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Review article

On the use of liposome controls in studies investigating the clinical potential of extracellular vesicle-based drug delivery systems – A commentary

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ABSTRACT

The field of extracellular vesicle (EV)-based drug delivery systems has evolved significantly through the recent years, and numerous studies suggest that these endogenous nanoparticles can function as efficient drug delivery vehicles in a variety of diseases. Many characteristics of these EV-based drug delivery vehicles suggest them to be superior at residing in the systemic circulation and possibly at mediating therapeutic effects compared to synthetic drug delivery vehicles, e.g. liposomes. In this Commentary, we discuss how some currently published head-to-head comparisons of EVs versus liposomes are weakened by the inadequate choice of liposomal formulation, and encourage researchers to implement better controls to show any potential superiority of EVs over other synthetic nanoparticles.

1. Introduction

The interest in using small extracellular vesicles (EVs) as drug delivery vehicles in different disease conditions has seen a steady increase in recent years [1,2]. A large body of studies have provided results that highlight a possible relevance for developing drug delivery platforms utilizing either autologous or heterologous EVs to transport drug compounds into an area of disease [1,3]. However, while the therapeutic effect obtained by EV-based drug delivery systems is well-documented in most studies published within this field, drawing conclusion on a possible superiority of EV-based drug delivery systems compared to traditional, synthetic, drug-loaded nanoparticles is currently based on much vaguer evidence (please refer to Fig. 1 for an illustrated comparison of the advantages of liposomes and EVs for drug delivery). In this Commentary, we would like to stress the importance of implementing a gold standard liposomal control (e.g. clinically approved) when evaluating the therapeutic benefits of EV-based drug delivery systems to ensure that these systems have a potential of being superior to the current best practice. We focus on the differences between liposomes and extracellular vesicles for drug delivery given their similarities with respective to the general composition and design, and thus, leave out comparisons to other self-assembling drug delivery system with great clinical potential [4–6].

2. Liposomes and extracellular vesicles

Liposomes are synthetic, enclosed phospholipid bilayer structures produced from a wide variety of phospholipids, and often stabilized by large molar fractions of cholesterol [7,8]. Using such a lipid formulation to produce liposomes facilitates stable encapsulation or loading of drugs depending on their chemical characteristics (hydrophilicity, molecular weight, pH-sensitivity etc.). Because of the production methods, liposomes can be produced as a highly homogenous population of nanoparticles with very small size polydispersity, which reduces the aspect of liposome size as a factor in any given experiment on their therapeutic efficacy [7]. While drug loading into liposomes is not trivial and has to be optimized for any new type of drug, the fact that liposomes are synthetic and produced by self-assembly makes it possible to encapsulate rather large hydrophilic drugs [9]. Liposomes can be endowed with targeting ligands in a controlled manner to produce a homogenous population of drug-loaded nanoparticles both with respect to size and surface-functionalization, hereby allowing for specific

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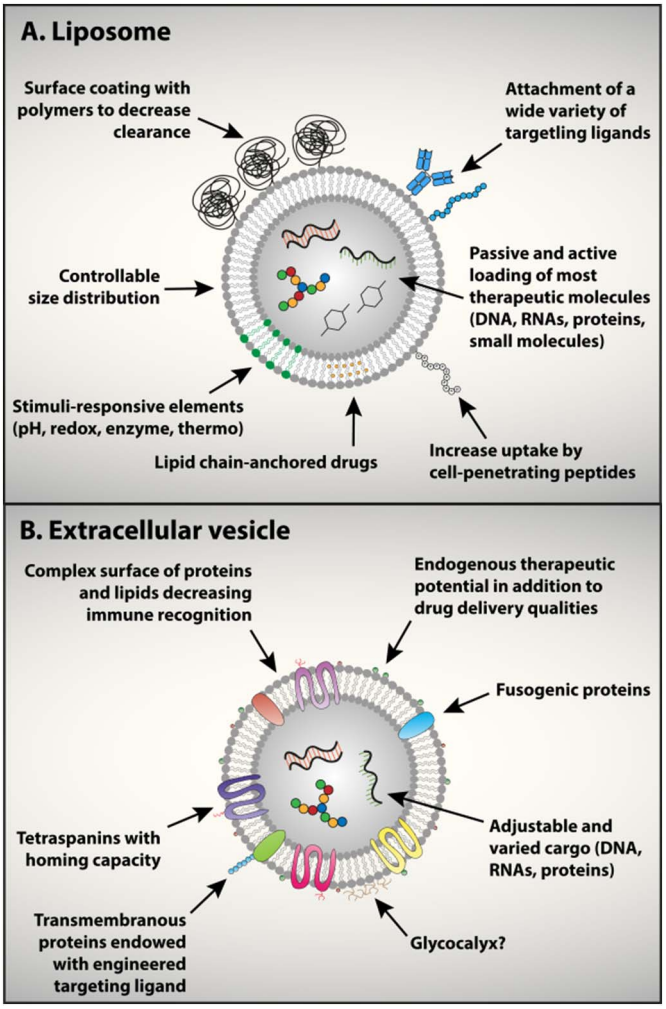

