Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Journal Name

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ARTICLE

Recent Progress of Liposomes in Nanomedicine

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Liposomes, spherical vesicles consisting of one or more lipid bilayer membrane(s) encapsulating an aqueous medium, are among the prominent players in the field of nanomedicine. Herein, we highlight the newest, atypical applications of liposomes towards their use in biomedicine. In particular, we put special emphasis on innovative chemical modification of liposomes, the interactions of liposomes with cells under the influence of shear stress, and the utilisation of liposomes as drug deposits in polymeric films and as components in synthetic cell mimics.

Introduction

Liposomes have been an integral component in drug delivery for more than 3 decades due to their ability to encapsulate hydrophilic drugs in the aqueous cavity within the liposome and lyophilic components between the lipid bilayer.[1] Their capability to protect the entrapped drugs against destructive conditions like pH changes, light and enzymes combined with their low toxicity and their enhanced permeability and retention effect proofed liposomes as efficient carriers for therapeutics.[2, 3] Yet still the application of liposomes involves difficulties, which need to be circumvented: the rapid sequestration of liposomes by macrophages,[3] drug leakage due to structural instability,[4] and active targeting is still limited to just a few specific tumors.[2] As a consequence, only few liposome-based formulations made it to clinics.[5-9] However, liposomes have found application in new emerging areas in the field of nanomedicine.

The aim of this highlight is to outline and discuss novel and promising findings from the last 3 years focusing on i) developments in the chemical modification of liposomes e.g., their use as carriers for nanoparticles, ii) the use of microfluidics to assess the interaction of liposomal formulations with cells, iii) liposomes as drug deposits in polymer films, and iv) liposomes in therapeutic cell mimicry. In highlighting the non-conventional application of liposomes in nanomedicine, we hope to demonstrate that these carriers have the potential to be successful in biomedicine beyond their typically envisioned applications.

Chemical modification of liposomes

This section highlights recent achievements in the (chemical) functionalisation of liposomes in the last 3 years. For comprehensive reviews on this topic covering prior findings refer to Perche et al.[10] or Maherani et al.[11]

Among the various bioconjugation methods available, e.g. amide or thiol-maleimide coupling, many of them suffer from a lack of specificity, since the corresponding functional group can be found in other membrane components or in the entrapped cargo, resulting in undesired side reactions. Herein, we highlight two recent elegant examples of chemically modified liposomes circumventing this issue. Vabbilisetty et al. utilised the specific Staudinger ligation in order to functionalise the surface of liposomes with an azo-derivative of lactose (model system for carbohydrate molecules, typical in bio-recognition processes, like metastasis or inflammation).[12] This bio-orthogonal chemical reaction was applied on 1,2-dipalmitoyl-sn-glycero-3-phosphocholine based liposomes co-assembled with an triphenylphosphine derivative of cholesterol. They demonstrated that the reaction proceeded in high yields, under mild conditions and without the use of any catalysts. In contrast to liposomes stabilised with cholesterol only, the glyco-functionalised liposomes revealed a significant increased structural integrity, combined with a sustained dye release rate over two weeks. Further, the grafted lactose was positively agglutinated to lectine and proofed the successful acquisition of a targeting property by the glycol-functionalisation. Best and coworker reported an alternative approach to generate light-disruptive liposomes.[13] They developed an ingenious molecular analogue based on the structure of the lipid phosphatidylcholine (PC). The architecture of the novel lipid (NB-PC) basically differed from the common PC by a modified sn-2 acyl chain and included a succinyl linker, connecting the PC headgroup to a photo-liable 2-nitrobenzyl group (NB), which in turn was linked to a hexyl group, to add an additional hydrophobic gain. The design allowed the formation of highly stable liposomes, co-assembled with PC. Further, the light-induced release of

encapsulated cargo depending on the amount of NB-PC used for the liposome assembly was confirmed.

