Design of Microbubbles for Gene/Drug Delivery

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Abstract

The role of ultrasound contrast agents (UCA) initially designed for diagnosis has evolved towards a therapeutic use. Ultrasound (US) for triggered drug delivery has many advantages. In particular, it enables a high spatial control of drug release, thus potentially allowing activation of drug delivery only in the targeted region, and not in surrounding healthy tissue. Moreover, UCA imaging can also be used firstly to precisely locate the target region to, and then used to monitor the drug delivery process by tracking the location of release occurrence. All these features make UCA and ultrasound attractive means to mediate drug delivery. The three main potential clinical indications for drug/gene US delivery are (i) the cardiovascular system, (ii) the central nervous system for small molecule delivery, and (iii) tumor therapy using cytotoxic drugs. Although promising results have been achieved in preclinical studies in various animal models, still very few examples of clinical use have been reported. In this chapter will be addressed the aspects pertaining to UCA formulation (chemical composition, mode of preparation, analytical methods…) and the requirement for a potential translation into the clinic following approval by regulatory authorities.

Keywords

Microbubbles • Gene and Drug Delivery

11.1 Introduction

Gas microbubbles have long been used as ultrasound contrast agents (UCA). These can be made of phospholipids, albumin, surfactant or synthetic polymer shells filled with gas displaying low aqueous solubility, such as fluorinated gases

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(*e.g.,* sulfurhexafluoride or perfluorobutane). Their size ranges typically between 2–8 μm (*i.e.,* below red blood size diameter), and thus are considered as pure blood pool agent. These stabilized gas bubbles proved to be useful in many different clinical contexts where imaging of perfusion is desired, such as for the characterization of liver diseases (Burns et al. 2000; Tranquart et al. 2008), left ventricular opacification (Senior et al. 2000; Kitzman et al. 2000) or response of malignant tumors to therapy (Lassau et al. 2007).

In recent years, the role of these microbubbles has expanded beyond their primary role in diagnostics to potentially provide new options for therapeutic purposes. The three main clinical indications for drug/gene US delivery are (i) the cardiovascular system (Unger et al. 2014), (ii) the central nervous system for small molecule delivery (Aryal et al. 2014; Liu et al. 2014), and (iii) tumor therapy using cytotoxic drugs (Ibsen et al. 2013a). All these applications rely on the mechanical properties of UCA when exposed to ultrasound. Indeed, when insonified under specific ultrasound regimen, UCA undergo oscillations inducing acoustic cavitation, allowing an increase in cell membrane permeability in the close vicinity of the UCA. This enables drugs to cross the membrane, thus penetrating into cells and ultimately inducing a therapeutic effect. On the same principle, UCA can also act on vessel permeability for promoting drug access to the extracellular space. Recent promising studies exploiting this property showed that UCA were able to transiently open the blood brain barrier (BBB) for drug delivery to the brain (Liu et al. 2014). Another emerging application is sonothrombolysis (STL), where UCA are used to promote blood clot lysis, leading to recanalization of previously occluded vessels and ultimately improved long-term outcome. This could become of a major clinical interest in the context of ischemic stroke or myocardial ischemia.

Besides UCA, use of ultrasound (US) for triggered drug delivery has many advantages. In particular, it enables a high spatial control of drug release; since the US beam can be focused as such that it represents only a few cubic millimeters of volume. Thus, it potentially allows activation of the drug delivery only in the targeted region, and not in the surrounding healthy tissue. Moreover, imaging of UCA can be used to firstly precisely locate the target region, and then used to monitor the drug delivery process by tracking the location of release occurrence. All these features make UCA and ultrasound an attractive means to mediate drug delivery.

In the course of all these developments, it appeared obvious that requirement for an efficient therapeutic agent based on UCA could be different than what is needed for an imaging agent. In particular, known limitations of UCA are limited payload, short half-life, size restraining their action exclusively to the vascular bed and poor response to ultrasound exposure due to a polydisperse diameter. Whereas these features are typically less critical for imaging purposes due to the exquisite sensitivity of imaging systems, they are prone to affecting the potency of UCA for drug/gene delivery. This has prompted different groups to focus on the design of UCA for therapeutic use. The topic of drug delivery using microbubble-assisted ultrasound has been addressed in several recent reviews (Lentacker et al. 2014; Sirsi and Borden 2014; Rychak and Klibanov 2014). This is also addressed in detail in different chapters of this book.

The purpose of this chapter is more to focus on the aspects of UCA formulation (chemical composition, mode of preparation, analytical methods…) and the requirement for a potential translation into the clinic following approval by regulatory authorities.

