisation can be achieved with the inclusion of PEGylated phospholipid.

Other phospholipid compounds or phospholipid adjuvants impart an extended range of characteristics to these microbubble vectors. The inclusion of PEGylated or non-bilayer phospholipids can prolong circulation and gene payload transfection, respectively. Alteration of the condensed and disordered phase domains within the phospholipid layers changes the miscibility of components within the shell, with a result in its characteristics.

The heterogeneity of constituent components changes the degree of phase separation, which itself alters the capacity for drug/ligand loading, stability, and acoustic characteristics. Heterogeneity of phase separation can confer desirable characteristics to the microbubble formulations. One such advantage imparted by the incorporation of brush moieties is through the steric protection they can provide. The major disadvantage is the threat to formulation quality, which may be the result of phase separation in the lateral orientation. It has been suggested that the degree lateral phase separation may be the limited through adjustment of the fabrication process and through manipulation of the shells composition.

The condensation state is a crucial in determining the *in vivo* stability and is reliant upon on the temperature. To ensure that an agent has sufficient in vivo stability the physiological body temperature must be below the threshold phase-transition temperature (Borden et al., 2005).

When considering phospholipid constructs the stability is very dependent upon the hydrophobic carbon chain lengths. Longer carbon chained microbubble phospholipid shells possess less surface tension and an increased tendency to resist gaseous permeation. Increased viscosity with enhanced durability, but reduced acoustic characteristics with diminished echogenicity (Duncan P.B. & Needham D. 2004).

The ideal compromise to ensure optimal phospholipid microbubble stability and the existence of the minimal phase-shift characteristics, which are achieved by ensuring the equivalence of the various constituents phase-transition temperatures and phospholipid fatty-acid residue lengths. Avoidance of unsaturated phospholipid components possessing a large degree of conformational liberty would also improve stability. These 'loose' phospholipids destabilise the order of the otherwise densely packed monolayer. Microbubble monolayer destabilising and disorganising abundant surface electrostatic charges should also be avoided because they induce electrostatic repulsions within a layer orientation (Borden et al., 2006).

Non-conventional production of the phospholipid can be undertaken using a flow-focused method, which encompasses the passage of the inert gaseous core and shell material through a fine nozzled device and into a water reservoir.

7.2 Polymeric

In terms of polymeric microcapsule formulations characteristics are dependent upon material biocompatibility polymeric component weight, which possesses a direct relation to the shell thickness. Variability to the polymer constituents influences the stability, echogenic, time to destruction and acoustic characteristics (Forsberg et al., 2004).

Inappropriate selection of the incorporated of Proteinaceous excipients into the microbubble shell can also embarrass the functional stability of therapeutic microbubble formulations. Exposural factors, which may disrupt the self-association forces between shell components, which must be mitigate**d** against, include heat and free-radicals. Adhesive shell building factors include thiol-rich proteins including HSA. These forces are imparted through the sonication of the shell constituents' results in intensive cavitation and free-radical production, which result in the production of HSA linking thiol bridges. These bridges can also be formed with the addition of glutaraldehyde and formaldehyde during the shell formation process (Tinkov et al., 2009).

7.3 Mechanical agitation

The most frequently utilised method for the production of acoustically active lipsomes and phospholipid-shelled microbubbles. The initial phase of the process involves the formation of a thin hydrated phospholipid sheet, which is then either infiltrated with an alcoholic component or phase inverted by an alternate approach. A gaseous component is then pressed in the space above the liposomal dispersion and then agitated at least several

thousand oscillations every second. Loading of the active agents can take place at initial microbubble production or after this second gaseous core forming stage. To ensure a predictable desire loading pattern it essential to take steps to ensure loading of the active molecular compounds are bound to the liposomal shell instead of encapsulated within the water soluble liposomal centre (Tinkov et al., 2009).