The incorporation of nanoparticles into liposomes gained particular interest recently, due to the potential enhancement and expansion of the scope in controlled release drug delivery.[14] In this context Sun and coworker communicated in 2014 about an example for stimuli-triggered liposomal drug release.[12] Based on the fact that silver nanoparticles (AgNP) exhibit surface plasmon resonance and convert most of the energy into heat, they embedded 3.5 nm sized AgNP into the lipid bilayer of liposomes using super critical carbon dioxide. The photothermal effect of the AgNP caused the liposomes to disrupt upon irradiation with UV light and led to an enhanced release of the encapsulated drug. In another study Amstad et al. reported on the preparation of liposomes, comprising self-assembled iron oxide nanoparticles (FeNP) embedded in a lipid membrane.[15] They found that the permeability of the membrane could be modulated, as a consequence of the direct injection of heat into the vesicles, due to a local heating of the FeNPs when an alternating magnetic field was applied. Petri-Fink and coworkers developed a novel fundamental approach towards the incorporation of large nanoparticle clusters within the liposome bilayer.[16] They showed that β-octylglucoside (OG) stabilised superparamagnetic iron oxide nanoparticles (SPION), form cluster-liposome hybrids when they were mixed with liposomes followed by the controlled depletion of the OG. These hybrid structures featured a Janus-like appearance with all incorporated SPION clusters being concentrated at one pole of the liposomes. In magnetic resonance imaging (MRI), a significantly higher sensitivity was suggested compared to the utilization of single particles.

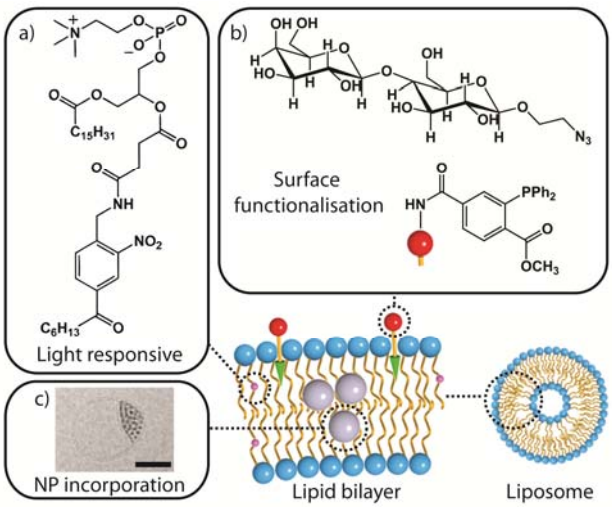

Figure 1. Illustration of a liposome highlighting the domains which were chemically modified recently. a) Modification of the sn-2 acyl chain with a photo-cleavable group.[13] b) Staudinger ligation at a doped liposome surface.[12] c) Incorporation of nanoparticle cluster within the bilayer structure (scale bar 500 nm).[16] c) is partially adapted and reprinted with permission from ref [16].

Shear forces based liposomal formulations for drug delivery

This section highlights the recent advances in the application of an alternative approach (i.e. the use of microfluidic devices) in the evaluation of liposomal formulations as drug delivery systems by taking into consideration the dynamic flow conditions in the body.

In vivo, cells are exposed to several biomechanical stimuli such as shear stress, pressure, and hoop stretch as a result of continuous blood flow circulating within our body. Mechanical stimuli are therefore important factors that influence the behaviour of cells e.g., their differentiation, proliferation, migration, and apoptosis.[17] Due to that, the ability to study cellular responses to mechanical stimuli *in vitro* is an important issue for fundamental studies in cellular biology but also for the development of novel therapeutic nanomaterials for drug delivery. Traditional cell culture studies (i.e., cell culture performed under static conditions in well-plates) are limited in simulating the complexity of the microenvironment of *in vivo* conditions. Devices that allow for the creation of fluid flows and mechanical forces by mimicking physiological environment not only facilitate a more efficient and accurate prediction of therapeutic efficacy and toxicity of drug carriers, they also decrease the reliance of *in vivo* characterisation in early stages, which is often tied to ethical and financial considerations. Microfluidic devices essentially deal with flow under microscopic environment. This system allows for the simulation of the fluidic aspects of the *in vivo* environment with perfect control of flow rate on the surfaces of culture cell, facilitating the study of the relationship between shear stress and drug carrier uptake. In tissue engineering, shear stress has been shown to be dynamically improving the quality of tissues, particularly for blood vessels and cartilage.[18, 19] In the recent years, the importance of shear stress in the cellular uptake/association of liposomal drug delivery systems has also been started to be considered in a few reports.[20-24] For instance in a study on shear stress regulated cellular uptake of liposomes, Strijkers and coworkers assembled an intercellular adhesion molecule-1 (ICAM-1) binding liposomal MRI contrast agent and investigated the association efficiency of these liposomes with mouse brain endothelioma cells in competing presence of mouse leukocytes under physiological shear stress conditions.[25] They found that the efficiency of *in vitro* binding of antiICAM-1 liposomes to ICAM-1 was reduced with increasing shear stress within the physiological relevant range. We have recently evaluated the effect of shear stress on the interaction of myoblast cells with several common drug carriers in the presence of low shear stress.[26] An important finding was that the interaction/association of myoblast cells with positively charged liposomes was more increased in the presence of shear stress while it remained unaffected for negatively charged and neutral ones. As a result, the therapeutic response in terms of cell viability and translocation efficiency

