

**MICROENCAPSULATION TECHNOLOGY BY NATURE: CELL DERIVED
EXTRACELLULAR VESICLES WITH THERAPEUTIC POTENTIAL**

Running Title: Extracellular vesicles with therapeutic potential

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Cell derived extracellular vesicles are submicron structures surrounded by phospholipid bilayer, and released by both prokaryotic and eukaryotic cells. The sizes of these vesicles roughly fall into the size ranges of microbes, and they represent efficient delivery platforms targeting complex molecular information to professional antigen presenting cells. Critical roles of these naturally formulated units of information have been described in many physiological and pathological processes. Extracellular vesicles are not only potential biomarkers and possible pathogenic factors in numerous diseases, but they are also considered as emerging therapeutic targets and therapeutic vehicles. Strikingly, current drug delivery systems, designed to convey therapeutic proteins and peptides (such as liposomes), show many similarities to extracellular vesicles. Here we review some aspects of therapeutic implementation of natural, cell-derived extracellular vesicles in human diseases. Exploration of molecular and functional details of extracellular vesicle release and action may provide important lessons for the design of future drug delivery systems.

Keywords: microparticles; exosomes; vaccination

Abbreviations: EVs (extracellular vesicles), MVs (microvesicles),

Introduction

Over the past few decades experimental data started to accumulate in support of the existence of extracellular vesicles (EVs) in the size range earlier thought to be occupied only by microorganisms. It is only recently that the universality of EV secretion has been recognized by the exploration of vesiculation also in prokaryotes [1-3]. The microorganism-sized EVs are readily taken up by cells of the immune system. Of note, many of these vesicles are released during apoptosis, and the immune system's "high throughput" homeostatic clearance machinery for the uptake of vesicles of apoptotic origin is highly efficient. This process is mediated by phagocyte receptors (e.g. phosphatidyl serine receptor, TIM-4 or TAM receptors [4, 5] ensuring the rapid internalization of EVs by cells. Importantly, endocytosis is not the only uptake mechanism of EVs. Direct fusion of their membrane with the plasma membrane of specifically recognized cells has been suggested as another uptake mechanism (for recent review see [6]. In this case the content of EVs is released directly into the cytoplasm of the targeted cell [7]. The efficient cellular uptake renders EVs attractive candidate vehicles to deliver selected molecules to cells.

There is a striking analogy between currently used pharmaceutical drug delivery systems such as liposomes or microparticles designed to deliver proteins or peptides (Figures 1 and 2). In general, encapsulation of molecules for targeted delivery provides protection against enzymatic degradation, aggregation, or precipitation. Also, encapsulation ensures high local concentration of substances at a distant, targeted site. It is tempting to speculate that the phospholipid bilayer "capsule" of cell-derived EVs serves similar biological purposes. In line with this hypothesis, recently, EVs have been suggested to function as multipurpose carriers i) to deliver complex information to other cells ii) for safe removal of potentially harmful molecules and iii) aiding and extending functions of the donor cells [8].

In contrast to liposomes, EVs are derived from cells of the body, thus, they are composed of self molecules tolerated by the immune system. They can be used for prolonged time safely. On the other hand, liposomes may be manufactured in large scale synthetically at relatively low expenses.

In the current article, we aim at briefly summarizing some basic concepts and therapeutic implications of EVs to attract attention to a rapidly evolving field that emerges in parallel with development of nanomedicines as drug delivery systems. We propose that novel therapeutic strategies may benefit from lessons of the evolutionary conserved, natural vesicular structures.

Overview of EVs

Classification and nomenclature of cell derived EVs

It has been suggested that major subpopulations of EVs include exosomes, microvesicles and apoptotic bodies [9].

