Micro- and nanobubbles: A versatile non-viral platform for gene delivery

Micro- and Nanobubbles: a versatile non viral platform for gene delivery

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ABSTRACT

Micro- and nanobubbles have been proposed as non viral strategy for ultrasound mediated gene delivery. Microbubbles are spherical core/shell structure filled by a gas, with a mean diameter between 1 and 8 micrometer, able to circulate in the bloodstream after their intravenous injection. They undergo volumetric oscillations or acoustic cavitation when insonified with ultrasound and particularly they are able to resonate at diagnostic frequency. For this behavior microbubbles are currently used as ultrasound contrast agents, but they are under investigation for therapeutic purposes**.** Microbubbles can a play a role in enhancing cell delivery. When combined with clinical ultrasound exposure they are able to favour the gene entry in cells by cavitation. Two strategies are possible: DNA can be co-administered with commercial contrast agents or loaded in purposely tuned bubble systems. Different technological approaches have been proposed to associate genes within microbubble structure. Nanobubbles, bubbles with sizes in the nanometer order of magnitude, have been recently developed with the aim of obtaining more efficient gene delivery systems. Their small sizes might permit the extravasation and ultrasound targeted site-specific release with minimal invasivness. By contrast, due to their sizes, microbubbles are not able to extravasate and their targeting capacity is limited to specific antigen present in the vasculature lumen. This review is an overview on the use of microbubbles as gene delivery systems, with a specific focus on recent researches regarding the development of nano systems. Particularly, the ultrasound delivery mechanisms, the formulation parameters, the gene loading approaches and the advantages of nanometric systems will be described.

Keywords: microbubbles, nanobubbles, nanodroplets, gene delivery, ultrasound, non viral carriers

1. INTRODUCTION

Gene therapy, a promising therapeutic option for the treatment of genetic or acquired diseases, is based on the ability to introduce new genetic materials to hosts. A major obstacle to gene transfer is the fact that naked nucleic acids are not taken up by cells efficiently due to the negatively charged phosphate groups and to their large size and hydrophilic nature. Moreover, they easily undergo to nuclease-mediated degradation. To overcome this limitations a number of gene delivery vectors have been developed all falling into two categories, viral and non-viral.

Micro- and nanobubbles might be a good non-viral strategy for site specific gene delivery, due to the potential to be activated in the presence of ultrasound (US) for a targeted delivery of DNA. Microbubbles are spherical core/shell vescicles filled by a gas (Fig.1). The core is a single chamber of low density and comprises a large majority of the total particle volume. Perfluorocarbons, sulfur hexafluoride, air, carbon dioxide have been used as gas core. The shell acts as a barrier between the encapsulated gas and the surrounding aqueous medium avoiding the gas dissipation. Various shells with different thickness can be obtained according to the component used, including lipids (~3 nm thick), proteins (15–20 nm thick) and polymers (100–200 nm thick) (Sirsi et al. 2009). The composition of the shell determines the stiffness of the bubbles, their resistance to rupture in the ultrasound pressure field, and the ease with which they are recognized and cleared by the reticuloendothelial system. The presence of a surfactant layer can increase the half-life of the bubble by decreasing the gas/water interfacial tension, in some instances, to near-zero values.

Figure 1 here

Microbubbles are under investigation for therapeutic applications, but currently they are used as injectable contrast agents for US imaging because they can interact with US waves. Due to the presence of a central gas core, microbubbles suspended in a liquid are exceptionally efficient US

reflectors. After the injection of aqueous suspensions of gas microbubbles into the bloodstream a strongly reinforced ultrasonic echography imaging occurred, thus aiding in the visualization of internal organs as for the detection of cardiovascular disorders and other diseases (Tilcock 1999, Lindner et al. 2000a, Lindner et al. 2000b, Lindner et al. 2000c, Cavalieri et al. 2006, Paradossi 2012). Due to their structure microbubbles are able to oscillate in the US pressure with the expansion and contraction of their volume. This phenomenon is called acoustic cavitation.

Microbubbles used for clinical imaging are typically between about 1 and 10 μm in diameter (the upper limit for passage through the lung capillaries), sizes that limit their extravasation from the bloodstream. Generally, the diameter of a microbubble is approximately equal to the size of a red blood cell, which allows it to display similar rheology in the microvessels and capillaries throughout the body after intravenous administration.

Recently, drug and gene delivery with micro- and nanobubble systems in the presence of US is gaining more attention in the nanomedicine field as this technique presents several advantages including non invasiveness, local applicability, and proven safety (Suzuki 2012). US itself can improve the delivery of DNA but a carrier is needed to increase the amount available for cell uptake, to protect genes from degradations and to obtain a targeted release**.**

US can play various roles in the presence of bubbles. Microbubbles can be injected upstream of a target region. When US is precisely focused on the target tissue, then the ultrasonic intensity can be increased to create bubble collapse cavitation. The collapse events appear to permeabilize the vessel walls and provide pathways for extravasations of DNA that can be either freely floating along with the microbubbles or associated with the microbubble surface.

Targeted gene-loaded microbubbles can be designed modifying their surface with targeting ligands with the aim to collapse under US releasing DNA in specific cells and protecting other tissues from side effects. An additional advantage of this US-targeted strategy is the possibility to visualize the delivery and release of the loaded compound with real-time US (ad un lettore non esperto come me

questa frase non dice molto: che cos'è la real time US?). This review is focused on the design of bubbles and reports case studies on microbubbles as well as on systems with nanometric sizes.