11.2 Formulation

11.2.1 General Consideration

11.2.1.1 Shell Components

Shell component selection is important. Semi-synthetic lipids will be favored because of their non-animal origin. Addition of a lipopolymer molecule (*e.g.*, PEGylated lipids) will improve blood circulation lifetime, but will potentially also impair drug loading mediated through non-covalent adsorption, such as hydrophobic or electrostatic

associations. Of interest, manufacturing process and nature of the shell components can also impact the shell membrane homogeneity. Indeed, this was nicely illustrated when microdomain, resulting from sequestration of some lipids, could promote heterogeneity in the UCA shell (Kim et al. 2003). In the same trend, it was recently demonstrated that replacing distearoylphosphatidylcholine (DSPC) by diphosphorylphosphatidylcholine (DPPC) in DSPE-PEG2000-biotin microbubbles yielded a more homogenous distribution of biotin ligands, as probed with fluorescently labeled streptavidin (Kooiman et al. 2014). As a consequence, this could affect the actual loading efficiency of the prepared UCA by restraining drug loading to a limited surface area at the UCA surface. Specific lipid mixtures or manufacturing conditions (heating) could be investigated in order to alter this heterogeneity.

11.2.1.2 UCA Stability and Lifetime

UCA bloodstream lifetime has considerably improved over the past years, now providing good imaging time frames in the range of a few minutes. However, for drug delivery this lifetime might not be optimal to allow release of the therapeutic agent, and thus to mediate the desired biological effect. In that sense, polymeric UCA may provide improved circulation time, but conversely, release of drug load from the polymeric shell must be carefully assessed. Indeed, upon destruction most of the shell remains intact (see below for details). Moreover, UCA with thick and rigid shells (such as protein or polymer-shelled) usually display poor oscillation behavior, unlike lipid-shelled UCA. This will likely have an impact on the cavitation effect induced by ultrasound, and thus their potency to promote a therapeutic effect. It has also been shown that these thick shelled-agents might display a higher loading capacity compared to soft shell agent.

Gas nature is an important player for the lifetime and stability, and a mixture of perfluorocarbon and nitrogen could be preferable to limit the risks of UCA size modification resulting from exchange with soluble nitrogen present in biological fluids. As such, these size changes might induce different responses to the same driven acoustic frequency, and thus might become less sensitive to ultrasound activation.

Since multiple passages are necessary to allow destruction of sufficient UCA for increasing accumulation of the therapeutic agent, the mode of administration might be considered. As an example, using continuous infusion in place of bolus injection of UCA would help in maintaining a constant concentration for a longer time, even at a lower range than that obtained with a bolus. The combination of UCA amount and US pulse characteristics should be carefully tuned according to the selected indication. This could require a high concentration for an instant, or a lower concentration for a longer time. This can significantly enhance drug delivery efficacy, while addressing possible safety issues.

11.2.2 Conditions Allowing Drug Delivery

Generally, UCA loaded with drug or in combination with circulating therapeutic agent can be activated in two manners for delivery: stable cavitation or inertial cavitation. Stable cavitation is usually achieved at relatively low acoustic pressure and is generated when UCA undergo repetitive oscillation. These expansions and contractions generate shear stress to cell membranes, affecting their permeability (van Bavel 2007). In contrast, inertial cavitation is obtained at higher acoustic pressure and results from violent UCA destruction, causing strong biophysical effects, microjets and microstreaming in the immediate surrounding environment (Husseini et al. 2005). This behavior is valid for soft-shelled UCA. In the case of hard shell agents, higher levels of energy are required to generate cavitation due to their inherent shell stiffness. Thus, under these high acoustic pressures, these UCA undergo violent rupture releasing free gas microbubbles through small defects in the shell, or so-called ‘sonic cracks’ (Bloch et al. 2003) (see Fig. 11.1 for illustration). This complex interaction between ultrasound waves, microbubbles and tissue will not be described in detail in the present chapter, but it is the object of a different dedicated chapter (see Chap. 9).