was also improved. In a followed up work, we investigated the response of hepatocytes and myoblasts to different polymer coated liposomes (Figure 2a).[27] PEGylated liposomes showed significant difference in cell association/uptake depending on the different cell types, different exposure time and shear stress, while poly(dopamine) (PDA) coated liposomes associate to a greater extent with hepatocytes than with myoblasts for different exposure time and shear stress.

In the field of drug delivery, it is important for drugs and their carriers to go straight to their targets. This can be achieved by active and/or passive targeting. In an elegant study by Zumbuehl and coworkers, a novel approach of passive targeting of tissues with a leaky vasculature was presented by creating unique lentil-shaped phospholipid liposomes that were sensitive to mechanical stress.[28] The cargo of these drug carriers could be targeted to and released in regions with changes in hydrostatic pressure such as inside a clogged artery without the need for any external trigger (Figure 2bi). This unique behaviour was achieved because the lentil shaped liposomes composed of a special type of synthetic phospholipid that are unstable to shearing forces along their equator (Figure 2bii). As a result, there was a significant high amount of dye release from the vesicles in the constricted artery model (Figure 2biv) than in the healthy artery model (Figure 2biii).

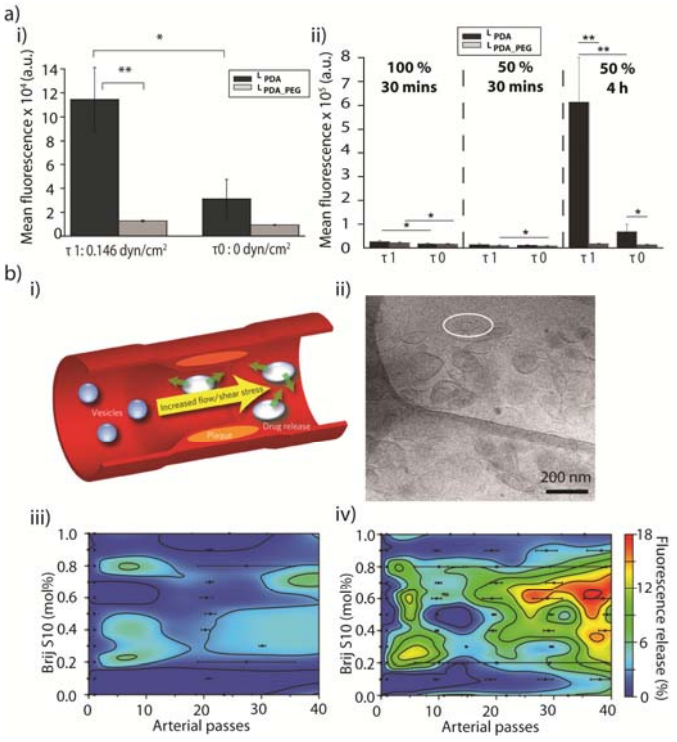

Figure 2. a) Interaction of liposomes with cells in the presence of shear stress: i) Response of hepatocytes exposed to fluorescently labelled poly(dopamine) coated liposomes (L_{PDA}) and PEGylated liposomes (L_{PDA_PEG}) for 30 min under static (τ0) and shear stress (τ1) conditions. ii) Myoblasts exposed to L_{PDA} and L_{PDA_PEG} (both either 100% (non-diluted) or 50% (2× -diluted)) for 30 min and 4 h at τ0 or τ1. Reprinted with permission from ref [27]. b) i) Schematic drawing of the hypothesis using changes in endogenous shear stresses as a physical trigger for drug delivery. ii) TEM image of the lentil shaped liposomes, iii,iv) fluorescence release patterns of these liposomes at 37 °C. Release in the healthy arterial model (iii) and release in the constricted artery model (iv). Reprinted with permission from ref [28].

Liposomes embedded in polymer films

Embedding and retaining small hydrophobic molecules or preserving the activity of structural dependent biomolecules such as enzymes when designing coatings for drug eluting implants remains challenging. Due to their ability to entrap both hydrophilic and hydrophobic molecules, intact liposomes have recently been embedded as deposits in polymer films for surface-mediated drug delivery (SMDD) applications.[29] SMDD refers to a concept where therapeutic cargo is delivered to adhering cells or the surrounding tissue/cells from a substrate. This approach has multiple advantages including that the drug is administered locally, which avoids systemic dilution and side effects, that the release of the therapeutic cargo can be tailored by engineering the coatings, or that the films can be deposited on different substrates, making the same coating amendable for different bulk materials such as metals, ceramics or polymers, etc.