Development of bubble formulations: theoretical aspects.

There are a wide variety of microbubble systems reported in the literature**.** Nanotechnological approaches can be used to prepare stable systems, but it is necessary to examine peculiar parameters **to stabilize the carrier and** the DNA loaded

Microbubbles can promote gene delivery into cells because they are able to increase cell membrane permeability in the presence of US using suitable conditions, that must be selected to avoid cell suffering. In general, cell damage is dependent on various parameters, i.e. US intensity, concentration of microbubbles and cell type; especially, US intensity and exposure time are key factors. Therefore, it is important to optimize the condition of US exposure in US-mediated gene delivery (Feril et al. 2006, Suzuki et al. 2008,Pislaru et al. 2003, Li et al. 2003). Low and intermediate acoustic pressure resulted in linear and non-linear oscillations of microbubbles, respectively, whereas high pressure US (MI >1.0) caused forced expansion and compression of microbubbles, leading to bubble destruction and to increase of cell membrane permeation. Therefore, microbubble acoustic cavitation can be used as a trigger to release DNA in the tissues exposed to US (Figure 2). Figure 2 illustrates a microbubbles carrying an active molecules once administered into the vasculature before and after US application to destroy microbubbles in the target region on the endothelial cells, (because microbubbles are not able to extravasate for their micrometer sizes-gia detto, forse è utile ricordarlo nella legenda della figura per non appesantire questa frase) Un'altra possibilità e usare tutta questa frase come legenda alla figura 2 e richiamare la figura nella frase precedente(Bekeredjian et al. 2005).

Figure 2 here

Many researches showed that the interaction of US and microbubbles can affect cell permeability with various mechanism: cavitation, generation of reactive oxygen species, rise in temperature, endocytosis or phagocytosis of the bubbles and fusion of the lipid bubble shell with cell membrane.

The acoustic cavitation of microbubbles, also called sonoporation, is one of the most important mechanisms to explain the increase on cell permeability. Generally, US applied to fluid causes cavitation, i.e. the creation, vibration and collapse of small gas-filled bubbles by the ultrasound beam. US alone may increase cell permeabilization on its own, but the addition persistent (???) microbubbles have an additional effect in increasing cell permeability. First, by acting as cavitation nuclei, microbubbles can lower the threshold of the acoustic power needed for cavitation. Then the destruction of microbubbles may cause high-energy microstreams or microjets, that will cause shear stress on the cell membrane enhancing cell uptake, probably due to transient holes. This phenomenon has been deeply studied as a way to mediate the transfer of different molecules (Tlaxca et al. 2010, Bao et al. 1997, Greenleaf et al. 1998, Guzman et al. 2001a, Kim et al. 1996, Tata et al. 1997). Sonoporation has been shown to enhance the intracellular delivery of small molecules, macromolecules (Guzmán et al. 2001b, Guzmán et al. 2002,Karshafian et al. 2009, Miller et al. 1999), (Yu et al. 2006) and genetic materials (Bao et al. 1997). **ineserire new ref.**).

Lawrie et al. (2003) showed that transfection was not related to free radical production from collapse cavitation, but it is mainly related to transient holes in the cell membrane produced by other cavitation phenomena.

Another interesting aspect to take into account is the rise in temperature in tissue after the application of high pressure US. Particularly, the bubble collapse can create high velocity jet streams that may cause a local and transient increase in temperature affecting the membrane permeability. The temperature increase can also affect the microbubble structure; Paradossi designed temperature sensitive microbubbles using cross-linked NIPAM (Capece et al, 2013). The commercial US contrast agent Sonovue® changed microbubble sizes in dependence of temperature

(Guiot et al. 2006). Endocytosis or phagocytosis, active membrane transport mechanisms, may also be involved in the uptake of whole bubble, bubble fragments or substances entrapped in microbubble structure..

Finally, lipid-based microbubbles could facilitate the deposition of genes in a cell is the fusion with the phospholipid bilayer of a cell membrane (frase non chiara!). This could result in delivery of the cargo of the microbubble directly into the cytoplasm of the cell with the possibility of further uptake in endosomes or delivery into the cell nucleus (Dijkmans et al. 2004).

Schemes of the hypothesized mechanisms for enhanced delivery using microbubbles and US are reported in Figure 3.

Figure 3 here

Continuare di qui

An important parameter to be considered in order to develop stable bubble systems is the Laplace pressure, which can govern the dissolution rate of the microbubbles in vivo. The gas pressure within a bubble is the sum of the equilibrium pressure, the Laplace pressure and the blood pressure.

The Laplace pressure is the pressure difference between the inside and the outside of a bubble (or a droplet) in a bloodstream. (Schutt et al., 2003) The Laplace pressure is given as

$$\Delta P = P_{inside} - P_{outside} = \frac{2\sigma}{r}$$

where P_{inside} and $P_{outside}$ are the pressures inside and outside a bubble respectively, σ is the interfacial tension, and r is bubble radius. A surfactant layer on the surface of the bubbles can reduce the interfacial tension and decrease the Lapalce pressure.