The key to the production of the acoustically active liposomes revolves around the production of the appropriate micro-emulsion. This can be undertaken through the combination of the perpetual aqueous phase and injectable oil phase. Suitable constituents of the injectable oil phase include castor or triacetin oil. Successful emulsion formation requires the incorporation of co-solubilizers, such as synthetic block copolymers, because of the defoaming characteristics of the oil phase. The adequately lipophilic agent can then be dissolved and remain persistently encapsulated within the oily phase and acoustically active liposomes (Fang et al 2007)

This versatile modus operandi for the production of therapeutic microbubbles is advantageous when integrating delicate targeting ligands and drugs molecules. Successful utilisation of this drug-loading approach requires meticulous control of important variable production parameters. Components concentrations, viscosity, Agitation time and temperature must be all thoroughly controlled to ensure predictable characteristics.

7.4 Emulsification

Some phospholipid-shelled microbubble and many polymer shelled microcapsule formulations are produced with this oil-in-water freeze-drying emulsification production method. Some of the commonly used later generations of contrast enhanced ultrasound agents have been manufactured in this manner. The inner emulsion phase consists of a volatile solid compound (such as camphor), an organic solvent phase, which has the characteristic of being lyophilizable water-immiscibility and polymeric shell material. The water-water-insoluble benzene compound based solvents, such as toluene or p-xylene. The polymeric shell material consists of compounds similar to the highly biodegradable and biocompatibile co-polymer poly-lactide-co-glycolide (PLGA). The emulsion matrix is the residual after the removal of the volatile aqueous and organic phases remaining after the freeze-drying process. The gaseous cores are formed after reconstitution of the gaseous vial and emulsion matrix with the injection solvent medium. Coverage of these microbubbles with gelatin or albumin compounds included in the aqueous phase of this emulsification production method may significantly improve their biocompatibility profile (Schneider et al., 1991; El-Sherif et al, 2003; Short 2005).

Lipophilic active products can be easily accommodated within organic emulsion phase of polymeric shelled microbubbles compounds. Water-soluble drugs and biological agents can be accommodated in these formulations through encapsulation. Incorporation of hydrophilic compounds within the mixed-phase emulsion is undertaken through their dispersion within the shell containing the organic phase. The next step of the process involves the production of a double emulsion, which consists of water in organic in water emulsion. The lypophilization of the double emulsion is the final synthetic mechanism resulting in hollow shell drug-loaded microbubbles. The important factors that need to be

controlled include the emulsion droplet sizes; quantity and molecular weight of the shell material monomers (Tinkov et al, 2009).

7.5 Probe-type sonication

Low frequency and high intensity ultrasound is the commonest technique for the production of protein-shelled microbubbles and less commonly phospholipid microbubbles. Ultrasound induces exuberant convection streams to disperse the inert gaseous phase into the aqueous phase and produces the microbubble gaseous core. The process generates free radicals and temperature rises in excess of seventy degrees centigrade, which itself produces insoluble-shell microbubble shells. The proteins can be denatured with unveiling of their thiol-groups with the subsequent formation of covalent and non-covalent bridging bonds. Phospholipid microbubbles do not benefit from higher temperatures as their exceeding their phase-transition temperature can be deleterious to the formulation.

The most commonly utilised shell building component was HSA because of its exceptional affinity to a wide spectrum of active agents, including nucleic acid products. Drug loading to only the outer shell can be undertaken with the cell surface loading approach. This involves the incubation of the active consignment compounds to composed microbubbles. The disadvantage of this approach is a relatively low concentration of active product can be achieved through this process of surface absorption. This limitation can be overcome by the inclusion of the compound throughout the depth of the shell. This can be achieved through the application of sonication to the shell active product mixture prior to the microbubble formulation. Fragile active compounds may not be amenable to the entire shell loading approach as they will be damaged by the harsh cavitation and chemical and thermal stresses.

7.6 Spray-drying method

This versatile approach is essentially the formation of hollow particles through a spray-drying process. Encapsulation with the spraying technique is commonly utilised for the production conventional microbubbles. It provides an opportunity for a reliable method by which drug-loaded polymeric- and protein-microbubbles can be manufactured. This spray-drying method provides drug volume loading, a stable dried product and a final composition produced in favourable conditions.

