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Review

Extracellular vesicles as drug delivery systems: Lessons from the liposome field

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ABSTRACT

Extracellular vesicles (EVs) are membrane-derived particles surrounded by a (phospho)lipid bilayer that are released by cells in the human body. In addition to direct cell-to-cell contact and the secretion of soluble factors, EVs function as another mechanism of intercellular communication. These vesicles are able to efficiently deliver their parental cell-derived molecular cargo to recipient cells, which can result in structural changes at an RNA, protein, or even phenotypic level. For this reason, EVs have recently gained much interest for drug delivery purposes. In contrast to these 'natural delivery systems', synthetic (phospho)lipid vesicles, or liposomes, have been employed as drug carriers for decades, resulting in several approved liposomal nanomedicines used in the clinic. This review discusses the similarities and differences between EVs and liposomes with the focus on features that are relevant for drug delivery purposes such as circulation time, biodistribution, cellular interactions and cargo loading. By applying beneficial features of EVs to liposomes and *vice versa*, improved drug carriers can be developed which will advance the field of nanomedicines and ultimately improve patient outcomes. While the application of EVs for therapeutic drug delivery is still in its infancy, issues regarding the understanding of EV biogenesis, large-scale production and *in vivo* interactions need to be addressed in order to develop successful and cost-effective EV-based drug delivery systems.

Contents

Abbreviations: AKI, acute kidney injury; ApoE, apolipoprotein E; a.u., arbitrary units; AUC, area under the curve; Alix, ALG-2-interacting protein X; Chol, cholesterol; DLin-MC3-DMA, heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; EGFR, epidermal growth factor receptor; EPC, egg phosphatidylcholine; EVs, extracellular vesicles; ILVs, intraluminal vesicles; LNP, lipid nanoparticle; mRNA, messenger RNA; miRNA, micro RNA; MPS, mononuclear phagocyte system; MVB, multivesicular body; PEG, polyethylene glycol; PEG-DMG, polyethylene glycol-dimyristolglycerol; PHEPC, partially hydrogenated egg phosphatidylcholine; PS, phosphatidylserine; RBC, red blood cell; RNAi, RNA interference; SHM, staggered herringbone mixer; siRNA, small interfering RNA; TL, total lipid; TSG101, tumor susceptibility gene 101.

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1. Introduction

Scientific and technological breakthroughs in the 20th century have led to the development of synthetic lipid- or polymer-based carrier systems for the entrapment of therapeutically active molecules to yield nanomedicines [1]. When compared to the administration of free drugs, advantages such as improved stability, solubility and *in vivo* pharmacokinetics have resulted in approximately 250 nanomedicines that are currently approved or in various stages of (pre)clinical evaluation [2,3]. The ultimate goal of nanomedicines is to improve patient outcomes by increasing the drug concentration in the target tissue or cell to enhance therapeutic efficacy, while simultaneously decreasing exposure of healthy tissues to reduce toxicity. Particularly in the field of oncology, altering the pharmacokinetic parameters and shifting the tissue distribution of chemotherapeutic drugs by formulating them as nanomedicines has shown to reduce dose-limiting adverse effects associated with these drugs, while maintaining or even improving efficacy [4]. While technology has enabled the production of high-grade synthetic nanomedicines in sufficient quantities to treat patients, clinical impact has been relatively modest due to a lack of understanding of *in vivo* interactions and fate of nanomedicines in the human body.

Nature's own 'carrier systems' such as bacteria, viruses or cells, have also been employed either as drug carriers or to study the underlying mechanisms responsible for their efficient delivery. Attractive features of natural carriers that could be applied to improve synthetic carriers include cellular tropism, efficient cell (organelle) entry, physicochemical properties and the ability to circulate without detection by the immune system [5].