As the Laplace pressure is reversely related to the size according to the equation, smaller bubbles will have higher pressure values than larger ones. When the inner gas leaves the core, the bubbles shrink and the Lapalce pressure increases thereby accelerating the rate of gas dissipation and the

resulting bubble shrinkage until the system rupture. The microbubble formulations can be stabilized by reducing the dissolution of the gas in the bloodstream. This can be obtained using a gas with a very poor aqueous solubility for the bubble core. For examples the encapsulation of appropriate perfluorocarbons which are gases not soluble in water can increase the lifetime of bubble systems. Van liew and Kabanov studied the kinetics of dissolution of microbubbles containing a sparingly soluble gas (van Liew et al., J. Applied Physiol, 1995; Kabanov, Ultrasound in Biol and Medic, 1998) Questa frase suona isolata: andrebbe legata meglio.

A recent strategy to obtain stable bubble systems is to develop nanoemulsions containing perfluorocarbons that can convert in bubbles in the presence of US. The phenomenon called Acoustic Droplet Vaporization (ADV) allows nanodroplets to be converted in larger bubbles by the pulse of US, permitting the liquid to gas transition in the core. (klipfgang, 2000). If formulated properly, prefluorocarbons emulsions can remain stable systems also at temperatures (i.e. 37 °C) higher that the ones of their normal boiling points. Such meta-stability is due to the high droplet curved interface that can raise the internal pressure within emulsion droplets. Consequently, perfluoropentane (bp=29 °C) can be used to prepare stable nanoemulsion at body temperature. When insonated the perfluoropentane nanodroplets change the internal phase from liquid to gas and can be used to trigger the release of the loaded genes.

A further factor to consider for bubble stability is that the exposure to intense energy of US can lead to rapid bubble destruction by cavitation due to their rapid contraction and expansion at resonance mechanical stresses and weakens the bubble shell (il finale di questa frase non è chiaro). Therefore, the shell components affect markedly the bubble stability. The shell composition should be designed in order to obtain systems with a prolonged shelflife. An additional strategy to produce more stable system is the cross-linking of the bubble shell. The shells of the bubbles can be also modified for targeting purposes.

Shells incorporating positively charged lipids or polyelectrolytes are the more suitable to take up genetic materials why? È il posto giusto per questa frase? Va contestualizzata meglio?

Microbubbles as gene delivery systems

In recent times the popularity of insonated-microbubbles technique has increased for the versatility of the application and ease of the use.

The effect of US on cell permeability and and their capacity to facilitate the entry of naked DNA in the cells was described in the last decades (Fechheimer, 1987). The first studies used a US frequency in the range of 20-50 kHz. These frequencies, with cavitation, are able to induce tissue damages (Miller et al, 2002; Wei et al, 2004). To overcome this limitation, the delivery of genes using US applied for clinical imaging, which operates at frequencies between 1 and 3 MHz, have been deeply investigated. Consequently, the addition of the clinical contrast agents was examined. It was shown that the combination of US and microbubbles used as echo contrast agents increased the transfection efficiency of naked DNA (Shohetet al 2000). Indeed, this approach can directly transfer genes in the cell cytosol as proved by various researches. (capitolo di libro).

Subsequently, to improve the delivery and to localize the transfection different bubble systems loaded with DNA have been designed***. Indeed.*** the main advantage of US-mediated microbubble-assisted transfection is the ability to achieve DNA delivery and expression only in the insonated areas and not in the non-targeted tissues. **Particularly targeting ligands can be covalently bound on the bubble shell to promote the attachment of DNA-loaded microbubbles to specific cells.**.

Two different strategies can be used to vehiculate genes with bubble systems. Commercial microbubbles and DNA can be co-administered and then after the US application naked genes are facilitated to enter into cells (Figure 4A). Alternatively, it is possible the adminstration of microbubbles containing DNA within their structure (Figure 4B). Much research attention is focused on this strategy, which allowed also the protection of DNA from degradation.

Figure 4 here

A number of technological approaches can be used to associate DNA within microbubble structure. A schematic representation of the different gene loading strategies is depicted in Figure 5. DNA could be attached to the external surface of the monolayer bubble by covalent or non-covalent bonds (Figure 5A) or it could be incorporated in the shell of a multilayer polymer microbubble, increasing the quantity of DNA vehiculated (Figure 5B). Moreover, DNA can be previously incorporated in lipoplexes, polyplexes, liposomes or nanoparticles that can be subsequently associated with the bubble surface using either physical or chemical interactions (BIBLIO) (Figure 5C).

Figure 5 here

For the gene loading approach by non covalent bonds (figure 5A), it is necessary to design microbubbles with a positive surface charge to favour the electrostatic interactions with the negatively charged nucleic acids. Indeed, several studies report on cationic microbubbles for enhancing ultrasound-mediated gene transfer using electrostatic interaction between plasmid DNA and the surface of the microbubble (Christiansen et al. 2003, Tlaxca et al. 2010).

Cationic phospholipid-based microbubbles represented a popular avenue for gene transfer because their distinct advantages including, (i) ease in handling and preparation techniques, (ii) ability to deliver many DNA molecules, and (iii) low immunogenic response. In contrast with other delivery systems, ultrasound combined with phospholipid-based microbubbles can provide real-time images of soft tissue structures without ionizing radiation and transfer genes to target issue with high efficiency and low level of danger (Zhao et al. 2008).

Albumin microbubbles have been intensively studied for their application in gene delivery (Porter et al. 1996, Cheng et al. 2011, Duvshani-Eshet et al. 2006, Frenkel et al. 2002). Neverless, due to

negative surface potential, albumin microbubbles present low transgene efficiency. An increase of gene transfection efficiency was observed modifying albumin microbubbles by polyethylenimine (Dang et al. 2011).