Fig. 1. Comparison of liposomes and extracellular vesicles (EVs) as drug delivery vehicles. (A) Liposomes are characterized by high homogeneity and low polydispersity, which is mostly due to the easily controlled production (which also facilitates loading of many types of drug cargoes). They can be modified to have polymers attached to their surface that increase their circulatory properties (curled black lines). This is important for therapeutic efficacy. To target certain cell populations in a different tissues or secure specific uptake in such tissues, the liposomes can be endowed with targeting ligands (e.g. antibodies). Furthermore, liposomes can be designed to possess specific controlled release mechanisms (due to inclusion of sensitive elements like lipids or peptide linkers in the formulation) or improved cellular uptake capabilities (by endowing the liposomes with cationic lipids or cell-penetrating peptides). (B) EVs generally vary in their size distribution, and the surface protein and RNA cargo composition is also very heterogeneous. This illustrates how these drug delivery vehicles are produced from cells. They can carry different types of cargo, including DNA, RNA, proteins, and maybe even exogenously loaded small molecule drugs. Due to their endogenous origin, the production of EVs for drug delivery purposes can be difficult in terms of controlling the design, drug loading, and yield. Still, because EVs are so advanced in their lipid and surface protein composition, they may be better fit for being present in different body compartments including the systemic circulation. The complex surface of the EVs also suggests improved capabilities of targeting and cellular uptake (due to the expression of tetraspanins and fusogenic proteins, respectively), together with a theoretical reduction in immune recognition.

accumulation in areas of disease and/or improved uptake into cells expressing the receptor for the attached ligand [7,10,11]. Like most types of nanoparticles, liposomes are readily taken up by the mononuclear phagocyte system (MPS) in the liver and spleen after opsonisation, which reduces their circulatory properties substantially [12–16]. However, years of development has discovered that surface-functionalization with polymers like polyethylene glycol (PEG) improves the circulatory properties significantly, paving the way for the so-called long-circulating liposomes. These long-circulating liposomes have proven important for efficient accumulation of liposome-encapsulated drug in areas of disease [7].

EVs are produced by most cell types in the human body and have been proven to be important mediators of paracrine signalling, and hence, they are crucial for the continuous communication between cells in different tissues [17]. They are composed of a complex mixture of lipids and proteins that differs with respect to the pathway of biogenesis and cell type of origin [17,18]. While some of these differences may be explained by differences in isolation methodology, lipid analyses even point towards a large variation in the composition of EVs with a similar size distribution [18]. As for the protein composition, the same kind of variation can be observed [18,19]. Importantly, the protein pattern on the EV surface depicts the homing capacity of the individual EV, i.e. the surface proteins function as targeting ligands that ensures preferential accumulation of the EVs at specific sites in the body (Fig. 1) [20]. The variety of EV types and their corresponding compositions are therefore of great interest as so-called liquid biopsies to search for biomarkers of normo- and pathophysiological conditions and as drug delivery vehicles [2,21]. Compared to liposomes, EVs are much more diverse in their lipid composition (Fig. 1), and hence, together with the large number of different proteins, some suggest that the complex EV surface may make them more suitable for being in the body (e.g. in the systemic circulation) [1,18]. Membrane proteins on the EV surface can also be modified to express exogenous targeting ligands for controlled accumulation or uptake into cells [22], although the process leading to successful expression of peptides on the EV surface is not well understood and difficult to control [23]. Drug loading remains an issue for the use of EVs as drug delivery vehicles [24]. Because EV-based drug delivery vehicles are isolated as completed bilayer spheres, the types of drug that can be passively loaded into the EV core is also very limited. Loading of an exogenous cargo, e.g. via electroporation, has been attempted in several cases of EV-based drug delivery, but these methods likely also lead to adverse effects on both the cargo and the EVs [1,3,25,26]. Instead, nucleic acids (like siRNA, miRNA etc.) can be overexpressed in the EV-producing cell, which in principle will lead to a high concentration of a given RNA-fragment as the EV cargo [24,27]. A circumvention of the loading issue has been to produce EV-mimetics from the plasma membrane fractions of cells, hereby obtaining the favourable, advanced surface composition of the drug delivery vehicle, while still allowing for encapsulation of water-soluble drugs [28].

