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Article

Nanobubbles: A Novel Targeted Drug Delivery System

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Nanobubbles are nanometer size bubbles having different constituents of varying physicochemical characteristic for the inner core and outer shell. Nanobubbles are mainly fabricated to improve the stability, bioavailability and improve the biodistribution of the delivered drug to the specific targeted site. Their small sizes bubbles allow the possibility of extravasation from blood vessels into the surrounding tissues and ultrasound-targeted site-specific release with minimal invasiveness. Nanobubbles are developing as important contrast agents for imaging and carriers for drug delivery at targeted region. Sonication is the primary method for preparation of nanobubbles followed by thin-layer evaporation, high shear emulsification, mechanical agitation and coacervation or coalescence. With exposure to ultrasound/extracorporeal shock waves, the drug is liberated from the nanobubbles into the target cells. This review paper is an effort to reveal the different formulation development techniques briefly and varying shell and core content for developing nanobubbles.

Keywords: Nanobubbles. Target drug delivery. Gene therapy. Thrombolysis.

INTRODUCTION

Nanobubbles are gas carrying concavities in aqueous solution with the size range of below 1μm. Bubbles are round, globular particles with a shell and a gas-filled core structure that enables them to perceptibly dynamic properties (Unger *et al*., 2004; Ferrara, 2008; Sirsi, Borden, 2009). The shell mostly composed of lipids, polymers, proteins, surfactant and polyelectrolyte multilayer, whereas core base can be charged up with different gases, such as perfluorocarbon, carbon dioxide, sulfur hexafluoride and air (Figure 1) (Cavalli, Soster, Argenziano, 2016). Their shell constitution can ensure a significant impact on the half-life of a bubble, as it regulates the interchange of gas from the shell core to the surrounding medium. Indeed, shell thickness and elasticity are the contributing parameters in overall nanobubble stability (Delalande *et al*., 2012). A variation on chemical components of ultrasound contrast agents (UCA), acoustic pressure, fluid viscosity, ambient temperature and pressure, gas diffusivity, and size distribution can all affect echogenicity and stability of UCA which affects shell properties and stability of UCA which in turn affect the threshold of nonlinear bubble activity (Perera *et al*., 2018).

These nanobubbles (NBs) are sub-micron sized and they are under development with the aim to extend stability, bioavailability and to augment the biodistribution of the drug to be delivered at the targeted pathological region. Devilish of Microbubbles is not possible from the bloodstream due to its large size (Bisazza *et al*., 2011; Nomikou, McHale, 2012; Cavalli, Bisazza, Lembo, 2013). Nanobubbles pave the route for extravasation from blood vessels into surrounding tissues, thus improving delivery efficiency and localization (Cavalli, Bisazza, Lembo, 2013). The fabrication and initial application of nanobubbles have shown promising results in recent years, with improved stability and loading compared to microbubbles (MBs) due to the size range between 400-800 nm in diameter, as well as the achievement of system extravasation. Conversely,

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MBs are unable to leave the vasculature, even in solid tumors, which often have leaky vasculature and poor lymphatic drainage which leads to extravasation and retention of macromolecules, also known as the EPR effect (enhanced permeability and retention) (Maeda *et al.*, 2000; Oeffinger, Wheatley, 2004).

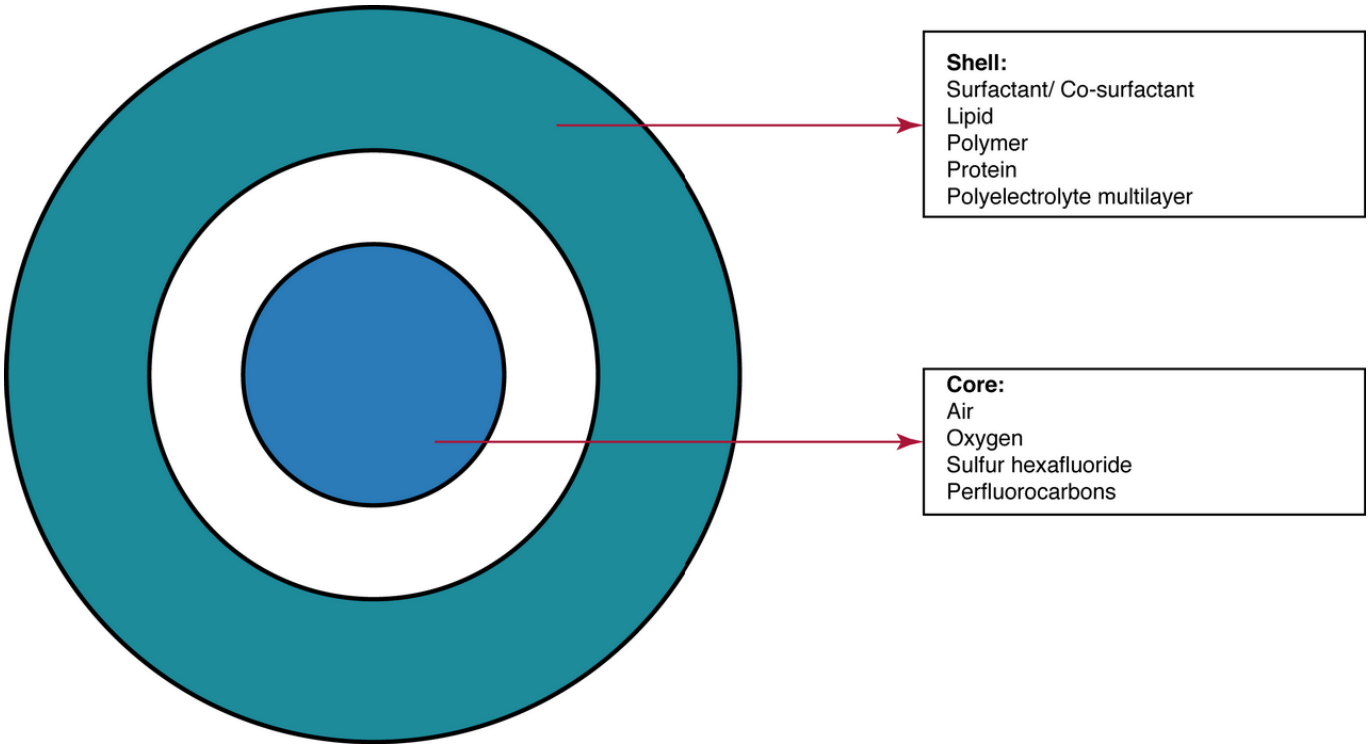

FIGURE 1 - Schematic representation of nanobubble structure.

In the view of enhanced nuclear envelope (NE) rupture, optical methods specifically, laser induced 'Photoblation technique' has shown an inflict damage or minute mechanical damage to the NE using vapour nanobubble (VNB)-mediated photoporation. This technique exploits the optical properties of sensitizing plasmonic nanoparticles such as gold nanoparticles (AUNPs) in significant enhancement of efficiency and throughput. Nanobubbles were initially fabricated as contrast agents, ultrasonic energy reflectors, inducing oscillations asymmetrically in the provision of exposure to acoustic fields in given diameters (Houthaeve *et al.*, 2018).

Ultrasound imaging has become a successful and well established diagnostic technique for clinical decision making (Frinking *et al.*, 2000). It is due to ultrasound imaging is non-invasive, less expensive and provides real-time images of soft tissue structures such as heart and blood flow without the use of ionizing radiation. Ultrasound beam reduces the toxic side effect of anti-cancer drugs used in cancer treatment (Liu, Miyoshi, Nakamura, 2006; Husseini, Pitt, 2008). The delivery of drugs to organs and tissues are affected by two main ultrasound effects including the cavitation and sonoporation effects. The cavitation effect results in the reduction of bubble size, while the sonoporation effect leads to uptake of the reduced bubble (Ayodele *et al.*, 2017). Therapeutic ultrasound was considered a tool for hyperthermia or thermal ablation of tumors. Ultrasound-assisted drug delivery system can help, the drug to penetrate through various tissues, change in permeability or absorption of the drug into the cells and tissue, converting specific drug into a therapeutically active form its inactive state (Wu, 1998; Husseini, Runyan, Pitt, 2002; Smith *et al.*, 2003; Dijkmans *et al.*, 2004).