Phospholipid-, protein- and polymer- microbubble formation utilises volatile ammonium derivatives and organic liquids (e.g. halogenated hydrocarbons) to induce pores and cavities in spray-dried particles. The commonly utilised HSA components of microbubble shells can be thermally stabilised and undergo chemical, but not free-radical cross- processes. The premise of this approach is the application of an evaporation process to a solvent mixture containing the dissolved shell material. The resultant saturation of the air-water interface with solvent-mixture forms an elastic film layer. On the droplets surface the shell components within the film solidifies, whilst the associated solvent completely evaporates. The particle formation is determined by the pressure induced fluid conversion into solid. The optimal acoustic characteristics are exhibited by microbubbles with the largest possible core cavity volumes. This spray-drying technique does not result in a uniform population of microbubble particle characteristics. The core cavity sizes can vary dramatically from

exceptionally large cavities with fragile shells to multiple small voids (Porter 1997; Lentacker et al., 2007).

8. Stability

The shell is fundamental for microbubble stability is maintains the integrity of the gaseous core by preventing the leaching of the gaseous core and minimising interface tensions. There is a complex gaseous balance reached between the solvent saturated gases and the gaseous core content. The passage of gas occurs from the core into the plasma solvent and simultaneously from the solvent into the gaseous core. This continues until the gaseous partial pressure equilibrium has been achieved. When the net gaseous movement is static the equilibrium osmotic gradient is zero. The aqueous insoluble Perfluorocarbon microbubbles vivo have negative osmotic gradient, so initially tend to increase in size because the net passage of aqueous dissolved gases into the gaseous core. Formulation techniques to improve stability include the incorporation of mixed gaseous cores, such core have been termed osmotically stabilized. Specifically engineered shells may reduce the degree of gaseous passage, but will be unlikely completely ameliorate it. Alteration of the gaseous core constituents will prove a more robust modification (Tinkov et al., 2009).

Air-filled microbubbles consisting of proteineous shells possess inferior echogenicity characteristics than fluorinated gas core microbubbles. The water insoluble perfluorobutane and sulphur hexafluoride consisting gaseous core microbubbles confer some superior properties to more advanced microbubble generations. Perfluorocarbon containing third generation microbubbles are disadvantaged by their tendency to absorb blood containing gases resulting in an inconsistent size profile and potentially reduced stability due to swelling. Perfluorocarbon and nitrogen mixtures may fare better and possess inherently superior pharmacodynamics properties (Tinkov et al 2009).

9. Quality control

Quality assurance requires a detailed understanding of microbubble properties. The formulation characteristics, which complicate analysis, revolve around the dynamic nature of microbubbles and their delicate quiescent phases. Their complicated characteristics include their unpredictable buoyancy and excessive sensitivity to shear-stress, pressure, temperature. These drug-loaded microbubbles are relatively complex composite mixture of molecular interacting structures, which can behave in a broad manner at any one time. Despite many of these unique properties many conventional assays can be utilised in an efficacious manner. Specific analytic tests are necessary to characterise therapeutic microbubble destructibility and echogenicity (Tinkov et al 2009).

9.1 Size

The terminological dogma underlying therapeutic microbubbles dictates size limitations of between 500 nm and 1μm in size. This size ranges enables optimal compromise between drug-loading in the gaseous core and echo signal. Nanobubble preparations have a greater predilection to take advantage of the permeability and retention effect. Nanoparticles more easily cross leaky capillary walls. Nanobubble units can have a tendency to coalescence into

a microbubble conformation, thus ameliorating this advantageous property of the smaller bubbles (Tinkov et al 2009).