Multivesicular bodies (MVBs) of the endocytotic compartment fuse with the plasma membrane, and release vesicles (50-100 nm in diameter) designated as exosomes [10, 11]. Another pathway of vesicle release involves budding of the plasma membrane with ultimate release of membrane surrounded vesicles referred to as microvesicles (MVs) often referred to as microparticles or ectosomes [12]. While the size range of exosomes roughly overlaps with that of viruses, vesicles, generated by budding, have larger diameter (100-1000 nm) corresponding approximately to the size range of bacteria or insoluble immune complexes [9, 13]. While exosomes are generated both constitutively and upon activation, the release of microvesicles is induced during apoptosis and activation [7, 9]. Both major types of EVs are encapsulated by a phospholipid bilayer membrane rich in cholesterol.

However, recent evidences support that there are numerous further subtypes of EVs. As an example, Théry et al. provided evidence for the existence of diverse populations secreted by different intracellular mechanisms [14].

As yet there is no international consensus regarding the terminology of EVs [15].

Detection methods of EVs

Detection of EVs imposes significant challenge on cell biologists, since conventional/ routine cell biology methodologies can not be applied for the investigation of these subcellular structures. Fluorescence microscopy and flow cytometry cannot be used for the analysis of exosomes, unless the vesicles are bound onto the surface of beads [16]. The analysis of not only exosomes but also of larger sized MVs, have limitations with conventional techniques. Flow cytometry fails to detect structures with less than 2-300 nm in diameter. Methodologies, used to characterize pharmaceutical liposome or microparticle/nanoparticle preparations, are more appropriate for studying cell derived EVs.

Transmission electron microscopy (in particular immune electron microscopy) has proven very useful for the detection and analysis of cell-derived vesicles irrespective of their size [17, 18]. Electron microscopy of exosomes shows a so called “cup shape” after isolation by sucrose gradient/cushion ultracentrifugation (that was suggested to be an artifact of preparation), while microvesicles are characterized by spherical shape with cryo- transmission electron microscopy [19]. Cryo-electron tomography microscopy was shown to be useful to avoid such types of artifacts [20, 21]. Also, scanning electron microscopy [22] single particle electron microscopy [23] and atomic force microscopy was used successfully to visualize individual EVs [13, 22]. Further non-conventional techniques of analysis (suitable for the analysis of EVs and liposomes) include dynamic light scattering analysis (DLS), nanoparticle

tracking analysis (NTA) [24, 25] and Fluorescence Nanoparticle Tracking Analysis (F-NTA) [26], Raman spectroscopy based techniques, Stimulated Emission Depletion (STED) microscopy, Impedance-based flow cytometry and resistive pulse sensing [27]. Presumably, the increasing demands of this research field will boost the development of further specific methodologies and user-friendly laboratory instruments fitted to the size range of EVs.

Biological functions

EVs are important recently recognized players of intercellular communication [12, 27, 28]. They are known to disseminate, support, and protect basic biological functions of the releasing cells. Exosomes have been shown to mediate horizontal transfer of mRNA, miRNA [29] and different types of cell surface receptors such as an oncogenic receptor [30] or purinergic P2X7 receptor [31]. One of the most important functions described in association with exosomes, is antigen presentation, a function earlier attributed to antigen presenting cells only [32]. Exosomes display both MHC-I and MHC-II molecules on their surface assembled with antigenic peptides. This feature has significant impact on the ability of EVs (such as exosomes) to induce immune responses upon injection as vaccines [33, 34]. Consequently, immunoregulation (including either stimulation or inhibition) is a principal function of exosomes, depending on the cellular source and target of the vesicles [12]. This feature raises the intriguing possibility of therapeutic immune modulation by exosomes.

Although immune regulatory functions of the larger sized MVs have also been reported (e.g. in the fetomaternal communication [35]), their best characterized function is the one they play in blood coagulation: they have significant procoagulant activity [36-39]. Similarly to exosomes, MVs represent a form of secretion of IL1 beta [31, 40] and have been suggested to contribute to the pathogenesis of rheumatoid arthritis [41, 42]. By their protease [43], and

possibly also by their glycosidase expression [44], MVs may contribute to the proinvasive character of tumors.