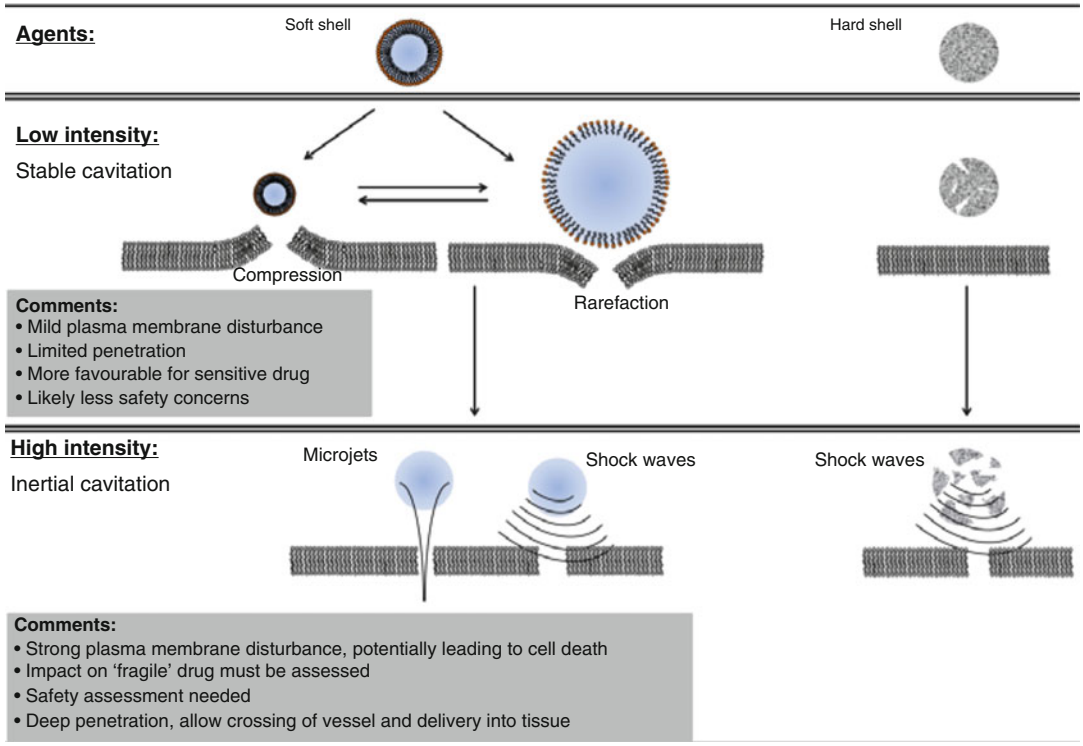

Fig. 11.1 Illustration of the physical mechanisms underlying the biological effect of soft or hard shell Ultrasound Contrast Agents (Adapted from Kiessling et al. 2014)

11.2.3 Drug Delivery with UCA and Ultrasound

11.2.3.1 Plain UCA with Free Drug

There are several reports describing the use of a mixture of UCA and drug for therapeutic purposes. This is a very convenient approach due to the immediate availability of both microbubbles and drug agents. It is also seen as a straightforward continuation of the current diagnostic use of clinically approved agents. Thus, many of these reports have used marketed UCA (Definity®, SonoVue®, Optison®…), and most advanced clinical studies have been conducted for the purpose of clot lysis, so-called sonothrombolysis (de Saint Victor et al. 2014), and more recently for pancreatic cancer (Kotopoulis et al. 2014). Conditions described were in presence or absence of fibrinolytic agent r-tPa, and under the regime of various ultrasound settings (various frequencies, acoustic pressure, pulse repetition frequency…). Some clinical trials have shown the potency of this approach to treat patients suffering from stroke by enabling faster and more complete recanalization (Molina et al. 2006). However, current trials did not provide significant improvement in patient conditions at 3 months post-treatment, partially due to the limited number of patients enrolled. Moreover, one of the main lessons to retrieve from these studies is that still little is known of the mechanism underlying UCA-enhanced sonothrombolysis (STL), and in some cases, lack of control of the biological effects of this new therapeutic approach has triggered some safety concerns. It is thought that design of UCA specific for STL indication has the potential to further improve not only their effect as treatment enhancers, but also their safety profile.

The approach of administering a mixture of UCA and drug has also been exploited for other therapeutic applications, such as delivery of cytotoxic drug (Ibsen et al. 2013a; Escoffre et al. 2013), BBB opening (Aryal et al. 2014) or gene delivery