In 2011 Nomikou explored the influence of microbubble surface modifications on their interaction with plasmid DNA and target cells, and the functional consequences of those interactions in terms of ultrasound-mediated gene transfer. Polyethylene glycol-stabilized lipid-shelled microbubbles with neutral, cationic and biotinylated cationic surfaces were compared in terms of their abilities to interact with a luciferase-encoding reporter plasmid DNA and with target cells in vitro. The results demonstrate that the biotinylated cationic microbubble > cationic microbubble > neutral microbubble, in terms of their abilities to interact with target cells and to enhance ultrasound-mediated gene transfer (Nomikou et al. 2011).

In 2010 Tlaxca et al. examined the role of acoustic power intensity and microbubble and plasmid concentrations on transfection efficiency in HEK-293 cells using a sonoporator with a 1-MHz transducer. A green fluorescent protein (GFP) reporter plasmid was delivered in as much as 80% of treated cells, and expression of the GFP protein was observed in as much as 75% of cells, using a power intensity of 2 W/cm^2 with a 25% duty cycle. In addition, the relative transfection abilities of a lipid non cationic and cationic microbubble platform were investigated. Cell survival and transfection efficiency were inversely proportional to acoustic power and microbubble concentration. Moreover, direct conjugation of plasmid to the microbubble did not appear to significantly enhance transfection efficiency under the examined conditions, although this strategy may be important for targeted transfection in vivo (Tlazca et al. 2012).

The possibility to deliver therapeutic genes to target cells, like tumor cells and not normal cells, was also investigated using ultrasound-targeted microbubble destruction.

To reduce tumor invasion and metastasis by inhibiting angiogenesis the Vascular Endothelial Growth Factor (VEGF) receptor, called KDR, was studied for suicide gene therapy. The KDR promoter that lead the suicide gene to be expressed specifically in tumor vascular endothelial cells

and tumor cells, were transfected using the commercial Sonovue bubble in a breast cancer model. Results demonstrate that ultrasound targeted microbubble destruction is a novel, safe, targeted, and effective gene transfection system, and genes modified by tumor tissue-specific promoters have shed a new light on the targeted gene therapy field (Xing-Hua et al. 2012).

Other studies investigated the use of ultrasound microbubble carrying gene and transactivating transcriptional activator (Tat) peptide to improve its transfection effect in vivo. Promising results were obtained without observing noxious effects on cell viability (Ren et al. 2008, Ren et al. 2009).

All the studies mentioned before concerned microbubbles carrying DNA on their structure. The second strategy involving microbubbles for gene delivery consists in the administration of DNA and microbubbles separately. Genes can be administered to the cells in a first time, and only in a second time microbubbles, acting as cavitation nuclei can be added into the cell media. This different approach was investigated by Escoffre et al. in 2010 using electrosonoporation. They first electroporated cells with plasmid DNA encoding green fluorescent protein and than sonoporated in presence of contrast microbubbles. After 24 h an increase of trasfection efficiency was observed only after electroporation and a maximized effect was observed using electrosonoporation in the presence of microbubbles (Escoffre et al. 2010).

Lipoplex- or polyplex-loaded nanobubbles represent another technological approach to carry genes Forse qui andrebbe spiegato per il lettore medio cos'è un lipoplex o un polyplex). Lentaker developed lipoplex-loaded microbubbles to increase the transfection efficiency of PEGylated liposomes. PEGylated lipoplexes were attached to microbubbles via biotin-avidin-biotin linkages: the lipoplex-loaded microbubbles transfected cells only after insonation and in a greater extent than that of free-lipoplexes (Lentaker, advanced functional materials)

Recently, a polyplex-microbubble hybrid formulation was developed attaching branched polyethylenimine (PEI 25 kDa) to lipid-coated microbubbles: This formulation increased the positive surface charge and the DNA loading. In vivo site specific delivery was achieved by

application of US to mice tumors (Sirsi et al., J, Controlled Rel, 2012).

Finally, a new transfection agent, Targesphere® SA, was marketed by Targeson. Targesphere SA is a targeted cationic microsphere indicated for transfection *in vitro* and *in vivo*. It is coated with streptavidin to allow conjugation of any biotinylated ligand to the surface of the microsphere. Plasmid, siRNA, and other anionic payloads can be added on the surface of Targesphere SA by simple incubation. Intracellular delivery is mediated by activation of Targesphere SA with ultrasound energy. Targesphere SA provides targeted cell specific transfection and is a complement to Targesphere, which is organ specific (http://www.targeson.com).

4.0 Nanometric systems for gene delivery (forse qui una tabella comparativa tra micro e nano sarebbe utile per riassumere I vantaggi potenziali descritti sotto))

Microbubbles with submicron sizes are defined nanobubbles. The fabrication and initial application of nanobubbles has shown promising results in recent years to increase stability, loading and to achieve the system extravasation. Nanoscale systems were previously designed as contrast agent. Only recently they have been also studied for the delivery of drugs and genes (Oeffinger et al. 2004, nanobubbles tumor). Indeed, microbubbles present short circulation time (minutes) and their relatively large sizes do not allow extravasation from bloodstream. With nano-sized bubbles the accumulation in the desired tissues via the Enhanced Permeability and Retention (EPR) effect (Maeda et al. 2002) or through active targeting would be possible. However, a small size is a basic requirement for a bubble to penetrate blood vessel pores (Yin et al. 2012). The blood vessels in tumors and inflammatory tissues are leaky and defective, with large pore cut-off sizes, and the endothelial cells are misaligned or have large fenestrations. This tissues have also a poor lymphatic drainage compared with normal tissues. All of this features allow for the delivery of gene carriers to this tissues by the EPR effect (Skinner et al 1990, Matsumura et al 1986, Dai et al. 2011, Yang et al. 2008). Nanobubbles are generally prepared by sonication of by the formulation of a nanoemulsion.