A wide variety of applications for drug delivery with ultrasound are under investigation in numerous areas (Takeuchi, Sato, Kawashima, 2002). Recent advances in nanobubbles formulation in the treatment of specific targeted tissues with the application of US energy are summarized in the following the Table I.

TABLE I - Recent advances in nanobubbles formulation in the treatment of specific targeted tissues with the application of US energy

S.No.	Nanobubbles formulation	Targeted area	Therapy	Drug/gene	Reference
1	Oxygen loaded drug	Hypoxic tissues,	Fluid therapy	-	(Matsuki *et al.*, 2014)
		Bladder	Chemo therapy	Mitomycin-C	(Bhandari *et al.*, 2018)
		Hypoxic tumor	Epigenetic Regulation	-	(Bhandari *et al.*, 2017)
2	Drug loaded nanobubble	HeLa cells (Cervical cancer)	Chemo therapy	Doxorubicin	(Deng *et al.*, 2014)
		MDA MB231 (Breast cancer tumors)	Chemo therapy	Doxorubicin	(Gao *et al.*, 2008)
		Monoclonal anti-HLA-G antibodies	Chemo therapy	Methotrexate	(Zhang *et al.*, 2014)
		HepG2 cells (Hepatocellular carcinoma cells)	Chemo therapy	Sorafenib	(Misra *et al.*, 2015)
		LS 174 T cells (Human colon adenocarcinoma)	Chemo therapy	Porphyrins chemical pigment	(Bosca *et al.*, 2018)
		HL60/ADM cells (adriamycin-resistant cell line)	Chemo therapy	Doxorubicin and Ciclosporin A	(Ding, Patel, Zhang, 2018)
		Tumorous cells in lungs cancer	Chemo therapy	Pemetrexed and pazopanib	(Şanlıer *et al.*, 2018)
3	Nanobubbles based Gene delivery system	COS7 cell line	Nonviral gene delivery	pDNA	(Cavalli *et al.*, 2012)
		MCF-7 (human breast cancer)	Chemo therapy	Doxorubicin and P-gp siRNA	(Yang *et al.*, 2015b)
		Gliomas	siRNA transfection	Anti-apoptosis gene sirtuin 2 (SIRT2)	(Yin *et al.*, 2013)

(continues on the next page...)

TABLE I - Recent advances in nanobubbles formulation in the treatment of specific targeted tissues with the application of US energy

S.No.	Nanobubbles formulation	Targeted area	Therapy	Drug/gene	Reference
4	Targeted (Site specific) nanobubble	Human epidermal growth factor receptor type 2 (HER2)	Inhibits overexpression of HER2 gene by anti-ErbB2 Affibody	Biotinylated anti-ErbB2 Affibody®	(Yang *et al*., 2015a)
		HepG2 cell	Chemo therapy	siRNA (BCL-2) and Paclitaxel	(Yin *et al*., 2014)
		Nucleus pulposus cells in spinal intervertebral disc	Anticatabolic and cell apoptosis	Resveratrol	(Shen *et al*., 2018)
		HepG2 cells (Hepatocellular carcinoma cells)	Antiangiogenetic medicine	Apatinib	(Tian *et al*., 2018)

PRINCIPLE

Nanobubbles have the advantage of modifying the normal water characteristics. These bubbles are minute or more specific nanoscopic than normal bubbles and do not float and have reduced buoyancy as the diameter is small. Due to its longevity, physical stability, they do not readily dissolve. With established Laplace pressure equation, the pressure difference within the inner and the outer side of a droplet (or a bubble) can be denoted as:

$$\Delta P = P_{inner} - P_{outer} = \frac{2\sigma}{r}$$

Whereand are the pressures inner and outer of the bubble respectively with interfacial tension "σ" and "r" as the radius of the bubble. The US energy is responsible in activating liquid to the gas phase inside the nanobubbles through the Acoustic Droplet Vaporization (ADV) mechanism (Kripfgans *et al*., 2004). The Laplace equation of pressure is inversely related to the bubble size and hence small sized bubbles contribute to high-pressure values in comparison with larger ones. The bubble gets shrinks with an increase in Laplace pressure as inner gas quits the core, navigated by the pressure gradient and hence acceleration of gas intemperance rate, followed with the shrinkage of the bubble until the rupturing of the system. As the surface tension is responsible for accelerating the pressure for dissolution of the bubble by diffusion, dissolution rate of the bubble *in vivo* is solely controlled by Laplace pressure (Van Liew, Raychaudhuri, 1997).

APPLICATIONS OF NANOBUBBLE

Targeted nanobubble

Nanobubbles are also compliant for modifications at surface to augment the signals and selectivity about tumor, and suppress nonspecific toxicity. Targeting nanobubbles through modifications at surface is also considered an interesting theranostic approach. A novel in targeted nanobubble system is a most favourable tool in diagnosis and evaluation for Her-2-positive breast cancer treatment response. Thin-film hydration and sonication method was used in fabrication of PEGylated phospholipid shell NBs to which the covalent bondage is established by Herceptin molecules. The NBs-Her linkage provides long-acting contrast enhancement, without toxic effects as proved in earlier performed vitro and in vivo experiments. Importantly, it is proven with efficient penetration in tumor and were retained longer in Her-2-expressing tumors by in vivo results (Jiang *et al*., 2016).

Drug Delivery

Nanobubbles drug delivery is potentially being exploited in the delivery of anticancer drugs like doxorubicin in both *in vitro* and *in vivo*. These NBs reach and get assembled at the site of a tumor followed by their amalgamation to form microbubbles. Further, these microbubbles undergo distortion at the target site with intense ultrasound and hence release of the drug is achieved which results in accumulating the higher proportion of drug within the targeted cells with increased efficacy and reduced toxicity. Further exploration is necessary for these method's utility while treating various malignancies.

A polymeric nanobubble system to increase the doxorubicin sensitivity of cancer cells associated with US. The systems developed include perfluorocarbon nanodroplets stabilized by a biodegradable block copolymer wall (PEG-PLLA or PEG-PCL). With increase in temperature at about to physiological temperatures, the nanodroplets get converted into nano/microbubbles. As per the received sonic wave signals by tumor-directed ultrasound, cavitations occurs in doxorubicin-loaded nanobubbles and finally get collapse, to release the encapsulated drug and increasing the effectiveness in tumor chemotherapy *in vivo* (Gao *et al*., 2008).

Nanobubbles have also been used for treatment of Parkinson's disease. The authors demonstrated that nanobubbles can be used to delivery apomorphine, a particularly beneficial but unstable drug for treating Parkinson's disease, through the blood barrier (Hwang *et al*., 2009).

The approach like 'Photoablation technique' can be used to promote the influx of various macromolecules that are too large to migrate passively through the NE. Thus, by providing unprecedented control over nuclear compartmentalization, nuclear photoporation offers a powerful tool over nanobubble based drug delivery applications (Houthaeve *et al*., 2018).

Another approach *viz.* Sonoporation technology is having an application in the transfer of drug or gene into the cell or tissue in the targeted region (Fan, Kumon, Deng, 2014). Presently, they are also under investigations in relation with gene and gas along with drug delivery (Marxer *et al*., 2011; O'Neill, Rapoport, 2011).

(Suzuki *et al*., 2005) reported on the use of nanobubbles combined with US to permeabilise four types cancer cells and potentiate the cytotoxic effect of anticancer drugs (cisplatin and 5-FU). Cell sensitivity to cisplatin and 5-FU was effectively increased with nanobubbles and ultrasound.