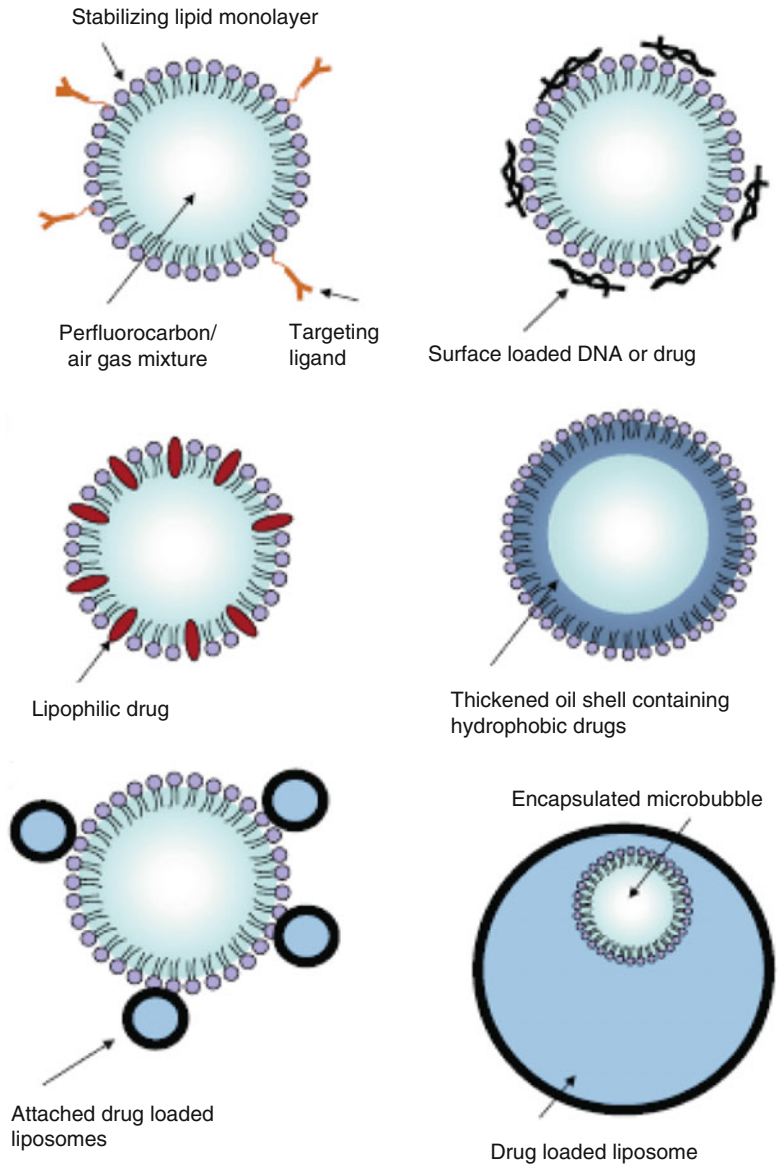

Fig. 11.2 Various options for the design of drug-loaded ultrasound contrast agent. (**a**) The basic microbubble design with a lipid monolayer stabilizing the gas core. Targeting ligands can be conjugated to the surface to help facilitate accumulation of the microbubble in desired tissues. These ligands can be antibodies or short peptide sequences. (**b**) The microbubble itself can be a vehicle by attaching drugs and even DNA to the surface through the use of electrostatic attractions. (**c**) Lipophilic drugs can be incorporated into the lipid monolayer shell of the microbubble. (**d**) The stabilizing shell can be thickened with an oil layer, allowing hydrophobic drugs to be carried within it. (**e**) Drug-filled liposomes can be attached to the surface of the microbubble. When the microbubble is exposed to ultrasound, the liposomes are disrupted by the mechanical actuation of drug release. (**f**) The microbubble can be encapsulated within a liposome along with the drug. When exposed to ultrasound, the microbubble ruptures the outer liposome, releasing the payload (Reproduced from Ibsen et al. 2013a)

(Rychak and Klibanov 2014; Newman and Bettinger 2007). The recent report of a successful chemotherapy improvement by using both ultrasound and UCA to treat pancreatic cancer has highlighted the clinical feasibility of such an approach, even though this has only been done on a limited number of patients (Kotopoulis et al. 2013).

In parallel to the co-administration approach of free drug and UCA, several groups have also investigated the potential of co-localization of drug and cavitational activity on therapeutic efficiency by preparing drug-loaded UCA. Indeed, it is speculated that incorporation of therapeutic agent close to the UCA shell would be more favorable since cavitation will likely drive delivery.

11.2.3.2 Drug-Loaded UCA

Drug loaded UCA have been formulated using different approaches, as illustrated in Fig. 11.2. Several strategies have been employed to incorporate therapeutic agent in UCA, such as chemical conjugation, electrostatic adsorption at the

outer surface and embedding in the shell. The choice for the best method of loading depends mainly on the nature of the drug.

One additional advantage of loading UCA with therapeutics is that it can act as a protective drug carrier. Thus, unstable drugs can be protected from degradation in biological fluids, thus prolonging their half-life. This was nicely illustrated by DNA molecules, for which enhanced resistance to nuclease degradation was measured (Lentacker et al. 2006). On top of that, the limited release outside the insonified area due to the relatively low peripheral microbubble destruction could help in preventing major adverse events related to the drug itself.

11.2.3.2.1 Drug in/on the Shell

Some studies have demonstrated that covalent attachment of the drug is more favorable to elicit a therapeutic effect. For example, conjugation of rose bengal onto UCA was proven to be more cytotoxic compared to un-conjugated sonosensitizer (Nomikou et al. 2012); so-called sonodynamic therapy (SDT). This approach could be of interest for the treatment of cancer lesions, and thanks to a relative good US tissue penetration, it appears better than typical light excitation used in photodynamic therapy (PDT).

However, careful evaluations are warranted to measure the impact of the loading on the actual drug activity. For example, r-tPA proved to be a quite complex molecule to formulate with the need to use specific conditions to maintain biological activity (risk of aggregation of the native protein, personal results). In addition, if chemical conjugation is employed, careful control of the conjugation process is needed to ensure that biological activity of the drug is not altered. Moreover, loading procedure could also impact the actual bioavailability of the drug. Thus, even though drug loading efficiency is obviously important, formula optimization should equally be considered to ensure a proper delivery of the active drug to the site of treatment.

Being inspired by cationic lipids or polymers used for non-viral gene delivery (so-called lipoplexes and polyplexes, respectively), some investigators have prepared UCA bearing cationic charges at the shell surface. For phospholipidic shells, this can be achieved by inserting cationic lipids (*e.g.*, DSTAP) to trigger a positive zeta potential of the microbubbles. This significantly increases the loading of nucleic acids, and several reports have demonstrated usefulness of these UCA constructs to promote gene delivery *in-vitro* and *in-vivo* (Wang et al. 2012; Rychak and Klibanov 2014).