We were among the first to demonstrate the uptake of fluorescent lipids by adhering cells from a liposome-containing PDA-based polymer film.[30] We demonstrated that the cell mean fluorescence depended on the polymer layer thickness and composition.[31] By pre-mixing the liposomes and the dopamine solution, we deposited these films in one-step.[32] These composite coatings exhibited sustained delivery of fluorescent lipids to adhering cells for 24 h. Further, we demonstrated their potential in SMDD by loading the liposomes with a hydrophobic cytotoxic compound, and observed a significant decrease in viability of the adhering cells. As an alternative to PDA, polymer films assembled via the layer-by-layer method (LbL) have been demonstrated as a matrix to embed structurally intact liposomes with triggered[33] or passive control over the interaction with the adhering cells.[32, 34] Taking the LbL approach a step further, DeMuth et al. demonstrated the use of liposomes embedded into multilayers to coat microneedles to be used *in vivo* as a locally administered vaccination.[35] The liposomes were loaded with an antigen, an adjuvant and a fluorescent tracer and the coated microneedles were inserted subcutaneously into mice. The liposomes were taken up by antigen presenting cells upon degradation of the polymer film and a significant increase in humoral immune response was observed. In an alternative approach, liposomes were embedded in a hydrogel composed of physically crosslinked poly(vinyl alcohol), and the delivery of the cytotoxic compound paclitaxel to adhering cells was demonstrated.[36]

Liposomes in cell mimicry

In this section we highlight some of the most recent and exciting accomplishments when using liposomes in cell mimicry. For a more detailed discussion of the concepts and the technology about semi-synthetic cells, the reader is referred to recent reviews in the field.[37-39]

The ultimate goal of minimal cell research is the creation of a compartmentalised biochemical system capable of self-reproducing all its molecular components (including the membrane) and eventually to divide. The most exciting accomplishments in this direction include: the achievement of liposome self-reproduction,[40, 41] the performance of biochemical reactions inside liposomes,[42, 43] the RNA replication,[44] the DNA amplification,[45] the production of lecithin molecules inside lecithin liposomes to induce compartment self-reproduction,[46] the ribosomal synthesis of poly(phenylalanine) or the synthesis of the functional protein GFP inside liposomes.[47] The most advanced research in this direction has been made using giant liposomes (GLs), where it has been possible to directly observe the GLs growth and division by light microscopy. Kurihara et al. used GLs as a microreactor to carry out internal DNA amplification *via* PCR mechanism and liposomes self-reproduction by the external addition of a membrane precursor.[48] In a different approach to stimulate the processes of liposomal growth and division, Terasawa et al., used an electric pulse to fuse GLs, which could be one of the stimuli to fuse membrane in the prebiotic environment.[49, 50] After gaining the excess membrane by fusion, a spontaneous division occurred (Figure 3a). The authors showed that the division resulted to be dependent on the presence of encapsulated inert polymer molecules which mimic cytosolic macromolecules. Another facet of this concept can be found in a recent work by Zhu et al.[51]