Therapeutic targeting of EVs

A few years after that the original concept of liposomes was raised by Bangham et al. in 1965 [45], these artificial lipid vesicles were suggested to be used as drug carriers [46], and a novel drug delivery system, liposomal encapsulation of drugs, has been introduced [47]. Currently, in the „nano era”, liposomes are frequently referred to as nanoparticles, and their use represents an organic part of nanomedicine. However, besides all benefits of engineered liposomes (in the case of which biocompatibility and biodegradability is evident), the use of EVs may be more favorable. These nature-encapsulated subcellular structures have been suggested for therapeutical delivery of molecules [48] and it may represent novel tools in future personalized medicine and in efficient and site-specific delivery of therapeutic drugs or nucleic acids.

Moreover, secreted EVs are not only nature-tailored carrier vehicles with potential therapeutic exploitation, but they also represent promising drug targets. As mentioned above, a wide variety of human diseases are characterized by elevated numbers and altered composition of circulating EVs. While in some cases their increased number may reflect general cellular activation or enhanced apoptosis, EVs may also substantially contribute as effectors to disease development. They were shown to contribute to tumor growth, migration and invasion, angiogenesis and tumor escape from immune responses (reviewed recently [49]). Therefore prevention of EV release or therapeutic removal of released vesicles from the circulation might also represent a therapeutic approach.

Tumor-derived exosomes are known biologic messengers in cancers, are mediators of tolerance induction and are shown to spread tumor growth signals that counteract the activity of therapeutic agents [50]. Therefore, therapeutic targeting of tumor cell derived exosomes represent an important therapeutic approach. The extracorporeal haemofiltration of circulating factors as a therapeutic strategy is already approved to be used in cancer patients [50].

EVs of pathogens in health and disease

Functional virus release has been reported to involve several elements from the EV biogenesis pathways [51]. EVs have been shown to play either enhancing or blocking roles in infections and represent removal systems for endogenous retroviruses or retrotransposons [51]. Recently, fraction of Adeno associated virus (AAV) vectors have been shown to be associated with EVs(vector-exosomes) and have been suggested for improved promising strategy to improved gene delivery [52].

EVs have been demonstrated to be secreted by Gram-negative [53, 54] and Gram-positive bacteria [55, 56], as well as eukaryotic parasites of the kinetoplast lineage and opportunistic fungi of both the ascomycetes and basidiomycetes lineages [57].

Outer membrane vesicles (OMVs) of many pathogenic bacteria contribute to the virulence of the releasing bacterial cells. Importantly, OMVs have been recently suggested to serve as a basis of non-replicating vaccines summarized by Ünal et al. [58].

A disease in which EV vaccination was proposed is sepsis, associated with increased proinflammatory cytokine levels and the accumulation of apoptotic cells. In the toxoplasmic model of sepsis, *Toxoplasma gondii*-pulsed Dex could stimulate a specific and protective T-cell response in CBA/J mice [59]. Although the mechanism remained unclear, and presumably activators of DCs, B-, T or NK cells may have contributed to the efficacy of

exosomes against *Toxoplasma gondii* infection in this congenital model, Dex appears to be a potentially useful tool for vaccination in sepsis.

In other models, exosomes derived from immature dendritic cells rescued septic animals because of the presence of milk fat globule epidermal growth factor (EGF)-factor VIII (MFG-E8) on their surface. MFG-E8 is required to opsonise cells for phagocytosis, which has to be promoted in septic animals to prevent the release of the potentially harmful substances from dying cells. An increased phagocytosis eventually reduces mortality, and attenuates the release of proinflammatory cytokines in the septic rats [60].

Conclusions

The ubiquitous feature of vesiculation by both eukaryotes and prokaryotes, has been established only recently. Both Gram negative and positive bacteria as well as fungi were shown to release these structure, and more recently, also the significance of plant derived apoplastic exosome-like vesicles have been suggested [61].