Due to the effective loading capacity being restricted to the UCA shell, drug-loading vehicles have mainly been developed with highly potent drugs, such as nucleic acid, known to be active in the μg range. It was estimated, on average, that loading capacity of UCA for nucleic acid complexation is typically in the range of 0.01 pg/μm^2. For a 2 μm UCA diameter, this corresponds to ca. 0.12 pg/microbubble. Thus, almost 10 million UCA are required to carry 1 μg of nucleic acid, illustrating the limited loading capacity of regular cationic UCA. As an example, the situation is similar for drugs when compared to usual circulating drug concentration during chemotherapy. This is why some groups have used the approach of pre-loading drug into nanoparticles prior to coupling them onto the UCA shell surface (Mullin et al. 2013).

11.2.3.2.2 Nanoparticles on UCA

These nanoparticles can be of different natures, such as liposomes (Kheirolomoom et al. 2007) or poly(lactic-co-glycolic acid) (PLGA) (Chappell et al. 2008). In particular, liposomal preparations of Doxorubicin are quite popular thanks to the availability of FDA approved products (*e.g.,* Doxil®). Properties of the drug to be formulated are of course very important, as it will have a direct impact on the loading efficiency. For example, highly hydrophilic drugs would be difficult to embed into the shell. Most importantly, impact of the drug loading on the UCA ultrasound responsiveness must be evaluated since modifications in UCA stability, risk of aggregation and shell properties (increase in stiffness) can be observed under certain conditions.

There are numerous possible approaches to associate the nanoparticles to the UCA. This can be achieved by biotin-avidin interaction, but this

approach is only valid for research or pre-clinical evaluation due to potential immune reaction with this protein (Chinol et al. 1998). An alternative mean to enable strong association relies on the use of chemical conjugation, such as amide, disulfide or thioether bond formation. Electrostatic attachment is another possibility leading to an easy preparation, but it does carry the risk of lack of reproducibility and lack of association control, impeding further development of this method towards clinical use.

11.2.3.2.3 UCA in Drug-Loaded Liposomes

Interestingly, a recent procedure has been described to increase UCA drug loading. This was accomplished by encapsulating the UCA within the internal aqueous space of a drug-loaded liposome (Ibsen et al. 2011). This rather unusual construct must be evaluated to assess not only the drug delivery potential compared to regular nanoparticle-loaded UCA, but also the US-responsiveness of the encapsulated UCA.

11.2.3.2.4 Hard Shell

In addition to the use of drug pre-formulated in nanoparticles, thick shelled UCA could also be a means to improve drug payload (Lensen et al. 2011). These agents are usually made of polymers and have shells of 20–100 nm thickness. Shell stiffness of these agents requires specific insonation parameters to mediate delivery. Indeed as these agents will not oscillate at low acoustic pressure, but require higher pressure to be activated leading to rupture and gas escape. For this reason, the potential issue that can be faced with this formulation is the drug release from the polymer shell; the shells remain in majority intact as only cracks are observed. Modification of shell properties, by altering shell thickness and composition, could improve both behaviors under US exposure and drug delivery efficiency. In addition, the nature of the polymer must be taken into consideration, particularly when dealing with safety linked to the biocomptability. In the same trend, it has been speculated that use of UCA composed of components with different shell properties can release a drug in a stepwise manner.

11.2.3.2.5 Nanoemulsion

Another possible approach is the use of drug-loaded nanoemulsion (Rapoport et al. 2009). These nanoparticles are made of liquid perfluorocarbon (*e.g.,* perfluoropentane). US exposure triggered heating causes a droplet-to-bubble phase shift, resulting in the *in-situ* formation of drug loaded UCA that can be further activated by distinct US conditions. The impact of phase shift has to be addressed in detail since it can alter the chemical integrity of the loaded drug. These particles display a particularly long circulation time, with up to 50 % of the injected dose still remaining in the circulation 2 h after administration (Rapoport et al. 2011). Moreover, thanks to their relatively small size (200–500 nm), they also have the opportunity to reach compartments not normally accessible to UCA. This is by escaping the blood space, so-called extravasation. Due to the narrow temperature range required for this transition phase, the treatment could be precisely tuned by modulating this temperature increase when focusing the ultrasound beam in the specified location. This will be extensively described in another chapter of this book (see Chap. 14).

11.2.3.2.6 Monosize

Recently, a new way to formulate drug in UCA relying on the use of microfluidics technology was described. Indeed, successful preparation of multilayer gas lipospheres was reported using flow-focusing geometry (Hettiarachchi et al. 2009). These rather complex constructs are composed of a phospholipid shell, an oil layer comprising cytotoxic drug doxorubicin, and an inner gas core. Moreover, this technology can address one additional critical parameter for an efficient drug delivery; control of UCA size so that it nicely matches the selected US frequency. Thus, devices allowing UCA preparation displaying a narrow size distribution were specifically designed. These developments will likely prove to be important for further improvement of drug delivery by ensuring that all the UCA exposed to US will be activated at a defined frequency, maximizing the delivery of the loaded drug. Use of monodisperse UCA may also improve the efficacy/safety balance by allowing use of lower

acoustic energy to elicit drug release, knowing that all UCA will eventually respond to US exposure at the selected frequency.