Most recent advances in the field of liposomes as synthetic cell mimics also include the study of liposome communities. Since literature on the origin of cells includes the relevance of interactions of primitive cells with each other,[52] the fusion of primitive cells could be seen as a way to increase the molecular metabolic complexity.[53, 54] A landmark study on liposome fusion was performed by Caschera et al. [55] who reported the synthesis of a functional protein inside liposomes obtained by the fusion of two different liposome populations. One of the most recent studies drives the idea to a higher complexity level. Carrara et al.[56] studied the possible advantages of liposome communities when compared to individuals. In particular, they constructed GLs colonies by attracting GLs to each other and to a solid support via electrostatic interactions, which can be seen as the first approximation as models for cells bound to each other. The study demonstrates how favourable properties and dynamics may emerge higher self-organisation levels. Other remarkable recent achievements in the field include the attempts for reconstructing cytoskeletal elements inside liposomes, in particular the minimal divisome machinery,[57] as well as the advancements in regulating protein synthesis in cell-free genetic circuits,[58] the cell-free reconstruction of ATP synthase,[59] or the tuning of the liposome membrane permeability.[60]

Finally, we would like to highlight one of the most recent advanced compartmentalisation approaches. Multicompartmentalisation is a key principle of eukaryotic cells which are able to perform multiple, spatially separated, chemical processes simultaneously with high accuracy and specificity.[61] The two most advanced systems are vesosome, liposomes entrapped in a larger carrier liposome,[62] and capsosomes, liposomes within a larger polymer carrier capsule. The most recent achievement in the latter case include the assembly of capsosomes with control over the spatial positioning of the subunits[63] or encapsulating both liposomes and polymeric capsules.[64] Additionally, this heterogeneous assembly was employed to conduct a triggered enzymatic reaction and to selectively address the degradation of the polymeric subunits, that is, without affecting the structural integrity of the liposomes (Figure 3b). Further, the functionality of capsosomes has advanced to perform an encapsulated couple enzymatic reaction,[65] as well as an encapsulated cascade reaction running simultaneously with an independent enzymatic conversion.[66]