Oxygen Delivery

Nanobubble can be utilized to create supersaturated fluids for oxygen delivery. In a previous study, it has been demonstrated and proved that oxygen nanobubbles are most effective therapy in reversing the hypoxia (Khan *et al*., 2018b). It also demonstrated that oxygen nanobubble could be prominent therapy in down regulation of an intermediate protein, hypoxia-inducible factor-1α (HIF-1α), with significant role in chemotherapy. Higher oxygen availability also favours the therapeutic efficacy of radiotherapy and photodynamic therapy (Khan *et al*., 2018a) and therefore, oxygen nanobubbles have many potential therapeutic applications.

(Matsuki *et al*., 2014) demonstrated that oxygen-filled nanobubbles can generate fluids with supersaturated oxygen that would not sufficiently accommodate the same to be safely infused into blood vessels. A normal saline solution containing oxygen-filled nanobubbles was found effective treatment in improvement of blood oxygenation. Thus, the use of oxygen-filled nanobubbles would be potentially effective and method of choice to improve blood oxygenation in disorders, ischemic heart diseases (IHD), anticancer chemoradiation therapies and in control of hypoxia related infection.

Theranostic Delivery

Another application is the development of a system that provides imaging support to therapeutic treatment. This approach, which is called theranostic, appears to be particularly effective in improving treatment of cancer or other important diseases at an early stage, as it can image the pathological tissues while also monitoring delivery kinetics and biodistribution of a drug, thereby obtaining important benefits in terms of tuning therapy and doses, and reducing adverse side effects (Shi, 2009;

Kelkar, Reineke, 2011; Lammers *et al*., 2011; Terreno, Uggeri, Aime, 2012).

Lipid nanobubbles for Ultrasound imaging detection have been proposed as an *in vivo* contrast enhanced system for imaging, and as a tumor drug delivery (Yin *et al*., 2012). Notably, the use of MRI detectable microbubbles for theranostic purposes has already been proposed. Moreover, PLGA micro/nanobubbles loaded with Gd-DTPA were fabricated as multimodal contrast agents for both MRI and US (Ao *et al*., 2010).

Gene therapy

Liposome-based nanobubbles and microbubbles are also under investigation for effective gene-based drug delivery with the application of non-viral vectors. Researches also have shown that ultrasound energy fused nanobubbles have shown improvement of gene transfer in diversified studies (Negishi *et al*., 2008; Suzuki *et al*., 2008).

Plasmid DNA encoding TNF-α, encapsulated in lipid NBs containing octafluoropropane (C3F8) gas, can be transfected to tumor cells in a spatial and temporal manner, as triggered by US exposure. This results in local production of TNF-α and thus causes antitumor action, including p53-dependent apoptosis activation, decrease in tumor vessel density and suppression of tumor size, but which reduces the acute toxic effects (Watanabe *et al*., 2010).

Thrombolysis

Various studies also indicate the application of nanobubbles in the removal of the clot in vascular tissues with induction of ultrasound. This process is widely known as sonothrombolysis. It is a non-invasive method causing less damage to endothelium (Iverson *et al*., 2008).

ADVANTAGES OF NANOBUBBLES

Till the date, chemotherapy is routinely being used as the prime modality in treatment of malignant neoplasms and substantial therapy in improving the rate of survival in case of cancer patients. In spite of that, further assessment is absolutely required to determine the efficacy of chemotherapeutic drugs to maximize drug toxicity in treating the cancer cells. The frequently found the associated adverse effect in such case is systemic toxicity. Moreover, the local delivery of chemotherapeutic drugs may minimise toxicity with increasing therapeutic dose at targeted sites and by lowering the plasma levels of circulating drugs. Ultrasound-targeted nano/microbubble destruction (UTN/MD) therapy has been widely used due to its non-invasiveness and targetability, as an effective drug delivery system (Zhou *et al*., 2019).

The ultrasound (US)-targeted nanobubble destruction (UTND) method has become a new trend as targeted drug delivery system to solid tumors which imminently lowers systemic drug exposure and enhances therapeutic efficacy. Thus, UTND has multiple significant advantages when compared with other drug delivery systems, Nanobubbles can be formulated easily by modified emulsification processes and employed as US contrast agents in visualising tumors (Tian *et al*., 2018).

Nanobubbles being the contrast agents may disrupt blood vessels and thus enhances the site-specific delivery of drugs. This could be the most effective approach employing the EPR effect in passive targeting of tumors. Moreover, NBs in combination with US could promote acoustic cavitation, improving cell membrane stimulation and ultimately permeabilization in drug uptake by tumor cells. The extensive use of the retention (EPR) effect with enhanced permeability in delivering drug at target tumor site is the motto that drug delivery by theranostics therapy involves. The EPR effect reasonably makes accumulate various types of nanomedicines in solid tumors, demonstrated by different studies. However, EPR is blamed as phenomenon with high variation due to tumor heterogeneity that ultimately results in delivering low drugs in clinical trials (Duan *et al*., 2020).

The earlier studies have thrown light on nontargeted NBs that are easily accumulated in the reticulo-endothelial system, causing lower drug concentration at the tumor site. To reduce systemic toxicity and increase therapeutic efficacy, it is crucial to construct drug-loaded and targeted NBs, carrying antibodies and peptides as tumor-specific ligands (Tian *et al*., 2018).

The formulations with echogenic bubble have wide applications in diagnosis of disease and as therapeutics. Therefore, nanobubbles were formulated and the evaluation of contrast agent was performed in determining property of nanosized bubble in ultrasonic imaging. Drug loaded NBs exhibit excellent ability to achieve ultrasound enhancement and effective drug loading/targeting. The release of drug from drug loaded-NBs at targeted site assure better efficacy with promotion of Ultrasound irradiation. The drug-loaded NBs could promote drug delivery to cells significantly and the process be analyzed with sigmoidal type pharmacokinetic curve. It further can be concluded that the formulation of nanobubble is most promising approach for both drug delivery enhancing. as well as ultrasound imaging (Wang *et al.*, 2010).

DISADVANTAGES OF NANOBUBBLES

The major drawback of the lipid-encapsulated NBs as a drug delivery vehicle is its low payload efficacy. To combat this, an oil shell can be incorporated to the interior of the lipid monolayer to enhance payload efficacy. The shell ingredients of NBs such as polydimethylsiloxane, Tween 80, polyethylene glycol stearate or polyvinyl pyrrolidone that were commonly added, reinforce the shell avoiding any gas escape and thus prolonging the liposome gas bubbles lifetime in the bloodstream. The avidin–biotin interaction method is frequently used to tether antibodies to micro/nanobubbles, leading to the development of a molecular targeting US imaging agent. However, avidin still has limitations due to its strong immunogenic character (Hamano *et al.*, 2019).

The use of cationic peptides or cell-penetratinge peptide (CPP) can enable ease for cellular internalization of therapeutic agents that assigned for interaction between positively charged carrier and the negatively charged plasma membrane. A CPP/cargo combination may inhibit the endocytosis pathway and translocate directly into the cells without consuming energy. Use of such CPPs is non-specific functional molecule that can penetrate any cell upon encountering it (Xie *et al.*, 2016).

Interaction of charged components of drugs could be easily coupled electrostatically with the shell when anionic/cationic lipids or polymers viz. electrostatic loading is the premature release of the drug cargo in the body after the intravenous injection. Indeed, blood components, like serum albumin could interact with the nanobubble shell favoring the displacement of the drug (Zelphati *et al.*, 1998).