Use of UCA for BBB opening has been described in several reports (Liu et al. 2014; Choi et al. 2010), most of the tested agents being approved UCA. However, UCA formulation with a defined diameter may improve drug delivery to the brain since it was shown that 4–5 μm size was more effective than 1–2 μm UCA, under tested conditions (Choi et al. 2010). This reinforces again the need to develop manufacturing processes allowing preparation of monodisperse UCA. However, currently the main limitation is the yield of such manufacturing which does not allow a high amount of UCA preparation, meaning that acoustic characterization and *in-vivo* tests are still challenging.

11.2.4 Optimization of Drug Delivery

Close contact between UCA and the target tissue should intuitively favor drug delivery, vessel permeability or clot lysis. Thus, a clever way to further improve drug delivery efficiency could be the use of targeting strategies such as either passive or active accumulation. Passive targeting can result from interaction of some shell components with the specific particle's clearance system. This is clearly illustrated by phosphatidylserine-containing UCA (*e.g.,* Sonazoid®), reported as being efficiently taken up by Kupffer cells.

The specificity of this UCA might be increased by adopting a specific targeting strategy for it. These UCA differ from those initially developed for blood pool imaging by the presence of a targeting moiety able to link the bubble to a selected cell biomarker. The general strategy is to link molecular entities to the phospholipid-stabilizing monolayer, allowing the bubbles to remain attached to selected sites in the vascular compartment. Once attached, these bubbles act as echo-enhancers, similarly to blood pool agents, since the signal is generated by the bubble itself and not the ligand. This raised important points.

As the bubbles remain strictly within the vascular compartment, targets must be selected that are accessible to them *i.e.* on the luminal side of endothelial cells (Bettinger et al. 2012; Pochon et al. 2010). Two application areas are well characterized: neoangiogenesis and inflammation, since both involve endothelial cells. Tumoral neoangiogenesis (Deshpande et al. 2010; Willmann et al. 2010), *i.e.,* the formation of new blood vessels, is a fundamental process occurring during tumor progression and is triggered by hypoxia. In the course of the inflammatory process, various cell surface markers are expressed or up-regulated on the endothelial luminal side, and are therefore accessible to targeted microbubbles. Site targeted microbubbles can also be used for the visualization of thrombi associated with stroke.

The attachment of bubbles to the surface of endothelial cells must be strong enough to withstand vascular areas where shear stress is high due to high blood velocity and viscosity. The attachment could be enhanced by adding radiation force. This improves the interactions between microbubbles themselves and their interactions with endothelial cells. Otherwise, incorporation of magnetic particles within the shell of the core could enhance attachment. By adopting such a strategy, we not only have access to highly specific areas to drive the desired therapeutic effect, but we can also significantly limit adverse events by limiting interaction with non-affected regions.

Efficient ligand-target interaction can be achieved by either using flexible spacers to present the targeting ligand in the most effective way, or by fine-tuning the ligand density at the UCA surface. To improve circulation time and binding specificity, a clever approach of a 'buried ligand' has also been recently proposed (Borden et al. 2006, 2008). Active accumulation could also be achieved by physical means, such as acoustic radiation force (Kheirolomoom et al. 2007; Frinking et al. 2012) or magnetic force to concentrate UCA at the site of treatment (Stride et al. 2009).

11.2.5 Characterization of UCA

An important aspect of UCA preparation is the quality control assessment (Fig. 11.3). This is

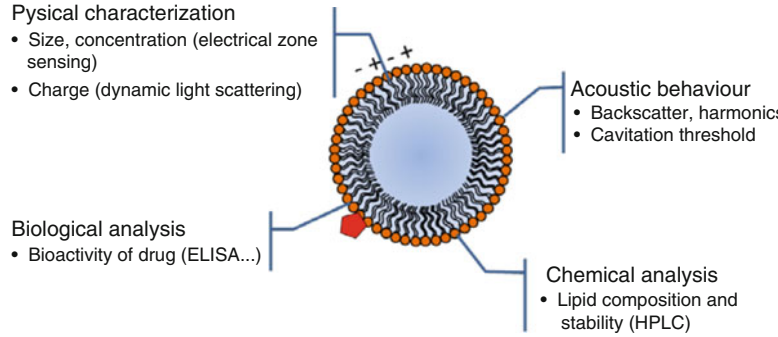

Fig. 11.3 Methods available for the quality control assessment of UCA (Red pentagon represents a drug molecule)

even more acute in the case of these rather complex UCA-drug constructs. As for regular UCA, size can be determined by electrical zone sensing, using the so-called Coulter Counter. This is a fast and accurate method to measure at the same time UCA concentration, gas volume, size distribution (in volume and number) and UCA surface. Other methods exist, but so far they have proven less suitable than electrical zone sensing due to technical limitation from the natural buoyancy behavior of UCA in liquid.

Zeta potential is also an important parameter to assess as it provides information on UCA surface charge. It relies on electrophoretic light scattering technology. The magnitude of the zeta potential gives an indication of the potential stability of the formed UCA. If all the UCA particles in suspension have a large negative or positive zeta potential, then they will tend to repel each other. On the other hand, if UCA particles display low zeta potential values, there will be a tendency for the particles to come together, promoting aggregation behavior. This measure could also be useful to monitor adsorption of nucleic acid molecules on positively charged UCA.