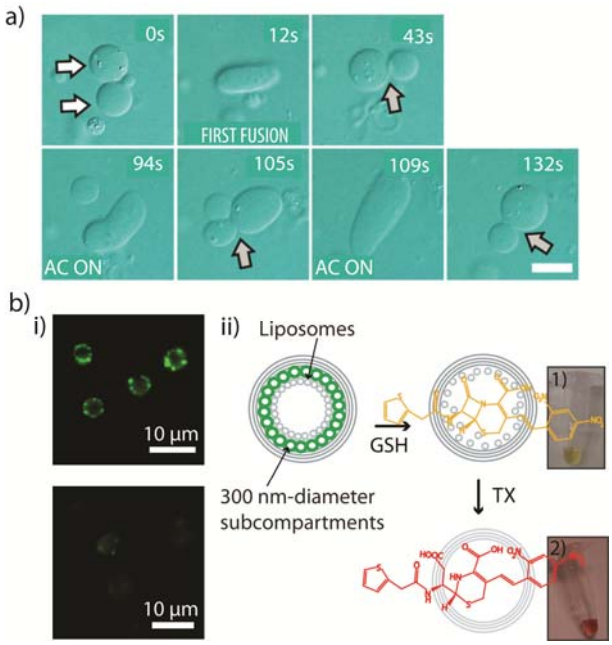

Figure 3. a) Repetitive cycles of fusion-to-budding transformation. After the first fusion (t=12 s), an AC signal was applied after each budding event (t=94 s and 132 s). The white arrows indicate the vesicles to be fused. Gray arrows show the neck formation. (Scale bar: 10 µm). Reproduced with permission from [50]. b) Degradation of polymeric subcompartments by reduced glutathione (GSH). i) Fluorescence microscopy images of microcarriers containing Alexa Fluor 488 labeled polymeric subcompartments before (left) and after (right) incubation with GSH. ii) Schematic illustration of the enzymatic microreactors containing ß-lactamase encapsulated in the liposomes after the degradation of the polymer subcompartments. After the addition of Triton X (TX), the yellow substrate nitrocefin (1) is converted into its red hydrolysed product (2). Reproduced with permission from [64].

Conclusions/Future Perspective

This highlight emphasises the impact liposomal formulations made in different areas in the field of nanomedicine. Recent chemical modifications have aimed to combine nanoparticles with liposomes since tracking or imaging sites directly attached to the liposomes is a powerful and promising strategy. Despite the progress achieved within the last 30 years in liposomal drug delivery, the demand towards a target-oriented functionalisation

of liposomes keeps in the focus of research in the 2010s. With good reason: modern polymerisation techniques and chemical strategies allow equipping liposomes with an unprecedented degree of complexity and promoted the simple drug carrier towards a complex biocompatible bioconjugate, featuring prolonged circulation time in the blood stream and control over both the location and time regarding the release of the entrapped cargo.

All the above studies on the consideration of shear stress for the characterisation of liposomal formulations, exemplified the fundamental importance of biomechanical stimuli in the design and evaluation of novel drug delivery systems, especially in the way new techniques are currently being developed to screen candidate drug vehicles in a high-throughput manner. We believe that dynamic cell culture studies taking into account the various biomechanical stimuli will not only revolutionise the way drug carriers will be characterized *in vitro* in the future, but will pave the way towards the continuous development of advanced devices for screening drug carriers in a high through put manner.

Liposomes have successfully been employed as drug deposits in polymer films for SMDD. In the last few years, the efforts have moved from assembly strategies to successful *in vitro* and first *in vivo* applications. However, large gaps in the fundamental understanding of liposomal formulations and their interaction with adhering cells remain and need to be addressed before these types of coating will/can be considered in translational medicine.

Finally, liposomes also play an important role in the cell mimicry field. Due to their phospholipidic bilayer structure which resembles that of biological cell membranes, liposomes are already considered as biomimetics. The construction of a semi-synthetic minimal cell by assembling separated components (e.g., nucleic acids or enzymes) using liposomes has already been achieved. One of the newest and unconventional contributions of liposomes in the cell mimicry field relies in their utilisation as compartments of a bigger carrier, with the aim to mimic eukaryotic cells, which can separate their functional processes in different/separated compartments (i.e., the organelles). Future directions will have to consider the interaction of these synthetic assemblies with biological cells and tissue to understand their potential in biomedicine.

We hope that we demonstrated in this highlight the important contribution of liposomes in several non-mainstream areas of the nanomedicine field.

Acknowledgements

This work was supported by a Marie Curie individual fellowship (L. H-R.), by a Sapere Aude Starting Grant from the Danish Council of Independent Research, Technology and Production Sciences, Denmark (B. M. T., M. E. L., B. S.), and a research grant from the Danish Council of Independent Research, Technology and Production Sciences, Denmark (P. S.).

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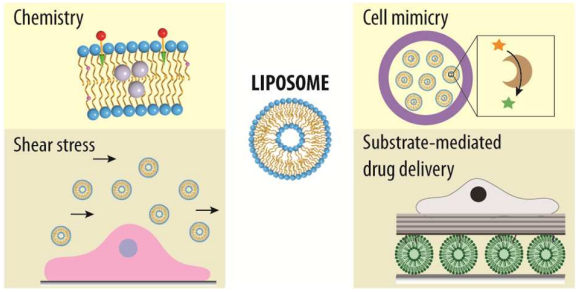

Liposome applications are highlighted focusing on chemical modifications, interaction with cells, and use in substrate-mediated drug delivery and cell mimicry.