The latter is used with perfluorocarbons that are liquid at room temperatuire to obtain perfluorocarbon nanodroplets that convert in nanobubbles by ADV. Another interesting preparation approach consists of the use of bubble liposomes to obtain nanobubbles (Bubble liposomesO).

The main challenge in the field of nanobubbles is the identification of formulation strategis to enhance the circulation time of bubbles thus allowing them to reach specific organs. Different technological approaches have been investigated to improve the lifetime of nanoscale systems including the concept of polyelectrolyte coatings obtained by the alternate deposition of polyanions and polycations for surface functionalization or the cross-linking of polymer shells. Of note, a recent study identified the relevance of polymeric modifications to extend the circulation time, which would be essential for application of these systems in passive drug or gene targeting (Du Toit et al. 2011). To reduce bubble size to the nanometer range without affecting echogenicity Krupka et al. in 2010 proposed the addition of surfactants or amphihilic polymers, such as Pluronics into the formulation of lipid bubbles (Krupka et al 2010). For this purpose five types of Pluronics, having different molecular weights (Mw 1100-4600 Da), were incorporated into the lipid bubble shell. Results demonstrate that Pluronic-lipid interactions lead to a marked reduction of bubble size.

The localized gene delivery using nanobubbles and a dual intensity ultrasound systems have been described (Horie et al. 2010). Polymer shell permit to chemically bind ligands to the bubble surface for active targeting.

In 2011 Lukianova-Hleb proposed plasmonic nanobubbles to obtain selective gene transfection. Plasmonic nanobubbles are a novel cellular agent with a dual and tunable mechanical and optical action that utilizes the formation of the vapor nanobubble around the plasmonic nanoparticle upon its transient heating with a short laser pulse through the mechanism of plasmon resonance it produces a localized mechanical, non-thermal impact to the cell. Plasmonic nanobubbles provided targeted gene delivery at the single cell level in a single pulse procedure that can be used for safe and effective gene therapy (Lukianova-Hleb et al. 2011).

Suzuki and colleagues developed bubble liposomes with sizes between 150- 200 nm. A liposomal system contain lipid nanobubble of perfluoropropane. A mixture of bubble liposomes and DNA injected in mice showed that the gene uptake is limited to the area exposed to US indicating that the systemmight be used to increase the DNA transduction.

In 2011 Bisazza et al. developed a new polymeric nanobubble formulation consisting of a diethylaminoethyl-dextran (DEAE) shell and a perfluoropentane core. Nanobubble diameters were less than 500 nm with a positive surface charge. DEAE nanobubbles were able to protect their cargo from the action of proteases and transfect plasmid DNA across the cell membrane without any resulting cytotoxic effects (Bisazza et al. 2011). The bubbles can be coated with stabilizer agents as Pluronic F68 to improve stability. The same reserch group developed in 2012 another polymer nanobubble formulation consisting of a chitosan shell and a perfluoropentane core for the DNA delivery. Chitosan was selected for the nanobubble shell because it is a polycationic polymer with low toxicity, low immunogenicity and excellent biocompatibility. DNA-loaded chitosan nanobubbles were formed with a mean diameter less than 300 nm and a positive surface charge. In vitro transfection experiments were performed by exposing adherent COS7 cells to US in the presence of different concentrations of plasmid DNA-loaded nanobubbles. In the absence of US, nanobubbles failed to trigger transfection at all concentrations tested. By contrast, 30 seconds of US promoted a moderate degree of transfection. Cell viability experiments demonstrated that neither US nor the nanobubbles affected cell viability under these experimental conditions. (Cavalli et al. 2012). Polyplex-loaded polymeric nanobubbles was also designed attaching DNA polyplexes, formed with AGMA1 a poly(amido)amine previously investigated as transfection agent (Cavalli 2010), by electrostatic interaction on dextran nanobubbles (Bisazza tesi di dottorato). This nanosytem showed a good transfection efficiency in vitro (Bisazza personal communication).

Recently, acoustic sensitive liposomes encapsulating emulsion, called eLiposomes was developed (2013 JJ- Controlled Rel). When US are applied to eLiposomes, the perfluorocarbon liquid droplets transform to gas, causing the rupture of the phospholipid bilayer and the release of plasmid.

6.0 Conclusions

US applications in medicine provide non invasive, well accepted and cost-effective techniques. The use of US-mediated gene delivery might be a promising tool for future localized therapies. Indeed, the application of US in the presence of micro- and nanobubbles might increase the transfection efficiency, favouring the DNA release on demand. Targeted-microbubbles with specific ligands attached on the shell surface might provide the delivery of genes in the target region. The limitation of microbubbles for gene therapy is their large sizes that did not allow their extravasation after i.v. administration. Consequently, they might be mainly proposed for superficial applications for the treatment of topical diseases. Recently more interest was addressed into the design of bubble systems in the nanometer order of magnitude to obtain an efficient gene carrier able to reach specific organs. Forse andrebbero meglio esplicitate le future perspectives a le limitazioni odierne che devono essere superate

References

- Bao, S., Thrall, B., Miller, D. 1997, Transfection of a reporter plasmid into cultured cells by sonoporation in vitro, Ultrasound Med Biol,; 23, 953–959.