In addition to these physical characterizations, chemical analysis of the shell lipid content is mandatory to ensure that the process of UCA preparation is robust and reproducible. For this purpose, specific analytical methods must be developed to allow titration of each UCA component, as well as the loaded drug. These methods mostly rely on reverse phase High-Performance Liquid Chromatography (RP-HPLC). For native lipids (not derivatized), the Evaporative Light-Scattering Detector (ELSD) is far more useful for lipid titration than the commonly used ultraviolet (UV) detector. Knowing the minute amount of material required for the formation of these stabilized gas microbubbles, this can be a challenging task, and usually requires extensive developments with robust and validated analytical methods. These methods will also be useful for controlling if loaded drug is affected by the manufacturing process. Indeed, drugs could be sensitive toward specific formulation conditions, such as heating and agitation (particularly for the case of probe sonication technique). This is why it is also important to assess whether biological activity of the drug remains unaffected by the formulation process. This is done using specific methods such as enzyme-linked immunosorbent assay (ELISA), or chromogenic assay for drugs with enzymatic activity (*e.g.,* r-tPa).

Besides these physico-chemical analyses, acoustic characterization is warranted to ensure that formulated UCA (with or without drug) are US-responsive. This can be achieved by performing backscatter measurement or determining cavitation threshold. In addition, some of these methods will also be useful to study the stability of UCA formulation. For example, evaluating the impact of long-term storage under stress conditions, such as temperature can be assessed on UCA concentration, chemical integrity of shell components, drug bioactivity and acoustic properties.

Finally, for the screening of drug-loaded UCA formulations in pre-clinical studies, development of analytical methods for assessing efficiency of drug delivery is required. In this trend, a recent report described a LC-MS/MS analysis method that allowed titration of doxorubicin in tumor

tissue extracts with a limit of detection of 7.8 pg (Ibsen et al. 2013b). Further to all these considerations and speculation, there is still some controversy about the relative advantage of administering (i) a mixture of drug and UCA or (ii) drug loaded-UCA. The second approach might prove easier, particularly when moving toward preparation of a Chemistry, Manufacturing and Controls (CMC) dossier for submission to regulatory authorities. This will be addressed in more detail in the last paragraph of this chapter.

11.3 Clinical Translation and Regulatory Issues

A major advantage of UCA is their capability to enable site-specific delivery of the drug by selective destruction of UCA with a non-invasive ultrasonic stimulus. However, this technique still presents many obstacles that need to be addressed and solved before moving into the clinic.

Firstly, a better understanding of the underlying mechanism of this approach is warranted to ensure safe delivery into patients. This is presented in another chapter (see Chap. 9), but so far, many concurrent or challenging hypotheses have been proposed without a unique theory. Even though it is not a pre-requisite to perform *in-vitro* or preclinical tests, the absence of a clear mechanism is a significant barrier for health authorities. This is reinforced by the observation of possible adverse events due to the UCA, the drug or the US beam, meaning that the contribution of each component is clarified. It is a prime importance since bioeffects have been observed in some studies (Vancraeynest et al. 2006). Safety assessment for the therapeutic application must be considered differently than for the diagnostic application. Indeed, whereas for diagnostic purposes, absence of or a few bioeffects is mandatory, it is however likely to be different for therapy to a certain extent. Secondly, one of the main limitations of UCA is their relatively low circulation time, typically ranging from 5–15 min, thus limiting their delivery potential. Thirdly, UCA are efficiently captured by organs, such as liver and spleen, raising the issue of unwanted accumulation in non-targeted organs/tissues that could become detrimental to the patient.

Preparing UCA loaded with a therapeutic agent, and possibly a ligand, for targeting can prove to be complex to manage for CMC dossier preparation. In particular, it is difficult to precisely measure the amount of drug loaded onto UCA, and to develop a process allowing reproducible preparation of such drug-loaded UCA. In that sense, a thorough evaluation of the advantage of loading drug onto UCA over co-injecting of free drug and UCA must be done. Preparation of drug-loaded UCA will lead to a new chemical entity, implying development of a complete process of manufacturing complying with GMP regulations. In contrast, use of approved drug and UCA could present some advantages in terms of development time and cost, if efficacy compared to drug-UCA complex is demonstrated in pre-clinical studies. However, it is also important to obtain authorities guidance on this approach since the use of targeting procedures for increasing local delivery will modify the natural metabolism of the approved drug. Therefore, this point must be carefully addressed during the course of the development of this new therapeutic method in terms of local and circulating concentration, changes in metabolic pathways and therapeutic dose.

In terms of quality control of drug-loaded UCA for chemical conjugation, it will be easier if conjugation occurs prior to UCA preparation. This will enable thorough chemical characterization of the conjugate, and ensure that the conjugation process did not alter biological activity of the therapeutic agent. One advantage of co-administrating the therapeutic agent and UCA is that there is no limitation in terms of drug dose, unlike what can be experienced with drug-loaded UCA.