NBs are Ultrasound-responsive drug delivery systems and the actual molecular mechanism behind the DNA damage induced by URDDS remains elusive and subtle and needs detailed study. (Furusawa *et al.*, 2012) found that Akt, a substrate of ataxia telangiectasia-mutated and DNA-dependent protein kinase (ATM/DNA-PK) when exposed to ultrasound, phosphorylated to the active form in U937 and Molt-4 cell lines without p53 (Zhao *et al.*, 2013).

Most of ultrasound-responsive materials require an ultrasound-responsive core (gaseous, PFC, or gas-generating). These ultrasound-responsive cores consume a lot of space in ultrasound-responsive materials (microbubbles, nanobubbles, or droplets), which makes lower drug/gene-loaded contents, and decrease the amount of drug/gene delivered to diseased tissues, and eventually lead to limited therapeutic efficacy. NBs induce cavitation by using ultrasound intensity in order to promote effective release of drugs/genes from nanomaterials base. But high intensity ultrasound may cause damage to neighbouring healthy cells. The rapid collapse of bubbles and hence rapid release of the drug/gene loaded in the bubbles is caused by high-intensity ultrasound waves induction, that may or may not meet the specifications for sustained release of certain drugs (e.g., insulin) (Cai *et al.*, 2020).

NBs employ US-energy and most important shortcoming of this energy is that it strongly attenuated or resisted by bone. The larger surface area phase arrays and other information gathered from sophisticated imaging techniques may rectify the ultrasound distortion of waves produced by the skull in exposing the brain tissue by means of trans-skull ultrasound (Zhao *et al.*, 2013).

Yet, the ultrasonic parameters are the noticeable issues. Any biological tissues may get damaged by

low- and high-frequency ultrasound, when heat related to induced sonication higher and the pore generation on cell membranes is not reversible (Mehier-Humbert *et al*., 2005). Hence, the duration and intensity of ultrasound sonication should be controlled. It also invented that pulsed-focused and ultrasound induced opening of the blood–brain barrier which also was accompanied by increased expression of heat-shock protein 70 (HsP70), interleukin-1, interleukin-18, tumor necrosis factor-α, and inflammation of brain tissues. It ultimate suggests that use of ultrasound-responsive materials in drug/gene delivery to the brain system should be performed with utmost care and extreme caution (Cai *et al*., 2020).

CLINICAL TRIALS EXAMPLES

Micro/ Nanobubbles have been applied in drug delivery in clinical trials as proven ultrasound-responsive materials (Hynynen *et al*., 2001; Dimcevski *et al*., 2016; He *et al*., 2016). The controllability of delivering the cargo like drugs and gene materials with ultrasonic switch and visualization of treatment was promoted by results of confirmed clinical trials.

MB/NBs as ultrasound contrast agents have been used in clinical for decades (Stride, 2015). Extensive pre-clinical studies also assure that the combination of microbubbles and ultrasound may result in enhanced drug delivery or gene expression at specific and selective sites (Martin, Dayton, 2013). Preclinical study in xenograft mouse models demonstrates that the anti-tumor effects of doxorubicin can be enhanced and at the same time the drug-induced heart damage in ATC be prevented by the combined treatment with doxorubicin-loaded glycol chitosan NBs and ESWs (Marano *et al*., 2017).

Several pre-clinical and clinical trials reported that apatinib, a novel and selective inhibitor of VEGF receptor 2, was efficient and safer for patients with advanced HCC as the first line therapy. A phase I study was undertaken for patients bearing advanced solid tumors, and turned with the maximum-tolerated dose of 850 mg once daily. In phase II study, patients with metastatic gastric cancer displayed that apatinib improved PFS obvious in comparing with placebo. The adverse effects included hypertension, fatigue and hand-foot syndrome, which were well tackled (Kou *et al*., 2017). In the recent clinical trial, (Wang *et al*., 2019) examined the beneficial effects of nanobubbles water applied on curcumin extract enhancing explosiveness without a training program. Nanobubbles water curcumin extract (NCE) supplementation may be helpful for athletes with antifatigue effect and in performing explosiveness or heavy -type exercises including overall physiological protective effects. More specifically, it reduces injury risks in case of women performing drop jumps.

FABRICATION OF NANOBUBBLE

Nanobubbles are fabricated predominantly by sonication, mechanical agitation, coacervation or coalescence thin-layer evaporation, and high shear emulsification techniques. The different processes involved in the construction of nanobubbles are given schematically illustrated in Figures 2, 3 and 4. Nanobubbles are currently under investigation in their application in targeted delivery of drugs, proteins and nucleic acids subjected to ultrasound exposure. The drug of choice is loaded or encapsulated in the inner core and with irradiation of ultrasound/extracorporeal shock waves the drug liberated from the nanobubbles into the target cells.

Different nanobubble formulations reported earlier are discussed and categorized here, based on inner gas used, there *in vitro*/*in vivo* behavior, interaction with ultrasound/extracorporeal shock waves (ESWs) used for delivery of drugs to target region, and their use for imaging, targeting and controlled the release.

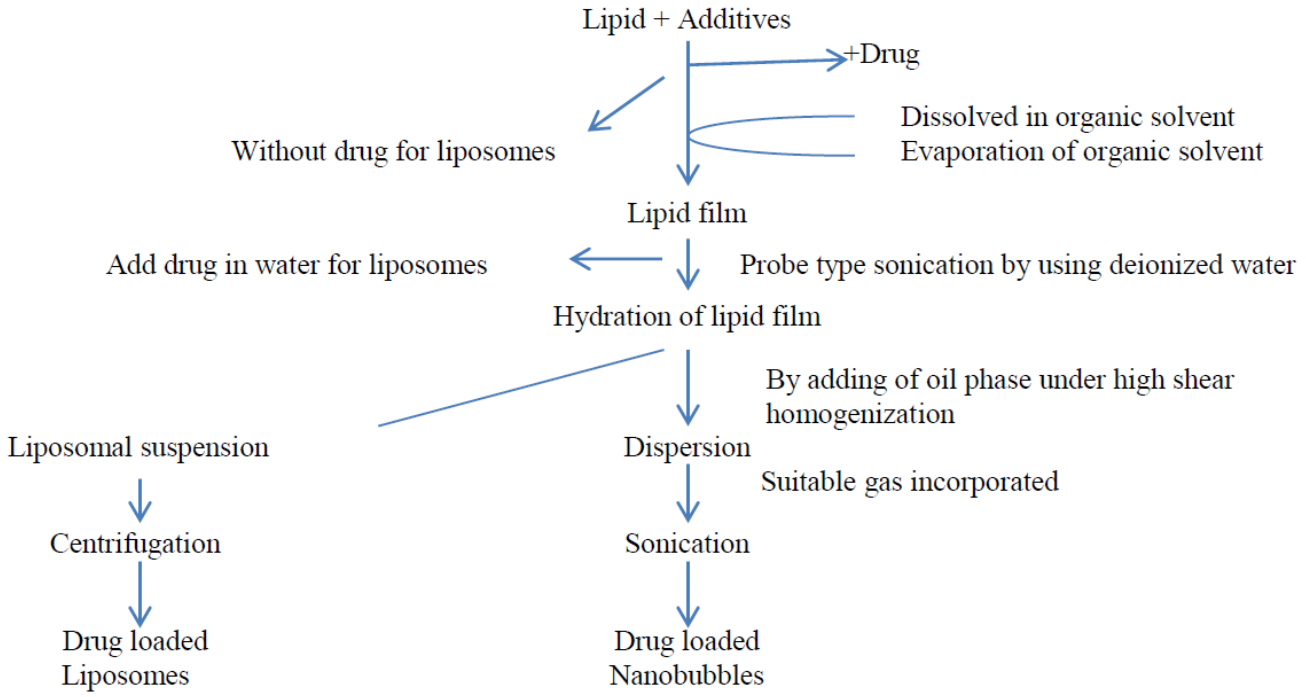

FIGURE 2 - Mechanical Agitation Method.