It is currently a unique situation that cell-derived EVs can be considered both as novel drug targets and natural drug delivery systems.

Unfolding diseases in which EVs play effector roles, may lead to development of EV targeting therapeutic strategies (such as prevention of vesicle release or to removal of secreted ones). On the other hand, manufacturing EVs for therapeutic applications is feasible *in vitro* inducing vesicle secretion by various stimuli. *In vitro* manipulation (e.g. transfection) of the releasing cells provides unique opportunity to produce tailored EVs with customized effector or targeting molecules. Vesicles, harvested from tissue culture supernatants, may be injected to modulate immune functions or to vaccinate against epitopes presented on vesicular surfaces in the context of MHC molecules. EVs are of proper size for uptake by cells, non-toxic, biodegradable, carry surface molecules that direct them to targeted cells, and carry complex

information. To date, especially exosomes have been shown to have a great potential. However, larger sized MVs, currently considered as biomarkers in body fluids such as blood plasma, urine or saliva, are far less characterized, and may hold yet unexplored therapeutic or vaccination potential. Vaccination by EVs or removal of circulating exosomes by haemofiltration are the types of exploitation of EVs have been already introduced to clinical practice.

A recent study has directly compared liposomes and exosomes as drug delivery systems for encapsulation curcumin in them. In a proof-of-principle study of Sun et al. it has been demonstrated that encapsulation of the antiinflammatory agent curcumin in exosomes was significantly superior to liposomal delivery as shown by the enhanced stability and higher concentration in the blood as well as higher therapeutic efficacy in (LPS)-induced septic shock mouse model [62].

Even though EVs may offer novel opportunities for prevention or therapeutic intervention in disease states in which patients do not respond to conventional therapies, one has to be aware of the risks also. Given that viruses and exosomes share size distribution and other biophysical parameteres, concerns center on potential contamination of exosome preparations with viruses. Development of safe technologies of large scale production of virus-free exosomal preparations is an absolute prerequisite of their therapeutic exploitation.

What appears to be clear is that is that researchers developing artificial drug delivery systems and those exploring EVs need to have an intense communication, and they should both follow the progress in the other field. The two scientific communities must recognize the possible mutual benefits of such an interaction. EV scientists have already taken advantage of methodologies originally used for the characterization of microbes, liposomes or other nanoparticles (such as DLS, AFM or NTA). Proof for the benefit for drug developers is best

exemplified by the recent development of artificial exosomes. These are liposomes coated with MHC/peptide complexes and Fab regions against T cell receptors to mediate cell surface adhesion [63, 64]. Presumably many further experiments will be inspired by lessons of natural EVs that successfully overcame evolutionary challenges. Studies focusing on nanomedicinal drug delivery systems and nature-tailored vesicles may cross-fertilize one another, and may lead to novel therapeutical solutions.