3. Are extracellular vesicles better than liposomes for drug delivery?

The main argument for utilizing EVs as drug delivery vehicles is the fact that they are suggested to be stable in the systemic circulation (possibly due to their low immunogenicity), have an endogenous homing capacity (although not yet fully elucidated), and the possibility of modulating the surface protein composition and cargo (e.g. by modulating the producing cell, Fig. 1) [1]. The endogenous homing capacity may make the EVs able to cross biological membranes that are not easily traversable by synthetic nanoparticles, especially if these are not functionalized with a targeting ligand [29–31]. These characteristics of EVs may in total infer a better pharmacokinetic profile compared to that of synthetic nanoparticles such as liposomes, however, this difference may also be severely affected by the heterogeneity of the EVs obtained from a biological sample and the process by which the EVs are isolated [32,33].

All of these positive aspects would in theory suggest an EV-based drug delivery system to be superior in therapeutic efficacy compared to liposomes [1]. Indeed, several studies have been published within the last few years presenting interesting results on differences between EVs and liposomes in relation to their uptake profile or therapeutic efficacy in vitro and in vivo [28,34–38]. Overall these papers suggest that EVs associate better with target cells in vitro and in vivo, which may be a testament to their special surface composition. For example, tumor

volume could more effectively be reduced by intratumoral administration of doxorubicin-loaded EVs compared to liposomes [34]. However, when analysing their circulatory properties after intravenous administration, EVs had the same pharmacokinetic profile as the control liposomes, thereby reducing one of the main arguments for using EVs for drug delivery, namely their native origin and the impact this should have on the circulatory properties [1,34]. Other pharmacokinetic and biodistribution studies have revealed that EVs, like liposomes, preferentially accumulate in the liver, spleen and lungs, and the current reported data on EV pharmacokinetics suggest a biphasic elimination pattern with a terminal half-life of 70–180 min [39–41]. In contrast, a classical stealth-like liposome formulation (i.e. PEGylated) will have a half-life in the order of several hours (depending on the species and disease model tested it ranges between 10 and 55 h [42–46]), whereas antibody- or peptide-functionalized liposomes (analogous to EVs) will follow a biphasic elimination pattern, e.g. with a terminal half-life of > 10 h [47,48]. The rapid clearance of EVs from the circulation is likely due to uptake by the MPS. To overcome this, the EV-field could take inspiration from early work on liposomes, where modulating the dose regimen to obtain a primary saturation of the MPS followed by additional administration improved the circulatory properties. However, the most substantial reduction in the clearance of liposomes came with the sterical stabilization imposed by the addition of polymers such as PEG on the liposome surface [7]. This resulted in a pharmacokinetic profile that (broadly speaking) was independent of the initial dose, and the improvement is well reflected by the difference in clearance between two clinically approved liposomal doxorubicin formulations, where *Doxil* (PEGylated) has a much longer circulatory half-life compared to *Myocet* (non-PEGylated) [49].

A good circulation profile of a drug delivery vehicle does not necessarily scale with good accumulation properties and subsequent therapeutic efficacy, especially if a certain drug requires entrance into the cell via a specific targeting ligand to exert its function. Therefore, the currently known circulation profile of EVs may not abolish their relevance as drug delivery vehicles, if their targeting and uptake capabilities are in favour of therapeutic efficacy [50]. Because the impact of the enhanced permeation and retention (EPR) effect on liposome accumulation in tumor tissues is poorly understood in humans and may be affected by the specific histology of the tumor, drug accumulation via EV-based drug delivery may even be higher due to the endogenous targeting potential [20,43,51–54]. Still, it is known for liposomes that even though ligands are attached to favour targeting of a specific epitope, the primary accumulation of the liposomes happens via a passive process with no input from the targeting ligand. Hence, in a tumor setting, targeted liposome formulations have to depend on the EPR effect (if it exists in the model studied) for initial accumulation in the tumor tissue before the impact of the targeting can happen, and therefore it is likely that EVs targeting a cancer cell epitope will have to depend on the EPR effect as well [7,8]. This also implies that the EVs (or liposomes) need to have a good circulation profile to obtain sufficient accumulation for therapeutic efficacy. While association to target cells may be improved for EVs compared to control liposomes, some have shown that EVs were essentially ineffective in delivering a therapeutic cargo into cells in vitro [35]. The reason for this may be that the cargo cannot escape the endosomal or lysosomal compartments and reach its target.

Some in vivo studies have proven EV-based drug delivery to be more effective than their liposomal counterparts [28,36]. For example, non-targeted liposomes were ineffective in delivering doxorubicin to tumors after intravenous injection, while EVs or EV-mimetic nanovesicles derived from the plasma membrane of the parent cell line could do so to reduce tumor growth. This was hypothesized to be due to the presence of the LFA-1 molecule on the EV/nanovesicle surface, which could target these particles to cell adhesion molecules on the tumor cell surface [28]. In addition, EVs outperformed liposomes (without cargo) on a wide variety of parameters related to the treatment of traumatic brain injury [36]. Of particular interest was the fact that EVs were able to improve the cognitive recovery, increase the amount of newborn neurons in the dentate gyrus, and reduce the level of neuroinflammation compared to liposomes designed to mimic the lipid composition of the EVs [36]. This indicates that in addition to acting as drug delivery vehicles, the EVs may themselves possess a therapeutic capacity, which could work in concert with the exogenously loaded drug.

