Perspective paper

Extracellular vesicles – a promising avenue for the detection and treatment of infectious diseases?

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Abstract

Extracellular vesicles (EVs) have gained increasing attention as novel disease biomarkers and as promising therapeutic agents. These cell-derived, phospholipid-based particles are present in many – if not all – physiological fluids. They have been shown to govern several physiological processes, such as cell-cell communication, but also to be involved in pathological conditions, for example tumour progression. In infectious diseases, EVs have been shown to induce host immune responses and to mediate transfer of virulence or resistance factors. Here, we discuss recent developments in using EVs as diagnostic tools for infectious diseases, the development of EV-based vaccines and the use of EVs as potential anti-infective entity. We illustrate how EV-based strategies could open a viable new avenue to tackle current challenges in the field of infections, including barrier penetration and growing resistance to antimicrobials.

Introduction

In the past decade, extracellular vesicles (EVs) have attracted significant attention in the fields of molecular biochemistry, biosensing and drug delivery due to their involvement in many physiological and pathological processes [1, 2]. EVs are cell-derived particles which are naturally produced by most eukaryotic and prokaryotic cells [3]. Based on their intracellular origin and biochemical composition the most common populations of EVs are classified exosomes (size 50-200 nm) or shedding microvesicles (100 nm - up to several µm) [4]. In this perspective, these will be collectively termed EVs as suggested by the International Society for Extracellular Vesicles (www.isev.org) and in recent reviews from the field [1, 4, 5]. EVs consist of a lipid bilayer membrane decorated with various surface and membrane proteins and are often referred to as natural liposomes. In recent years, EVs have attracted enormous scientific attention as novel therapeutic agents and biomarkers with first clinical applications already in the pipeline [6]. In nature, EVs are very efficient mediators of intercellular communication [3] and transfer protein and nucleic acid based cargoes (*e.g.*, micro RNA) highly selective from one cell to another [7], even over long distances [8]. For example, EVs from T cells are targeted unidirectional to antigen-presenting cells (APC) [9]. EVs may not only transport biomolecules to distant tissue, they are also postulated to bypass immune activation and to exhibit improved stability under physiological conditions [3]. The potential of utilising EVs for medical applications has been exploited, for example by developing EV-based selective anti-inflammatory drug vehicles in different mouse models of inflammation [10]. Furthermore, EVs from immune and non-immune cells are involved in immune regulation processes [11] and they have been employed as immune-modulators to treat severe forms of graft-*versus*-host disease in humans [12]. Although EV applications have resulted in first (pre)clinical trials, significant drawbacks such as their reproducible large-scale production under good manufacturing practice (GMP) standards, industrially applicable robust analytics and safety validation need to be overcome [5, 13]. Here, basic and industrial investigation is needed as discussed in this perspective paper and outlined in recent extensive reviews [1, 3, 4]

Interestingly, not only eukaryotic cells use EVs as messenger tools. Bacterial EVs (so-called outer membrane vesicles, OMVs) have been identified as key mediators in the communication of bacteria and within biofilms [14]. Indeed, *Pseudomonas aeruginosa* release signalling molecules into EVs to enable specific inter-bacterial communication by transfer of both soluble and insoluble factors, such as quorum sensing molecules [15]. OMVs are strongly involved in pathogen-mediated infections, such as bacterial, viral, fungal and parasite infections [16]. They can mediate host immune activation and have been shown to transfer resistance genes or virulence factors [15]. For example, OMVs from known pathogenic bacteria, such as *Staphylococcus aureus*, *Mycobacteria tuberculosis* and fungi, such as *Cryptococcus neoformans* contain hydrolytic factors, toxins, proteins, DNA or cell wall components [14]. The OMV release mechanism is currently discussed in three diverging hypotheses including pressure release from the cell wall, cell-wall modifying enzymes and EV-specific release

channels [17]. Once liberated, OMVs can contribute to the formation of biofilms [18, 19] and transfer virulence factors [20] or even DNA [21] to mammalian cells. When taken up into mammalian cells, OMVs may also transfer their toxic and pro-inflammatory cargoes and manipulate the host immune response, induce proliferation, DNA damage or other deteriorating effects. For a detailed discussion on these mechanisms and effects the reader is referred to a recent review [22]. In this perspective paper, we focus on recent efforts in developing novel EV-based detection and therapy avenues. These approaches may pose potent future strategies to counteract decreasing options for treating and diagnosing severe infections, especially when facing growing antimicrobial resistance.