Acoustic activity, pharmacodynamics and pharmacokinetics of microbubble preparations are highly dependent upon their size distrubtion. A Broad, mult- or bimodal distribution rather than a more conventional normal distributions have been found. The upper size limit should be between 5 to 10μm to enable safe clearance through the pulmonary vasculture. The size distribution can be sized and calculated by using electromagnetic impedance field calculation. As the particles pass through the 'coulter counter's' miniscule apertures located between two the complementary electrode the reading are produced. The detection and measurement of microbubble shadows cast as microbubble particles passage before a narrow uniform light source, is termed the 'light obscuration' method. The size range of this technique is has a maximum size tolerance of 400μm and requires low concentrations to be accurate. Laser diffraction approach can measure in the presence of high concentrations, is unaffected by buoyancy and can accurately assess any population distribution. Its particle size tolerance is 40-2 mm and requires calibration/optimisation for individual compounds. The dynamic light scattering method relies upon the 'Brownian motion of particles' is seldom utilised due its unreliability (Tinkov et al 2009).

9.2 Zeta potential

The zeta potential has important ramifications to both the intrinsic physiochemical characteristics of microbubble formulations and molecular pharmacokinetics. The active-product loading capacity, stability of the colloidal dispersion and the interaction with the endothelial cell wall properties can be usefully represented by the zeta potential behaviour. Preferable zeta potentials may significantly extend capillary retention time duration (Fisher et al., 2002).

The electrophoretic mobility can be determined by the degree of laser light scattering, which results from particle oscillation induced by an alternating electrical field. Using mathematical modelling the electrophoretic potential be translated into the zeta potential. The accuracy and precision of this presumptive calculation can be compromised by the inherent characteristics of microbubbles. The convection associated buoyancy compromises the reproducibility of original electrophoretic observations. Several environmental, physiochemical and electrochemical can affect the calculated zeta potential measurements (Tinkov et al 2009).

9.3 Mechanical properties

Critical to the modality of microbubbles is their characteristic acoustic behaviour on the application of an ultrasounic field. This behaviour is dictated by the shell characteristics. Advanced in the field of dynamic imaging has enabled the robust characterisation of ultrasound manipulated microbubbles.

The shell hardness plays a disproportionally large role in the determination of the ultrasonic threshold energy microbubble cracking threshold. Upon reaching their reaching their cracking threshold the encapsulating shell wall ruptures and the gaseous bubble is released

instantaneously leaving a partially maintained shell. Harder shells require in the order of twice to twenty-fold the ultrasonic energy to rupture and liberate is payload, in comparison to softer shelled formulations. These necessarily high energy levels and more pronounced harder-shell microbubble oscillations may have a deleterious effect upon the adjacent cells (Postma et al., 2004; Tinkov et al., 2009).

Soft more pliable shells, such as phospholipid shell components oscillate through the process of expansion and contraction. With the expansions lipid aggregates are expelled and then reseals. Significantly lower ultrasonic energies are necessary to rupture softer shells (Sboros et al., 2006; Tinkov et al., 2009)

9.4 Lateral phase separation

Microbubble shells can be significantly compromised by the phenomenon of phase separation. The molecular findings demonstrated within the shell during lateral phase-shift are an uneven distribution of constituents. The differentials in inherent forces include heterogeneous electrostatic binding and steric-shielding binding forces. Quality assurance methods are essential to ensure microbubble stability. Fluorescent dye tagged microbubble laser scanning confocal microscopy can employed to characterise the quality of dispersion and drug-loading (Borden et al., 2006).

9.5 Chemical integrity

Novel and broad characterisation techniques are necessary to ensure quality control of payload compounds incorporated within microbubble formulations.

Reversed-phase and normal-phase liquid chromatography are useful tools for quality assurance analysis of smaller molecular therapeutic compounds and small phospholipid compounds respectively (Lentacker et al., 2006; Hvattum et al., 2006).

10. Safety

There is relatively little clinical experience of the microbubble agents as a result the extent and spectrum of adverse events has not yet completely revealed themselves. Thus far, the clinical use of these agents as largely contrast ultrasound agents has proven to be safe. The complicity of the therapeutic adjuncts will probably introduce further adverse effects.

Myocardial exposure to microbubble agents may induce a physiological response of premature cardiac contractions. Microvascular damage, pectechial haemorrhage, free radical damage and single strand DNA fractures are also postulated side-effect of the focused ultrasound microbubble destruction process. In clinical practice these complications have been seldom encountered.