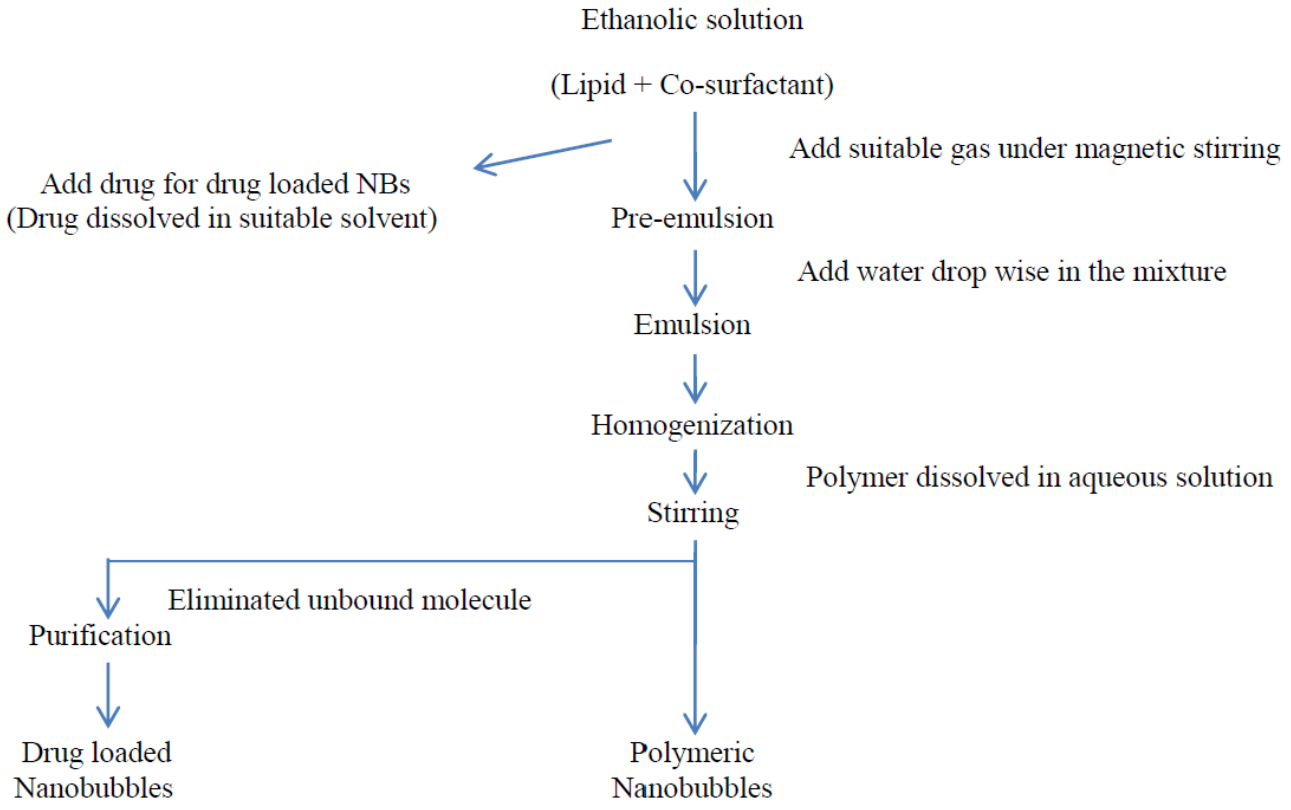

FIGURE 3 - High Shear Emulsification Method.

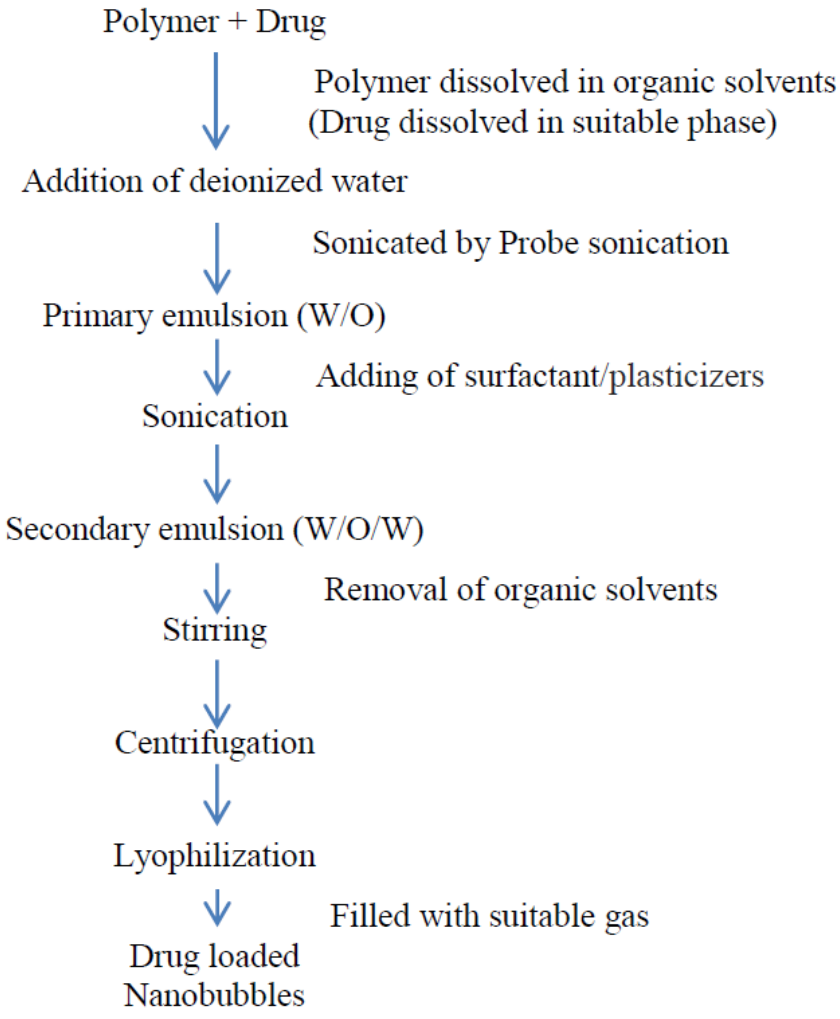

FIGURE 4 - Probe-Type Sonication Method.

Core Gas Based Nanobubbles

The various gases have employed in the preparation of gas filed nanobubble such as air, perfluorocarbon and sulfur hexafluoride. As per previous studies reported in the microbubbles designing, first-generation microbubbles were nothing but air-filled micro-cavities, with mean diameters of 1–8 μm which make them compatible to pass through the pulmonary capillaries. However, such air-charged microbubbles expelled from the circulatory system very rapidly cause of low resistance at different arterial pressure gradients and due to a greater extent of air solubility in blood (Kabalnov *et al.*, 1998). Therefore, different divergent agents as second-generation agents more specifically hydrophobic gases with high molecular weight *viz.* perfluorocarbons or sulfur hexafluoride has investigated. In this review, various gas-filled nanobubbles are discussed in Table II shown examples of various nanobubble used for drug delivery.