Extracellular vesicles (EVs) are cell-derived membrane vesicles characterized by a (phospho)lipid bilayer structure that function as a means of cell-to-cell communication, in addition to direct cell contact and the secretion of soluble factors [6]. They are natural carrier systems that have recently gained much interest due to their instrumental role in physiological as well as pathological processes. It appears that EVs can specifically transfer their content, which consists of complex biological molecules, from one cell to another even over longer distances. Consequently, EVs are investigated as therapeutic targets [7] and as diagnostic/prognostic biomarkers [8]. It is thought that virtually all cells in the human body release EVs, which is supported by the possibility of isolating EVs from all bodily fluids including blood, urine and saliva. EVs are usually classified based on their (intra)cellular origin, biogenesis, physicochemical properties and/or surface markers, (although there is little consensus about EV nomenclature in the field) and include apoptotic bodies, microvesicles and exosomes [9]. Apoptotic bodies are generated when cells undergo apoptosis and fragmentation. These vesicles can contain DNA, RNA and histones and broad size ranges between 50 and 5000 nm have been reported. Apoptotic bodies are characterized by the presence of phosphatidylserine (PS) on their surface which functions as an 'eat-me' signal for phagocyotic cells, thereby protecting healthy cells from exposure to possible harmful cellular debris [10]. Cells can release microvesicles (also referred to as ectosomes or microparticles) *via* outward budding of the plasma membrane. Microvesicles typically display sizes between 50 and 2000 nm. Although microvesicles can be enriched for a subset of proteins, current isolation protocols do not allow for a clear separation of circulating microvesicles and exosomes [9]. Exosomes seem to be the smallest type of EVs with reported diameters between ~40 and 150 nm. In general, it is thought that exosomes are generated *via* the formation of intraluminal vesicles (ILVs) in multivesicular bodies (MVBs). Fusion of MVBs with the plasma membrane causes the secretion of the ILVs, which are dubbed exosomes upon release in the extracellular environment. Exosomes are often characterized by their protein contents indicating an endosomal origin such as ALG-2-interacting protein X (Alix), tumor susceptibility gene 101 (TSG101) and tetraspanins (CD9, CD63) [11]. Nevertheless, it is possible that exosomes can also be released by cells *via* direct budding and fission of the plasma membrane. By virtue of their defined size and natural function, exosomes appear ideal candidates for drug delivery purposes [12,13]. Although many of the cited research articles in this review specifically mention the use of exosomes for their studies, consensus is yet to be reached regarding the isolation and detection techniques to accurately separate subpopulations of vesicles and we have therefore used the term 'EVs' throughout this paper to include all types of cell-derived membrane vesicles. The interest in understanding the delivery efficiency of EVs and harnessing their delivery potential for exogenous substances invites a critical reflection on vesicles as carrier systems. We particularly focus on the comparison with the current golden standard for drug delivery systems, liposomes, which share the phospholipid-bilayer structure with EVs (Fig. 1).

In contrast to EVs, liposomes have been employed as drug delivery systems for decades [14]. Important discoveries such as improved production using extrusion (and more recently microfluidic preparation), efficient drug entrapment by remote loading, enhanced stability by altering the lipid bilayer and prolonging liposomal circulation by modifying the surface with polyethylene glycol (PEG) have ultimately led to the approval of over a dozen liposomal nanomedicines since the nineties, with many more in clinical trials [15].

Examining the evolution of the 'mature' liposomes as drug carriers can contribute to the development of the yet 'immature' EVs for delivery purposes. For example, many technological methods used for the preparation and characterization of liposomal drug delivery systems may also be applied for EVs (Table 1). Conversely, by studying the biological mechanisms that underlie the efficient transfer of contents from one cell to another *via* EVs may yield advantageous knowledge that can be applied to improve current (liposomal) delivery systems. This review aims at discussing important drug delivery features of EVs and liposomes such as physical characteristics, *in vivo* behavior and fate, cellular interactions and cargo loading.

First-generation liposomal nanomedicines have been approved since the nineties and much knowledge has been obtained about their behavior in animal models and humans. As EVs are at the inception of being applied as drug carriers, many of the described observations have been made *in vitro* and great care should be taken when extrapolating these results to *in vivo* situations.

2 . Key features of drug delivery

Drug delivery by liposomes is mainly attributed to their ability to circulate over longer periods leading to accumulation in tissues that are characterized by permeable vasculature, which facilitates extravasation of liposomes. Although the mechanisms behind cargo delivery by EVs have only just started to be unraveled, it is believed that their surface properties, which influence circulation time and cell interactions, are the main factors underlying their efficient transfer of cellular material.

2.1 . Circulation kinetics and biodistribution

Liposomes are synthetic spherical vesicles. They self-assemble as a result of the hydrophobic effect when amphiphatic molecules, usually phospholipids, are brought in an aqueous environment. The minimal size of liposomes is ~30 nm, which is primarily determined by the difficulty of lipid-packing inside a strongly curved geometry. To obtain liposomes of such small sizes, substantial energy transfer to the membranes is required, for example by extrusion or sonication. The largest liposomes can measure up to several microns. Between sizes of ~40 and 900 nm, liposomes display opalescence (Fig. 2). Especially the 90° light scattering can be an accurate measure of particle size specifically in the 50–200 nm region where scattering is strongly dependent on the liposome size [28].

Liposomes can be uni-lamellar or multi-lamellar and incorporate both hydrophilic compounds in the aqueous compartment(s),

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