In regards to the reasonable ultrasound energies feasible for focused therapeutic ultrasound microbubble destruction, 'the principle of as low as reasonably achievable' has been deemed appropriate by radiological committee guidelines. For clinical ultrasound of microbubble agents the accepted safe mechanical index parameters are 0.05 to 0.5. For harmonic and non-harmonic clinical imaging modalities exceeding the mechanical index of 0.5 would result in microbubble destruction. The American Food and Drug

Administration (FDA) have set the maximum accepted mechanical index tolerance of 1.9 (Tinkov et al., 2009).

10.1 Immunogenicity

Although microbubbles themselves are not immunogenic, the incorporation of targeted ligands to their shells does render them immunogenic. Immunoreactive components are desirable to target delivery, but, undesirable for ability to attract unwanted immunological attention whilst in transit to the target site.

Conventional exposed spacer-linked or directly linked ligands loaded upon the gaseous microbubble cores may be inappropriately degraded or trigger an aggressive detrimental immunology response. Buried ligands within an overbrushed grafted polymeric-PEG layer may enable the camouflage of these immunoreactive elements. This stealth layer can then be unveiled and the immunoreactive layer underneath revealed with the application of focused ultrasound beam. The ultrasonic waves push the microbubbles towards the vessel walls and the polymer sheath is unbrushed and enables ligand binding to the specific receptor targeting sites. The caveat of these stealth particle modifications is reduced target receptor site affinity (Kilbanov et al., 1999).

11. Gene-loading microbubbles

Gene-therapies have proven an intriguing, but nonetheless frustrating proposition. Their potentially enduring therapeutic promise has been slighted by some critical inherent deficiencies. The absence of any effective conventional carriers has hindered the progression of more widely clinically applicable nucleic acid therapeutic options. The essential criteria an advanced gene carrier must possess an exceptional safety profile. The carriage of their genetic payload must be hostile and protect it from the otherwise deleterious effects of native enzymes, immunological elements and pharmacokinetic elimination. The aim is the controlled and timely liberation of the active agent followed by appropriate transfection of the target cells. Transfection is mediated by attenuated viruses, thus rendered incapable of replication and instigating disease. Amongst critics of gene-therapy options typical concerns over safety exist as with non-microbubble preparations. These safety concerns pertain to the risks of genetic mutations, pathogenesis and immunological reactions. Work utilising non-viral vectors aimed at excluding these viron-vector related risks has not yielded practical or efficacious alternatives. Alternative delivery systems investigated include microbubble, lipoplexes, electroporation and microinjection.

Genetic therapy delivery mediated through microbubble formulations is widely considered to be a safe prospect. The enhancement in transfected genetic material quantities is significant. In vitro models have demonstrated transfection magnitiude enhancement rates of 10-3,000 fold in comparison to naked plasmid DNA constructs alone. Despite the observation under in vivo conditions, which have demonstrated 1,000 fold transfection improvement, true proven in vivo transfection enhancement remains elusive. Serum nucleases are pose a significant risk upon naked and viron loaded nucleic acid compounds. The shielding effect of microbubbles has been proposed in microbubble constructs loaded with adenovirus-associated microbubbles (Tinkov et al., 2009).

Unlike other therapeutic opportunities the response to therapy rarely correlates with the quantity delivered. It is essential however, to deliver a threshold quantity to the appropriate area. The limited surface absorption binding capacity of plasmid DNA to produce maximally loaded gene loaded microbubble is in the order of approximately 0.001-0.005 pg/μm². Adjuvants can be utilised to increase the gene binding and loading properties. These include the use dipalmitoylphosphatidylethanolamine (DPPE) containing phospholipid microbubbles, which have a higher binding capacity to plasmid DNA. Layer-by-layer technique with poly-L-lysine may enable pDNA packing to decimal points higher than conventional microbubble loading. The absorbed loading of genes on to albumin microbubbles has been found not to enhance pDNA loading. Utilising a formulation approach a greater than 200-fold loading to entire shell volume has been reported. More recent developments are secondary-carriers associated microbubbles and the active nucleic acid components. These constitute nanoparticles, polyplexes, liposomes and lipoplexes. They have multiple functions, including enhancement of the transfection and microbubble strong shell attachment to the microbubble shell with improved loading capacity. They function through the condensation of large DNA molecules, thus protecting them from the action of serum nucleases. Entrance into the intracellular compartment involves both endocytosis and pinocytosis (Frenkel et al., 2002; Tinkov et al., 2009).