TABLE II - Various nanobubble used for drug delivery

S.No.	Core	Shell	Drug	Nanobubbles Applications	Shell excipients	References
1	Perfluoropentane	Lipid	Apomorphin	Therapeutic	Hydrogenated soybean phosphatidylcholine (SPC, Phospholipon® 80H), Coconut oil, and cholesterol.	(Hwang *et al*., 2009)
		Lipid + Polymer	Doxorubicin	Therapeutic	Glycol chitosan, Epikuron 200® (soy lecithin containing 95% of dipalmitoyl phosphatidylcholine), and Palmitic acid.	(Marano *et al*., 2016a)
			Prednisolone phosphate and a Gd(III) complex	Theranostic	Epikuron 200® (dipalmitoyl phosphatidylcholine 95%), Palmitic acid, Pluronic F68, and Chitosan (low molecular weight, 50–70 kDa, DD = 75–85%).	(Cavalli *et al*., 2015)
			Vancomycin	Therapeutic	Dextran sulfate, Epikuron 200®, and Palmitic acid.	(Argenziano *et al*., 2017)
			Taxanes (Paclitaxel and Docetaxel)	Therapeutic	Glycol chitosan, Epikuron 200®, and Palmitic acid.	(Marano *et al*., 2016b)
		Polymer	Doxorubicin	Therapeutic	PLGA, PVA	(Meng *et al*., 2016)
2	Air	Polymer	Doxorubicin	Therapeutic	PLGA, PVA	(Deng *et al*., 2014)
3	Sulphur hexafluoride	Lipid	Coumarin-6	Theranostic	Soybean lipid, Tween 80, Cholesterol.	(Wang *et al*., 2010)

Perfluorocarbon-based nanobubbles for drug delivery

Lipid-based nanobubbles

Apomorphine loaded on lipid-based nanobubble

Apomorphine was prepared using a perfluorocarbon and lipid-based a nanobubble which is aimed for the sustained type drug delivery. Drugs like apomorphine are known for inherent instability, low oral bioavailability and with a very short half-life which was required a suitable drug delivery carrier for targeting this drug at the specific site. These nanobubbles helped in the safety and enhanced stability of apomorphine (Li, Danhof, Bouwstra, 2001; Subramony, 2006; Hwang *et al*., 2009). Apomorphine microbubbles are unstable with the mean diameter of 1–6 μm which is too large for

their intravascular applications (Suzuki *et al*., 2007). The fabrication of nanobubble is depicted in Figure 2. The increased oil and perfluoropentane content ratio result in an enlarged size nanobubbles while an additional increase in the emulsifier content like cholesterol aids in lowering the particle size of the proposed nanobubbles. The increased value of oil and perfluoropentane helps in minimizing the surface charges of nanobubbles while cholesterol has zero effect on surface charges. Cholesterol helps in minimizing surface tension in contrast to oil and perfluoropentane which does not change the surface tension significantly. The nanobubbles loaded with high perfluorocarbon (PNs) protect the apomorphine base. However, no specific effect was studied in perfluorocarbon nanobubbles having high oil content in comparison with free form. High oil content PNs shows very moderate drug delivery rate, in comparison with the non-ultrasound effect and hence drug release percentage is decreased. Incorporation of ultrasound increased 3.9-fold of drug release when compared to the other formulation at the end of the experiment (8th hours). The clinical connection of this is to avoid dose repeatability of apomorphine which could be much favorable in those patients who require per day 10 to 15 injections (Ugwoke *et al*., 2000). The current studies also prove that the high percentage of oil or perfluoropentane system indicated lower drug release profiles in comparison to the low percentage oil and perfluoropentane in case of the apomorphine HCl and also for its base form. The hemolysis percentage decreases with the increased percentage of oil and perfluoropentane since almost of all phospholipids get occupied at the interface with the emulsified form within the inner phase containing a large volume with a consideration of the hemolysis results, the developed PNs in present study work to be considered as biologically safe and with minimal toxicological risk.

Lipid and polymer-based nanobubbles

Doxorubicin

Doxorubicin was prepared using perfluorocarbon and glycol Chitosan-based on nanobubbles was aimed for the controlled pattern of drug release at the targeted site. Nanobubbles filled with perfluorocarbon gas have used previously for preparation of contrast agent in consideration of their high stability and inert biological nature. Furthermore, its lower water solubility extent reduces the dissolution rate of gas from the inner bubble core into the blood circulation, and hence ultimately stabilizes the system with increasing the *in vivo* lifetime of the bubbles. Recent studies also have shown that the chitosan shelled perfluoropentane cored NBs have recently designed as agents for theranostic in nano-formulations (Cavalli *et al*., 2015; Marano *et al*., 2016).

These nanobubbles, with amalgamation of doxorubicin in anaplastic thyroid cancer (ATC) Cell lines and enhances its anti-tumor activity. In this method, extracorporeal shock waves (ESWs) are widely employed in the delivery of drug at the target site. Extracorporeal shock waves (ESWs) are nothing but acoustic waves which are used frequently in urology for lithotripsy (Jocham, Chaussy, Schmiedt, 1986). ESWs have to be concentrated with high precision in detail to estimate it's permeability through the plasma membranes (Constantinides *et al*., 2000; Fang *et al*., 2006; Ingram, Priston, Sewell, 2006). Doxorubicin also interferes with normal cells causing several adverse effects, among which cardiotoxicity is prominent. Notably, the severity of these effects and their incidence are dose-dependent (Carvalho *et al*., 2009; Chatterjee *et al*., 2010). These nanobubbles help in reducing the cumulative dose and targeting the drug at the tumor region. Doxorubicin-loaded nanobubble preparation as shown in Figure 3. For tuning the formulation, two different types of fluorescent glycol chitosan nanobubbles formulation was carried out.

The empty nanobubbles and the drug-loaded nanobubbles presented an average diameter lower than 500 nm with a positively charged surface. Doxorubicin-loaded nanobubbles do not significantly alter these values, in the presence or absence of an ESW treatment when compared to a doxorubicin hydrochloride aqueous solution, used in the therapy. After 3 hours, only 9.2 % of the drug was released in the absence of ESW with 40.3 % of free doxorubicin, also no initial burst effect

was observed showing NBs stability. Interestingly, doxorubicin was released to a greater extent following ESW treatment with 500 pulses at 0.59 mJ/mm2. Notably, after 3 hours, ESWs generated an increase of about 70 % of free doxorubicin nanobubbles without ESW treatment. Empty Nanobubbles affected cell viability both CAL-62 and 8305C cell lines and Glycol Chitosan Nanobubbles shells had no significant effect on cell viability. Cytotoxic effect of doxorubicin in both CAL-62 and 8305C cell lines only at levels hardly obtainable in patient receiving the doxorubicin dose of 60 mg/m^2 with a GI_{50} at 48 hours of 1.57 μM in CAL-62 and 3.93 μM in 8305C cells, ESW treatment increased the cytotoxic effect of the free drug with decrease in GI_{50} in both cell lines.

The combination of ESWs with doxorubicin-loaded nanobubbles had the highest cytotoxic effect. The cytotoxicity of CAL-62 treated with negative nanobubbles in combination with free doxorubicin and ESWs treatment was considered as more cytotoxic when compared to the free drug. However, combining ESWs with free doxorubicin plus empty nanobubbles was less effective than using ESWs with doxorubicin loaded into Nanobubbles. Empty nanobubbles were found to be safe in rat cardiomyocytes H9C2 cells as they entered the cells at a level comparable to that observed in cancer cells. The drug-loaded nanobubbles enhance the overall cytotoxic effect in comparison to the free drug. Mechanistically, ESWs triggered the intracellular release of drugs from NBs out-turned with the highest nuclear drug content.

Prednisolone phosphate and a Gd (III) complex

In this case, prednisolone is being loaded in chitosan nanobubble using perfluoropentane as an inert gas. This stable theranostic chitosan-based nanobubble contains the MR imaging gadolinium diethylenetriaminepentaacetic acid (Gd-DOTP) along with the drug (Prednisolone phosphate) companions have helped to reduce adverse effects of anticancer drugs and achieve prolonged drug release. The earlier research studies suggested that the lipid nanobubbles for US imaging detection were an application for the *in vivo* distinct enhanced imaging of tumor type tissues in essence of drug delivery field (Yin *et al*., 2012; Cavalli *et al*., 2015). The MRI detectable MB's are having application for theranostic purposes has already been proposed. Gd-DTPA loaded microbubbles of PLGA were fabricated as contrast agents for several modes both US and MRI (Ao *et al*., 2010). Albumin-based microbubbles charged with decafluorobutane gas and accommodating Gd-DTPA linked covalently with Human Serum Albumin (HSA) as a model protein drug was employed to enhance the diagnosis of aggravated sites within the vascular vessels (Anderson *et al*., 2012).