From a regulatory point of view, the gas microbubble is considered as the active entity, meaning that each of the microbubble components should be fully characterized. Furthermore, the manufacture of clinical materials should be carried out in compliance with the Good Manufacturing Practice. With respect to the formulation characteristics, the selection of the ingredients is of outmost importance since the use of specific components should be validated for these new drug delivery systems for parenteral administration. In that perspective,

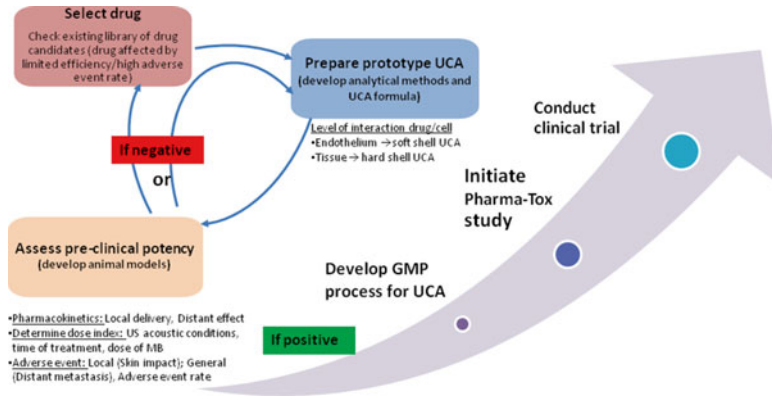

Fig. 11.4 Pre-clinical steps towards UCA clinical development for therapeutic use

the retained formulation for clinical trials must be challenged before finalization, as changing any of the components at a later stage could be difficult, even impossible, and costly.

Once the formulation is finalized, many steps must be accomplished before any clinical use: robustness of the manufacturing process, stability of the product and validation of the test methods. An additional requirement is a pharma-toxicology package that follows the International Conference on Harmonization (ICH) guidelines. Even though adverse events cannot be considered as a limiting factor for the use of UCA in ultrasound imaging, the introduction of therapeutic drugs in addition of other materials in the bubble requires specific toxicology assessment. For conventional agents, it is generally admitted that the rate of these events (around 0.01 % for serious adverse events based on post-marketing safety data, with no significant differences between agents) is below what is reported for iodinated compounds and MR agents. Even though the therapeutic field is exposed to higher rates of adverse events in relation to the intrinsic nature of the interaction with tissues, it is essential to demonstrate that the risk/benefit ratio remains positive and higher than without the introduction of UCA and US. Some key points need to be investigated, such as allergic reactions to foreign materials, maximal tolerable dose in animals to determine the maximal dose to be used in patients, and the absence of compromised blood flow after injection due to sticking of UCA to endothelial cells outside the therapeutic area. These different steps are time-consuming and expensive, and can be summarized as illustrated in Fig. 11.4.

Finally, when the steps above have been completed, the agent is suitable for clinical testing pending Investigational New Drug Application and Institutional Review Board (IRB) or ethical committee approval for the selected indication. Regulatory approval must be carefully considered since three components are closely interacting; the microbubble, the therapeutic drug and ultrasound waves. Whether we can precisely characterize the microbubble constituents and the acoustic parameters, the use of various therapeutic drugs is still a barrier. Indeed, do we need to get an approval of this device whatever the drug is, this being valid for the injection of free drug only, or do we need to get an approval for each individual drug to be used? The use of drug-loaded systems should expose us to a different regulatory pathway, which must be drug-specific.

A key component, which is not discussed in the present chapter, is the acoustic contribution to the desired effect. As such, there is a need to adapt the machines to this new modality, but in the same this is changing the nature of the equipment, which is moving from a pure diagnostic field to the therapeutic field. This means different requirements and higher regulatory constraints. In that perspective, a strong partnership is needed to exploit the potential of this therapeutic approach, and to strengthen the place of UCA in the diagnostic palette so they can be used by physicians according to their specific demand.

Conclusion

Drug delivery mediated using microbubble-assisted ultrasound is a promising therapeutic approach. The fact that ultrasound is a non-invasive technique, that can transmit energy deep into a discrete region of tissue or organ, is a key feature for enabling local drug delivery. In addition to the drug-delivery capabilities, one must bear in mind that UCA are also imaging agents, giving rise to the concept of therapy and diagnostics with one single agent, so-called 'theranostics'.

Future improvements are warranted to pave the way for a clinical translation of this innovative therapeutic approach; (i) manufacture of UCA for longer circulation time and higher loading capacity, (ii) development of specific US protocols and (iii) tackling regulatory hurdles for clinical use in safe conditions. This requires a multi-disciplinary approach, with a close collaboration with ultrasound and drug pharma companies in order to sequentially address the different challenges with a clinical objective. The limited number of drugs tested in these conditions, together with the absence of clinical results, represents a significant drawback, indicating that clinical approval of this method will require at least 5 additional years. Emerging formulation based on microfluidics could become a disruptive technology, by allowing the manufacturing of UCA on spot, using FDA-approved devices. This will completely change the game in the field of UCA, since some features considered as important when using regular manufacturing processes could be obsolete for microfluidics-based preparation (*e.g.,* long-term stability).

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