Although the total amount of currently available head-to-head comparisons is still very low, there are indications that for some diseases, EVs could prove a better choice as drug delivery vehicle compared to liposomes. The question is, however, if the outcome of these comparisons can be used to determine such a superiority?

4. Towards a fair comparison of EVs and currently used liposomal drug delivery systems

Regardless of the outcome of any comparison of EVs and liposomes, the analyses often suffer from not analysing the EVs and liposomes on comparable levels. While we acknowledge the choice of liposome composition in the context of the study (e.g. as being comparable to the EV composition), the resulting head-to-head comparison in a therapeutic setting still becomes heavily distorted since such a liposome composition rarely would have been chosen as a clinically relevant formulation for drug delivery [36]. This becomes even more problematic if the resulting study is cited in too general terms, which does not include a discussion of possible reasons as to why the control liposome formulation may not have been optimal for drug delivery at all (for example, a recent head-to-head comparison of EVs and liposomes was published in a high impact journal without reporting the lipid composition of the liposomes [55]). One could have a much more relevant control than just naked liposomes by including a polymer surface coating (e.g. PEG) on the liposomes to increase their circulatory properties [10]. Such a surface modification may in fact be relevant to increase the circulation time of EVs as well. This would, however, likely shield the complex surface of the EV, which could reduce its impact on the circulatory properties, and while the current efforts has made significant improvements on the EV circulatory properties by PEG coating, the EV circulatory half-life is still rather short [50]. It is important to stress that while there is no evidence to underscore an argument of only using PEGylated liposomes as controls in comparison studies from a downstream clinical perspective (given the clinical approval of several non-PEGylated liposome variants, e.g. the recent approval of *Vyxeos* [56,57]), inclusion of such a control would provide answer to the simple question: Is the improvement in the efficiency seen for a given drug merely based on an increase in the circulatory half-life of the drug rather than the effects imposed by the EV composition? If so, the liposome strategy may be an easier approach to obtain this or better increases in circulation properties.

If a particular ligand is hypothesized to mediate the observed improvement in therapeutic efficacy by way of EVs, it is inadequate to compare with only non-targeted liposomes if the aim of the study is to illustrate the superiority of EVs in comparison to other synthetic drug carriers [28]. In this case, one could produce liposomes targeted to the same surface receptor to analyse the exact relationship (and possible superiority) between the EV- or liposome-based therapies. This is especially important, because proteinase K treatment of the EV surface ameliorated the improved uptake seen for the EVs, and therefore there is little doubt that the proteins on the surface play a leading role in the association between EVs and target cells, which should also be modelled in the controls [34,55]. Furthermore, if a clinically approved liposome-based drug delivery system is available for any particular drug, this could be included in the study of EV-based delivery of the same drug [28].

The field of EV-based drug delivery is still young and thriving, and the coming years will most likely see new interesting studies showing the potential of this ‘new’ type of nanoparticle to treat a wide variety of

diseases. While the EVs have several theoretical advantages, the field of liposome-based drug delivery is still much more developed and liposomes have several actual advantages including easy composition design and scalability, tuneable size and surface charge, well-characterized surface coatings, the possibility of attaching targeting ligands in a controllable manner, efficient methods for cargo loading, and a bulk of evidence showing their therapeutic relevance in both preclinical and clinical settings. Therefore, EV-based drug delivery may be of greatest relevance in diseases where liposomes or other synthetic nanoparticles in general have failed to show improvements. If the field continues to progress in the direction of broader disease indication (e.g. cancer), we urge the future authors of preclinical EV-based drug delivery studies to choose gold standard liposome controls to describe this therapeutic potential best. First, this would result in a much fairer comparison between the different nanoparticle technologies analysed. Second, including the 'current best practice'/gold standard in the studies (some of which may even be clinically approved) and proving superiority to these with an EV-based drug delivery system would significantly highlight the clinical potential of the new treatment much better. In fact, wanting to claim superiority of EVs over liposomes as drug delivery vehicles or vice versa may not even be relevant. Instead, the two fields could facilitate a positive feedback mechanism, in which evidence from the EV-field regarding special lipid compositions favourable for circulation or accumulation, or targeting ligands that improves the uptake into specific cells of the diseased area, could be used to design new liposomal formulations with higher efficiency than what is known today. Using this strategy, one would benefit both from the endogenous characteristics of EVs and the ease of design/production and drug encapsulation of liposomes. With these factors in mind, we may soon see the clinical potential of EV-based drug delivery systems.

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