The preparation of theranostic nanobubbles is depicted in Figure 3. In order of tuning the sizes of formulation, two distinct co-surfactants was tested-tetradecylphosphoric acid (C14) and palmitic acid (C16). Prednisolone phosphate (PLP) and Gd-DOTP do possess distinct physicochemical characteristics and may have futuristic application in various areas of the nanobubble system. Being an amphiphilic molecule, PLP is detected mostly at the interface with the perfluoropentane core and the negative charged Gd-DOTP complex is bound electrostatically to the cationic surface of the nanobubble. Chitosan was a preferred as an essential component of the shell of nanobubble due to its better biocompatibility and the polycationic character that makes it as a unique compound to generate stable supramolecular ion pairs along with anions (Dash *et al*., 2011). Among two non-toxic co-bound slightly stronger to palmitic acid-based nanobubbles. All the preparations showed polydispersity index values ranging from 0.12 to 0.27, with a narrow dispersion observed in C16 containing nanobubbles.

Interestingly, C16 preparations showed a slightly smaller size (around 10%) than the C14 containing system, regardless of the presence of Gd-DOTP. Further additional experimental support to determine the effective binding of Gd-DOTP to the chitosan NBs were obtained by determining the zeta-potential. Instead, the presence of the anionic Gd-complex causes in overall neutralisation of partial charge of the bubbles, with a minimization of the zeta potential of ca. 30%; as the Gd-DOTP complex is being neutralized with help

of specific positives charges of the NBs, and thus the residual charges responsible for the established stability possessed by the nanobubbles.

Based on the enhanced imaging performance of MRI/US, smaller size, and improved polydispersity index, palmitic acid-based chitosan nanobubbles was the formulation of choice for loading PLP. Pluronic F68 is a non-ionic copolymer type amphiphile based on ethylene and propylene oxide units extensively employed in pharmaceuticals. The presence of Pluronic F68 in the formulation is to stop the NBs aggregation through steric stabilization that has been dominated by the effect of solvation. The chitosan-based nanobubble formulations disclosed good physical stability with incubation with the plasma at 37ºC for 3 hours, without deducing morphology or size changes and aggregation phenomena. This behavior is in proportionality with the presence of perfluoropentane core which would be still in a liquid state. This stabilization is a response of the reduced size of the nanodroplets and the existence of a phospholipid monolayer at the interface of the system.

The insonation parameters are not having any role in disrupting the nanobubble structure, as disclosed by maintaining low and controlled release kinetics. Preferably, the physical stability of the theranostic NBs could be visualized even after 5 minutes of insonation. The existence of lipid monolayer would be the significant factor for maintaining the structural preservation of the overall system after insonation.

Vancomycin

Vancomycin was loaded in dextran sulfate shell and perfluoropentane cored nanobubbles. This nanobubble was aimed at local delivery with the potential treatment of skin infections, with prolonged release at the site of the target region and had better stability. Previous studies reported the vancomycin with poor absorption via gastrointestinal tract along with a poor oral bioavailability which was often recommended with low intravenous infusion as another feasibility for drug administration, even though vancomycin instability in aqueous solutions at 37°C was a significant obstacle in rapid suppression in drug efficacy (Mawhinney *et al.*, 1992; Raverdy *et al.*, 2013; Argenziano *et al.*, 2017). Following parenteral administration displays a slow mode of action, a complex concentration-time profile, and a low penetration of tissues (Vandecasteele, De Vriese, Tacconelli, 2012). Moreover, systemic Vancomycin (Vm) administration might be the reason for several adverse effects (Vidal, González, Fuente, 1992). Vm topical application that would be much safer than the systemic administration is currently in the limit by several factors such as skin barrier properties and poor drug permeability (Giandalia *et al.*, 2001).

The vancomycin nanobubble preparation is as depicted in Figure 3. The Empty fluorescent NBs and drug-loaded nanobubbles have fabricated by the addition of 6-coumarin to the perfluoropentane core. Alternatively, fluorescent vancomycin formulated in a chemical reaction between fluorescein isothiocyanate (FITC) and vancomycin. All formulation bubbles sizes indicate around 300 nm as a value of average diameters rather polydispersity index is included in between 0.22 to 0.25 with zeta potentials ranged between -34.5 mV (Empty Nanobubble) to -29 mV (Vancomycin loaded nanobubbles). The loading of vancomycin in the Nanobubble structure does not affect the viscosity of the formulations. The Nanobubbles has loaded with vancomycin and encapsulation efficiency determined was 86% along with loading capacity of 29%. The stability of the empty nanobubbles and drug-loaded nanobubbles formulation has determined on a long-term basis with no significant variation up to six months. Vancomycin loaded nanobubbles also confirmed with significant toxic effect and viability of HaCaT cell lines was not affected by any formulation significantly. The Vancomycin loaded nanobubbles displayed slow and steady prolonged release drug kinetics when compared with vancomycin based aqueous solution, with only 16% of drug release from VmLNBs after 6 hours. The drug-loaded nanobubbles showed more effectivity and enhanced efficacy than vancomycin alone in killing MRSA (methicillin-resistant *Staphylococcus aureus*), with vancomycin loaded nanobubble the antibacterial activity is effectively sustained overtimes as a result of prolonged drug release.

Taxanes loaded on Glycol-chitosan Nanobubbles

Taxanes (Paclitaxel and Docetaxel) were loaded in perfluoropentane inner core and glycol chitosan shell based nanobubbles was aimed for prolonged-release and to reduce side effect. Previous studies reported the paclitaxel and docetaxel is the potentially treat for castration-resistant prostate cancer (CRPC), but unfortunately this shows little effect on prolonging survival (Darshan *et al.*, 2011; Marano *et al.*, 2016; Shiota, Eto, 2016) and is complicated with several side effects (Schutz, Buzaid, Sartor, 2014). These nanobubbles fabricated in the treatment of CRPC. In this method, extracorporeal shock waves (ESWs) are being used for delivery of the drug in the target region. Taxanes nanobubbles preparation as shown in Figure 3. The Fluorescent nanobubbles without any drug have obtained by the addition of green fluorescent 6-coumarin to the perfluoropentane core.

All formulation nanobubbles present an average diameter of about 300 nm along with a positive surface charge. The drug loading slightly affected the zeta potential values. The Glycol chitosan nanobubbles were able to incorporate the two taxanes in the core to a higher extent, paclitaxel were loaded into the nanobubble system with approximately 94% encapsulation efficiency and the loading capacity of about 5.8% approximately and the docetaxel was also loaded into the nanobubble system with an encapsulated efficiency of about 86% and the loading capacity of about 5.2% respectively. The release profiles of both drug loaded nanobubbles found prolonged duration *in vitro* release kinetics of the two taxanes from the drug-loaded nanobubbles has previously demonstrated and no initial burst effect has determined for both the drug formulations.

The paclitaxel results showed that about 15% and 22% of docetaxel has released from the nanobubbles within the first 8 hours. The stability of drug loaded nanobubbles formulation has confirmed by long-term and there was no significant variation up to three months after the fabrication of these formulations. The combination of ESWs and NBs enhances paclitaxel cytotoxicity, thus decreasing the drug GI_{50} of 52% in PC3 cells and 61% in DU145 cells, respectively. The similar effect was obtained in the docetaxel activity; that is, the drug GI_{50} is 48% in PC3 cells and 40% in DU145 cells. The Nanobubbles also works as a stable taxane reservoir in the CRPC cells. The ESWs increases the release of drug from nanobubbles, leading to a higher cytotoxicity and anti-migration effect.