In vivo microbubble transfection delivery studies have been undertaken in organ systems already validated for diagnostic contrast ultrasonography. Vascular paradigms are particular relevant to study therapeutic microbubble compounds. Numerous pathological vascular paradigms have indicated that the duration of action varies from condensed intensive therapeutic windows to the extremes of a month. No meaningful conclusions can be drawn from study of these gene-therapy microbubble therapies for atherosclerotic or intimal disease. Ultrasound induced sonoporation and of the microbubble containing genetic-material enables delivery into the cytoplastic compartment. Nanobubble delivery does not seem to enhance entry into the nuclear compartment resulting in its deactivation/destruction prior to activity (Newman & Bettinger 2007; Liu et al., 2006; Unger et al., 2006).

Elucidation of formulation, pharmacodynamics and pharmacokinetic principles is necessary to definitively ascertain the modality of this novel disease-specific therapeutic option. Establishing such studies are complicated by the absence of standardised models with comparable measurable parameters. Also more modern genetic therapy incorporated microbubble formulations have not been studied to the same extent as co-administration preparations. This leaves the door ajar for more modern formulations fulfilling their yet unrealised potential (Tinkov et al., 2009)

12. Conclusion

Safe *in vivo* utilisation of microbubble contrast enhanced ultrasound agents is well established and validated. The idea of directly translating microbubble into routine used therapeutic treatments is oversimplification of the challenge ahead. The premise of diagnostic microbubble is with low administrated doses to produce a sustained and clearly visible echogenic signal. Whereas, with therapeutic microbubbles the all the associated paradigm variables still remain too uncertain. The quantities of both ultrasonographic

energy and microbubble dosage are many magnitudes greater, which imparts numerous complications. The most serious of these is significant unintentional tissue damage related to cavitation-inducing ultrasound beams. A pattern of cellular damage has been demonstrated on myocardial tissue between the upper safe ultrasonic energy limit and 2.5 times of this limit. Such a degree of irreversible cellular destruction will prove detrimental to organ function and subsequently result in significant morbidity. Despite these concerns, it has been proven that efficacious microbubble therapeutics used at the effective dosages with safely ultrasonography energies.

Further studies on all aspects of formulation, production and trials of therapeutic microbubbles are necessary as a precursor to establishing these agents as realistic therapeutic options. The promise in their therapeutic role may lie as co-administration agents to optimise selective uptake of the active agents. Therapeutic microbubbles may prove the 'missing-link' in fixing the inherent shortfalls of promising therapies, particularly in reference to gene therapy.

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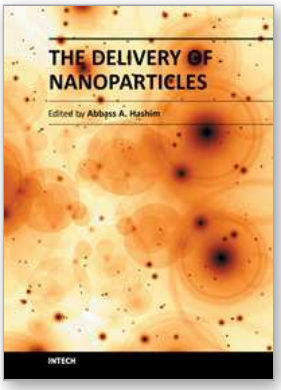

The Delivery of Nanoparticles

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Nanoparticle is a general challenge for today's technology and the near future observations of science. Nanoparticles cover mostly all types of sciences and manufacturing technologies. The properties of this particle are flying over today scientific barriers and have passed the limitations of conventional sciences. This is the reason why nanoparticles have been evaluated for the use in many fields. InTech publisher and the contributing authors of this book in nanoparticles are all overconfident to invite all scientists to read this new book. The book's potential was held until it was approached by the art of exploring the most advanced research in the field of nano-scale particles, preparation techniques and the way of reaching their destination. 25 reputable chapters were framed in this book and there were alienated into four altered sections; Toxic Nanoparticles, Drug Nanoparticles, Biological Activities and Nano-Technology.

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