- Bekeredjian, R., Grayburn, P., Shohet, R. et al., 2005, Use of Ultrasound Contrast Agents for Gene or Drug Delivery in Cardiovascular Medicine Journal of the American College of Cardiology, 45, 3, 329-335.

- Bisazza A, Civra A, Donalisio M, et al. The *in-vitro* characterization of dextran-based nanobubbles as possible DNA transfection agents, Soft Matter, 2011, 7, 10590-10593.

- Cavalieri, F., Hamassi, A., Chiessi, E. et al., 2006, Ligands Tethering to Biocompatible Ultrasound Active Polymeric Microbubbles Surface Macromol. Symp. , 2006, 234, 94-101.

- Cavalli, R., Bisazza, A., Trotta M. et al, 2012, New chitosan nanobubbles for ultrasound-mediated gene delivery: preparation and in vitro characterization Int J Nanomedicine, 7, 3309-3318.

- Cheng, Y. , Jiang, L. , Liu N. et al., 2011, P85, Optison microbubbles and ultrasound cooperate in mediating plasmid DNA transfection in mouse skeletal muscles in vivo, Ultrasonics Sonochemistry, 18, 513-519.

- Christiansen J.P., French B.A., Klibanov A.L.; et al. 2003, Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles, Ultrasound Med Biol; 29,1759-1767.

- Dai J, Zou, S, Pei, Y et al., 2011, Polyethylenimine-grafted copolymer of poly(l-lysine) and poly(ethylene glycol) for gene delivery, Biomaterials; 32(6), 1694-1705.

- Dang, S., Wang, R., Qin, M. et al., 2011, A novel transfection method for eukaryotic cells using polyethylenimine coated albumin microbubbles, Plasmid, 2011, 66, 19-25.

- Dijkmans, P.A., Juffermans, L.J.M., Musters R.J.P. et al, 2004, Microbubbles and ultrasound: from diagnosis to therapy, Eur J Echocardiography, 5, 245-256.

- Du Toit LC, Govender T, Pillay V et al.,2011, Investigating the effect of polymeric approaches on circulation time and physical properties of nanobubbles., Pharm. Res., 28, 494-504.

- Duvshani-Eshet, M., Adam, D., Machluf, M.,2006, The effects of albumin-coated microbubbles in DNA delivery mediated by therapeutic ultrasound, Journal of Controlled Release, 112, 156-166.

- Escoffre, J., Kaddur, K., Rols M. et al., 2010, In vitro gene transfer by electro-sonoporation.Ultrasound in Med & Biol., 36 (10), 1746-1755.

- Feril Jr., L.B., Ogawa, R., Tachibana, K. et al., 2006, Optimized ultrasound-mediated gene transfection in cancer cells, Cancer Sci. 97, 1111-1114.

- Frenkel P, Chen S, Thai T, et al. 2002, DNA-loaded albumin microbubbles enhance ultrasound-mediated transfection in-vitro, Ultrasound in Medicine and Biology, 28, 817-822.

- Greenleaff, WJ., Bolander, ME, Sarkar, G. et al.. 1998, Artificial cavitation nuclei significantly enhance acoustically induced cell transfection, Ultrasound Med Biol, 24, 587-595.

- Guiot, C., Pastore, G., Napoleone M.et al., 2006, Thermal response of contrast agent microbubbles: Preliminary results from physico-chemical and US-imaging characterization Ultrasonics, 44, e127-e130

- Guzman, HR, Nguyen, DX , Khan S. et al..,2001a, Ultrasound-mediated disruption of cell membranes. II. Heterogeneous effects on cells.J Acoust Soc Am, 2001;110, 597-606.

- Guzmán, H.R., Nguyen, D.X., Khan, S. et al., 2001b, Ultrasound-mediated disruption of cell membranes. I. Quantification of molecular uptake and cell viability, The Journal of the Acoustical Society of America; 110 (1), 588-596.

- Guzmán, H.R., Nguyen, D.X., McNamara, A.J. et al., 2002, Equilibrium loading of cells with macromolecules by ultrasound: effects of molecular size and acoustic energy, Journal of Pharmaceutical Sciences; 91 (7), 1693–1701.

- Horie S, Watanabe Y, Chen R, et al.2010, Development of localized gene delivery using a dual-intensity ultrasound system in the bladder, Ultrasound in Med. & Biol., 36, 1867-1875.

- Karshafian, R., Bevan, P.., 2009, Sonoporation by ultrasound-activated microbubble contrast agents: effect of acoustic exposure parameters on cell membrane permeability and cell viability, Williams et al. Ultrasound in Medicine & Biology, 35 (5), 847-860.

- Kim, HJ, Greenleaf, JF, Kinnick, RR et al, 1996, Ultrasound- mediated transfection of mammalian cells, Hum Gene Ther ; 7, 1339-1346.

- Krupka TM, Solorio L, Wilson RE,et al.., 2010, Formulation and characterization of echogenic lipid-Pluronic nanobubbles, Mol. Pharm., 7 (1), 49-59.