Polymer-based nanobubbles

Doxorubicin-loaded on PLGA

Doxorubicin was prepared using Perfluoropropane gas and PLGA, such nanobubbles are aimed for the controlled release of certain drugs at the target site and had better stability. These nanobubble increase ultrasound imaging of the tumor and improve its therapeutic effect. Nanobubble was formulated using a double emulsion (W/O/W) technique. The doxorubicin nanobubble formulation sketch is as shown in Figure 4.

A doxorubicin nanobubble increases the concentration of the drug in targeted tissues or tumors to achieve the tumorolytic effect. The perfluoropropane gas enhances the accuracy of ultrasound imaging and hence drug release. The average size of nanoparticles was 500 nm with the potential of -23 mV exhibit better stability. The encapsulation efficiency of the Doxorubicin nanobubbles was 78.6 % while the efficiency of drug loading was 7.4 %. Different researches on the drug release pattern from nanobubbles with or without ultrasonic effect concluded that the ultrasound is necessary for the drug release and also contribute to the controlled release effect for Doxorubicin nanobubbles. *In vivo* studies of the ultrasound imaging system on the VX2 liver cancer cells, in comparison to saline, Doxorubicin nanobubbles could augment the ultrasonic imaging remarkably at the tumor site.

After administration of the Doxorubicin nanobubble drug in the ear vein, the nanobubbles could quickly attain the tumor area and reach the peak after 17 seconds. The result confirmed that the Doxorubicin nanobubbles are used to strengthen the ultrasound imaging and release of drug in the targeted tissue. The drug loaded into PLGA helps to minimize its toxic effect on the body remarkably, thus improving the therapeutic efficacy of a tumor at a greater extent (Meng *et al.*, 2016).

Air-based nanobubbles for drug delivery

Doxorubicin-loaded on Poly (lactic-co-glycolic acid) Nanobubbles

The Doxorubicin was prepared using Poly (lactic-co-glycolic acid) and such nanobubble is under investigation of the sustained type of drug delivery. These nanobubbles help in improving specific targeting, increased delivery to the site of interest and decreases the incidence and intensity of the side effects (Betancourt, Brown, Brannon-Peppas, 2007; Tang *et al*., 2007; Wang *et al*., 2011; Deng *et al*., 2014). The doxorubicin loaded into PLGA nanoparticles is a feasible approach to decrease chemotherapy cytotoxicity to healthy tissues, which help to control undesirable adverse effects *viz*. impaired cardiac function (Lin, Ng, Wang, 2005; Jain *et al*., 2011). The drug routinely loaded employing double emulsion technique the preparation is as illustrated in Figure 4 without loading the gas used for this method.

The Doxorubicin free PLGA Nanobubbles do possess a negatively charged surface and smaller diameter ranges between 531 nm and 258 nm by controlling the ultrasound amplitude from 15% to 45%. The low polydispersity of Doxorubicin free PLGA Nanobubbles suggests a narrowed distribution of size. The doxorubicin free PLGA Nanobubbles are round, spheroid in shape with a smoother surface and a form of a dispersion.

The 45% ultrasound amplitude was selected to fabricate doxorubicin loaded with PLGA Nanobubbles. The doxorubicin concentration varies from 0 to 65 mg DOX/g PLGA, and other parameters for instance Poly (vinyl alcohol) concentration, sonication duration and ultrasound amplitude are kept constant. The diameter ranges from 258 nm to 317 nm. The initial DOX concentration has minimal effect on the particle size, surface charge and polydispersity. The drug payload and encapsulated efficiency are almost increased with doxorubicin concentration. There was a relative saturation when doxorubicin concentration was beyond 52 mg DOX/g PLGA. In actual the drug content in the PLGA NBs was interfered by drug-polymer interactions and the drug miscibility in the polymer.

The maximum drug-polymer miscibility would result in the higher drug incorporation (Panyam *et al*., 2004; Manchanda *et al*., 2010). The drug payload and encapsulation efficiencies are 3.6% and 70.9% respectively. The drug-loaded PLGA NBs are uniform and globular sphere with a smooth surfaced and in dispersion with water. The mean diameter is approximately 270 nm with narrow size distribution. The Stability studies were performed by re-dissolving the drug-loaded PLGA NBs in PBS at 37ºC for one month to assess their size change. The doxorubicin loaded nanobubbles were devoid of any remarkable changes in the average diameter within one month.

The advantage of this drug delivery system with the nanobubbles is the controlled drug release, which improves the drug bioavailability and reduces the side effects and toxicity of drugs in the normal tissues by the EPR (enhanced permeation and retention) effect (Santander-Ortega *et al*., 2010; Jia *et al*., 2012). The cell viability has shown higher cytotoxicity to kill HeLa cells when compared to the free DOX at the same DOX concentration.

Sulfur hexafluoride based nanobubbles for drug delivery

Coumarin-6 loaded on lipid-based nanobubble

Coumarin-6 model drug was loaded into the lipid-based nanobubble. The nanobubbles are under investigation of ultrasound imaging function and cellular delivery property. The contrast agent nanobubble preparation as depicted in Figure 2. In this method, coumarin-6 was selected as a model drug for cellular testing. The Emulsion, liposomes and chitosan nanoparticle were also made ready for the cell test. These can refer as the control agents for nanobubbles. The Emulsion is formulated using the technique of preparation of bubbles without introducing the gas. The fabrication of liposomes extensively employs “film” technique (Sezer, Baş, Akbuğa, 2004; Wang *et al*., 2010). The Chitosan nanoparticle (CNP) was developed through a crosslinking mechanism. In all the preparation, the overall concentration of coumarin-6 and lipid were 20 µg/ml and 3 mg/ml maintained respectively.

The nanobubbles are bright spots present in the focus region and the range of size of bubble used to be between 333 to 246 nm. The high ratio of tween 80 ensures improvement in the acoustic backscatter feature of bubbles. The nanobubbles multiplied from 3.002 to 16.110 as tween 80 changed from 0% to 3% while an excessive quantity of cholesterol blamed for negative contribution to the acoustic bubble property. When the lipid was around 2.5mg/ml, the bubbles represent the ultimate acoustic backscatter profile among the formulations tested. On comparison of overall pharmacokinetic task part within chitosan nanoparticles, emulsion and liposome, the nanobubbles scored with the highest final concentration (plateau concentration) 29.780 ng/ml while the most substantial value of $t_{1/2}$ found was 35.4 min.

CONCLUSION

Nanobubbles are novel systems of drug delivery specifically designed for targeting the drug to affected sites upon exposing to ultrasound/ESWs. Nanobubbles are gas composed hollow, spherical pouches or cavities in aqueous solution with size range below 1μm. Bubbles are sphere-shaped particles with a shell and core structure filled with gas to give the acoustically active properties. Nanobubbles help in reducing the cumulative frequency of dose and targets specifically at to site of interest. Nanobubbles formulation for drugs with gas entrapped in different core shields helps for sustained delivery, reduced frequency of administration and enhances bioavailability and efficacy in comparison with conventional dosage forms. The nature of gas like perfluorocarbon contributes for enhancing the stability of bubbles to augment release kinetics of the drug against air bubbles which gets dissolved quickly in the bloodstream before achieving actual therapeutic window.

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ABBREVIATIONS

NBs - Nanobubbles
MBs - Microbubbles
NPs - Nanoparticles
US - Ultrasound
NE - Nuclear envelope
NER - Nuclear envelope rupture
HCl - Hydrochloric acid
PNs - Perfluorocarbon Nanobubbles
ATC - Anaplastic Thyroid Cancer
ESWs - Extracorporeal shock waves
PLGA - Poly(Lactic-Co-Glycolic Acid)
MRI - Magnetic Resonance Imaging
DOX - Doxorubicin

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