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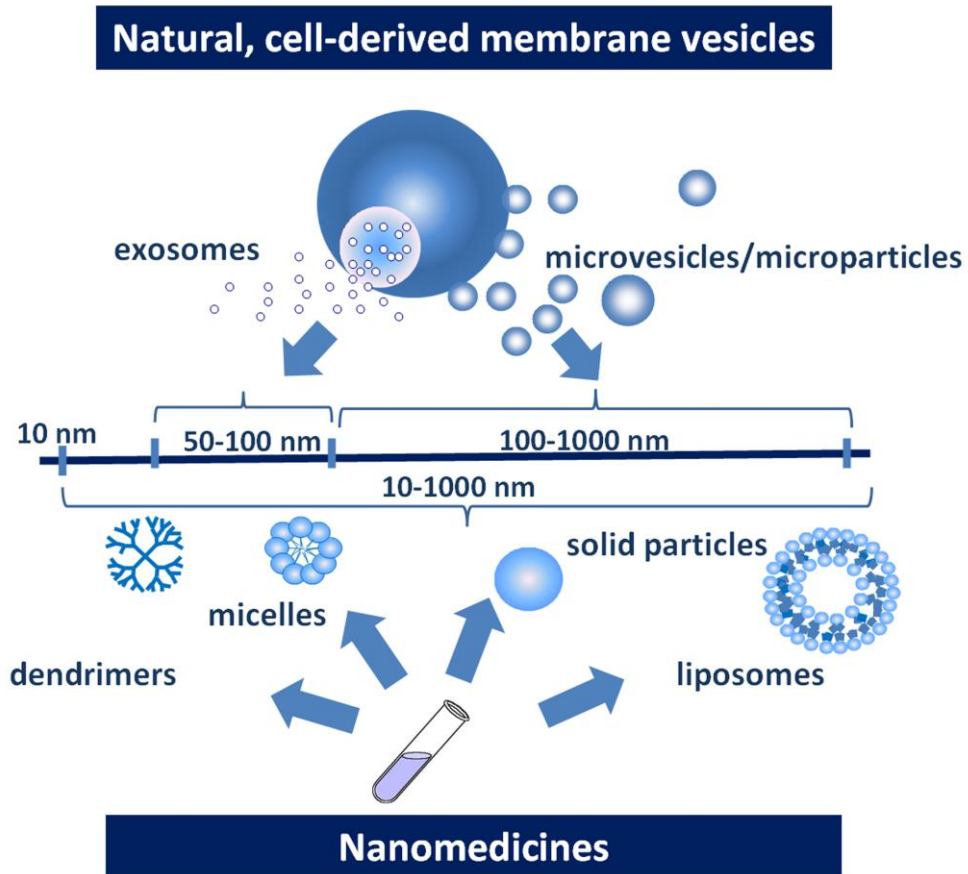
Figures and Figure Legends

Figure 1. Comparison of size ranges of different natural membrane vesicles and nanomedicines

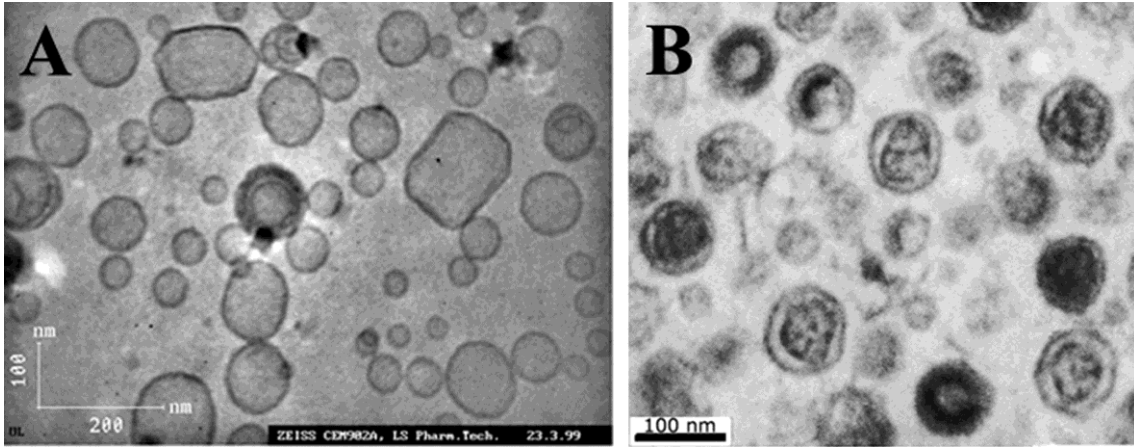


Figure.2. Cryo-electron microscopic image of liposomes (A) (http://www.mardre.com/homepage/mic/tem/samples/colloid/pc_samples/dmpc_liposome_cryo3.jpg) and 5/4 T hybridoma cell exosomes (B) show high similarity in both size distribution and their shape