- Li, T., Tachibana, K. , Kuroki, M. et al., 2003, Gene transfer with echo-enhanced contrast agents: comparison between Albunex, Optison, and Levovist in mice-initial results, Radiology 229, 423-428.

- Lindner, J.R., Coggins, M.P., Kaul, S. et al., 2000a, Microbubble persistence in the microcirculation during ischemia/reperfusion and inflammation is caused by integrin- and complement-mediated adherence to activated leukocytes Circulation, 101, 668-675.

- Lindner, J.R., Dayton, P.A., Coggins, M.P. et al., 2000b, Noninvasive imaging of inflammation by ultrasound detection of phagocytosed microbubbles, Circulation, 102, 531-538.

- Lindner, J.R., Song, J., Xu, F. et al., 2000c, Noninvasive ultrasound imaging of inflammation using microbubbles targeted to activated leukocytes, Circulation, 2000, 102, 2745-2750.

- Lukianova-Hleb E, Samaniego A, Wen J, Metelitsa L, et al. 2011, Selective gene transfection of individual cells in vitro with plasmonic nanobubbles. Journal of Controlled Release, 2011, 152, 286-293.

- Matsumura, Y , Maeda, H, 1986, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res. 46, 6387-6392.

- Miller, D.L., Bao S. and Morris J.E., 1999, Sonoporation of cultured cells in the rotating tube exposure system, Ultrasound in Medicine & Biology, 25 (1), 143-149.

- Nomikou, N., Tiwari, P., Trehan T. et al. , 2011, Studies on neutral, cationic and biotinylated cationic microbubbles in enhancing ultrasound-mediated gene delivery in vitro and in vivo, Acta Biomateralia, 55, 77.

- Oeffinger E, Wheatltley A., 2004, Development and characterization of a nano-scale contrast agent, Ultrasonics 42, 343-347.

- Pislaru, S.V., Pislaru, C., Kinnick, R.R. et al, 2003, Optimization of ultrasound-mediated gene transfer: comparison of contrast agents and ultrasound modalities, Eur. Heart J. 24, 1690-1698.

- Porter, TR., Iversen, PL, Li, S. Et al. 1996, Interaction of diagnostic ultrasound with synthetic oligonucleotide-labeled perfluorocarbon-exposed sonicated dextrose albumin microbubbles, J Ultrasound Med, 1996, 15, (8):577-584

- J. Ren, C. Xu, Z. Zhou et al. 2009, A novel ultrasound microbubble carrying gene and Tat peptide:preparation and characterization., Acad Radiol, 16, 1457-1465.

- Ren, J., Whang, Z., Zhang, Y. et al., 2008, Transfection Efficiency of TDL Compound in HUVEC Enhanced by Ultrasound-Targeted Microbubble Destruction, Ultrasound in Med & Biol. 2008, 34, 1857-1867.

- Sirsi, S., Borden, M., 2009, Microbubble Compositions, Properties and Biomedical Applications, Bubble Sci Eng Technol., 1 (1-2), 3-17.

- Skinner, SA, Tutton, PJ, O'Brien PE 1990, Microvascular architecture of experimental colon tumors in the rat, Cancer Res.;50 (8):2411-2417.

- Suzuki, R., Takizawa, T., Negishi Y. et al., 2008, Tumor specific ultrasound enhanced gene transfer in vivo with novel liposomal bubbles, J. Control. Release 125, 137-144.

- Tata DB, Dunn F, Tindall DJ. ,1997,Selective clinical ultrasound signals mediate differential gene transfer and expression in two human prostate cancer cell lines: LnCap and PC-3. Biochem Biophys Res Commun; 234, 64–67.

- Tilcock, C., 1999, Delivery of contrast agents for magnetic resonance imaging, computed tomography, nuclear medicine and ultrasound, Advanced Drug Delivery Reviews, 37, 33-51.

- Tlaxca, J., Anderson, C., Klibanov, A. et al., 2010, Analysis of in vitro transfection by sonoporation using cationic and neutral microbubbles, Ultrasound in Medicine and Biology , 36, 11, 1907-1918.

- Unger, E., Porter, T., Culp, W., et al., 2004,Therapeutic applications of lipid-coated microbubbles Advanced Drug Delivery Reviews, 56, 1291– 1314.

- Xing-Hua, L., Ping, Z., Li-hua W. et al. 2012, The targeted gene (KDRP-CD/TK) therapy of breast cancer mediated by SonoVue and ultrasound irradiation in vitro, Ultrasonics, 52, 186-191.

- Yang X, Zhu B, Dong T, et al. Interactions between an anticancer drug and polymeric micelles based on biodegradable polyesters, 2008, Macromol Biosci. 8 (12):1116-1125.

- Yin, T., Wang, P., Zheng, R. , 2012, Nanobubbles for enhanced ultra- sound imaging of tumors, Int J. Nanomedicine 2012. 895-904.

- Yu, T., Li, S.L., Zhao J.Z. et al., 2006, Ultrasound: a chemotherapy sensitizer, Technology in Cancer Research Treatment, 5 (1), 51-60.

- www.targeson.com

- Zhao, L., Lu, C., 2008, Factors that affect the efficiency of antisense oligodeoxyribonucleotide transfection by insonated gas-filled lipid microbubbles, J Nanopart Res, 10, 449-454.

FIGURE LEGENDS

1) Figure 1: Schematic rapresentation:of microbubble structure (A), different shell compositions(B).

2) Figure 2: Schematic representation of substance delivery using ultrasound targeted microbubble destruction

3) Figure 3: Hypothesized mechanisms for enhanced drug delivery with the insonation of microbubble contrast agents (Adapted from [23]).

4) Figure 4: Different strategies to delivery DNA with microbubbles

5) Figure 5: Different approaches of DNA loading on microbubbles

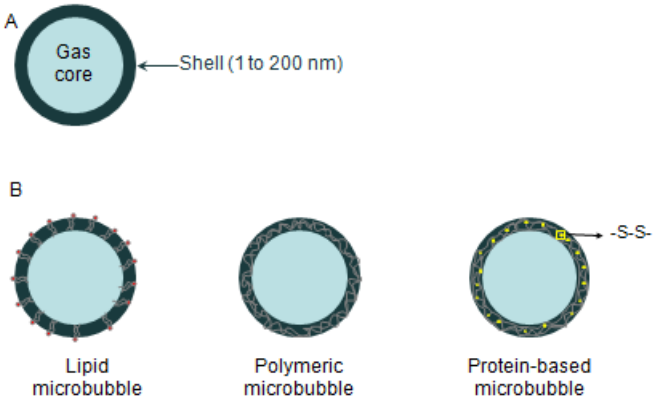

Figure 1

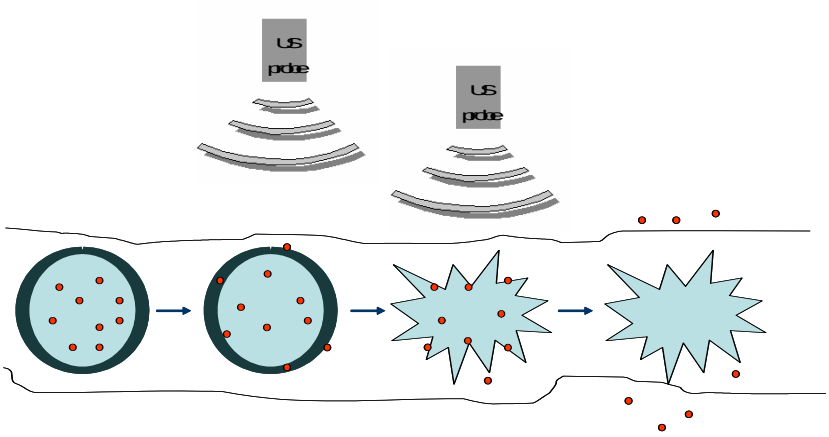

Figure 2

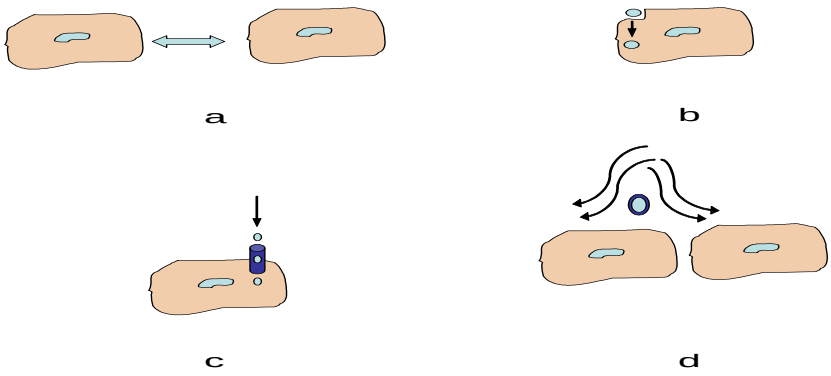

Figure 3

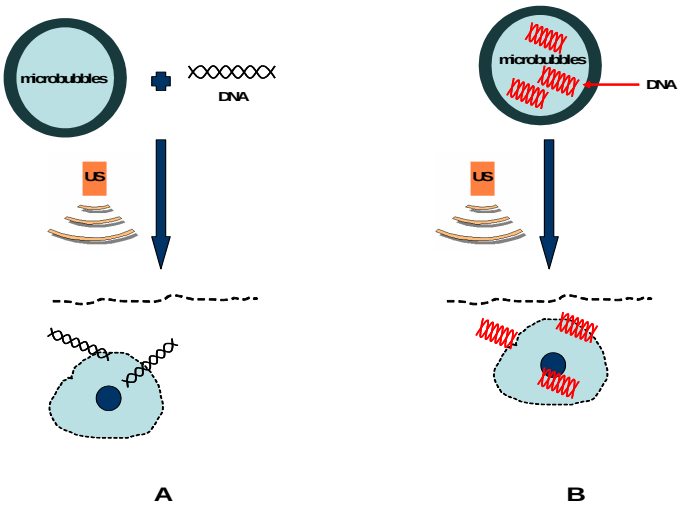

Figure 4

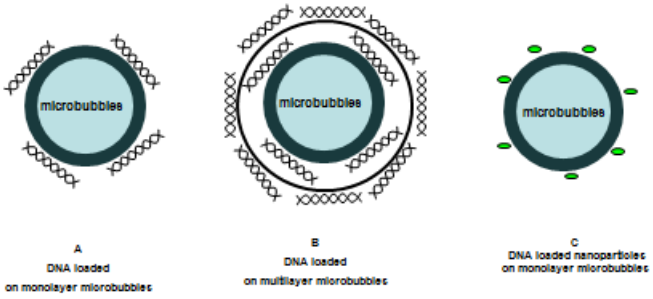

Figure 5