EV-based diagnostic approaches

Recent discoveries lead to increasing evidence that extracellular vesicles may serve as biomarkers for diverse pathological conditions, ranging from cancer to sepsis. Extracellular vesicles feature particularly interesting characteristics, as they contain selected information on the composition of the donor as well as the acceptor tissues. The fingerprint analysis of extracellular vesicles may hence allow probing of a much wider context and EV-based systems may outperform classical biomarkers and lead to better diagnostic performance (higher specificity and sensitivity). Profiling of secreted vesicles in body fluids, such as blood and urine, may give access to spatiotemporally resolved information and disease progression. The prospect of using EVs as biomarkers for both bacterial and viral diseases has been exploited in a number of studies [23, 24]. Recently, it has been shown that CD11β-positive EVs can potentially be used as sensitive and specific biomarkers to differentiate between infectious and non-infectious inflammatory conditions in intensive care unit patients [25]. Similarly, exosomal CD81 has been shown to be associated with inflammatory activity and severity of fibrosis in patients suffering from hepatitis C [26]. A study by Singh and colleagues pointed out that *M. tuberculosis* induces secretion of exosomes containing a unique set of host miRNA and mRNA from macrophages that could be used to diagnose tuberculosis [27]. Several recent studies highlight the potential of exosomal microRNA profiles for use as diagnostic biomarkers of disease through non-invasive blood tests [24]. Not only exosome composition but also the concentration of exosomes in body fluids may be indicative for the disease state. Interestingly, patients suffering from sarcoidosis presented with significantly higher exosome concentrations in broncho-alveolar lavage fluid (BALF) compared to healthy individuals, suggesting that exosomes may play a key role in disease progression and exosome concentration in BALF may thus be indicative for disease severity.

In addition to the potential of EVs as biomarkers, EV-based carriers may in future be applied as diagnostic imaging agents, either experimentally to work out tissue homing and specificity [28-30], or clinically to better diagnose pathogenic processes in distant tissues that cannot normally be imaged by classical contrast agents [29, 30]. Suitable labelling of exosomes without affecting their properties is pivotal. Recently, Hwang and colleagues have demonstrated rapid radiolabeling of macrophage-derived exosome-mimetic vesicles with ^{99m}Tc for *in vivo* radionuclide imaging via SPECT/CT in

living animals [28]. Hu and colleagues have employed electroporation to load exosomes with superparamagnetic iron oxide nanoparticles for magnetic resonance tracking [30]. Busato et al. [29] have labelled cells with ultra-small superparamagnetic iron nanoparticles and then isolated exosomes from previously labelled cells. These exosomes retained the nanoparticles and demonstrated preserved morphology and physiological characteristics. Detection limits of 3 µg and 5 µg were demonstrated by magnetic resonance imaging *in vitro* and *in vivo*, respectively. Surprisingly, there are very few studies directly comparing EVs to their synthetic liposome counterparts. Using labelled exosomes as imaging probes promises advantages including the bypassing of many issues such as immune activation, off-target effect and rapid *in vivo* degradation. However, at the same time, these apparent advantages need to justify the risks of bringing biologically active exosomes into the body for diagnostic purposes. Off target effects of such naturally derived imaging probes need to be excluded and more investigations on risks and benefits of EVs are needed. Nonetheless, the above studies pave the way for exosome based diagnostic imaging with the prospect to enable new insights into disease aetiology, *e.g.*, in the context inflammatory responses or biofilm formation. Additionally, these concepts may be further extended to theranostic applications, *e.g.*, using iron oxide labelled exosomes for magnetic hyperthermia.

EVs as anti-infective therapeutic tools

EVs as versatile vaccines

There are two potential avenues of using EVs for treatment of pathogen-mediated infections: EV-based vaccines as discussed in this section and EVs as anti-infective entities themselves as outlined in the next paragraph. EVs may be ideal candidates for vaccination, as they naturally transfer information between cells, are stable under biological conditions and show specific binding to immune-competent cells [3, 9]. EV-based vaccines may be derived from pathogen primed human cells or directly from pathogens. APC-derived EVs, in particular from dendritic cells (DC) that were pulsed with pathogens appear to be a promising source of EV-vaccines. *Leishmania* antigens transferred onto DC-derived exosomes conferred efficient protozoan infection protection by induction of T cell proliferation [31]. Furthermore, bone marrow DC-derived exosomes previously pulsed with pathogens elicited protective immunoglobulin production in mice which were lethally challenged with *Streptococcus pneumoniae* or *diphtheria toxoid* [32, 33]. EVs isolated from *Mycobacteria tuberculosis* treated macrophages induced antigen-specific interferon and interleukin production in T cells and thus a protective immunisation in an aerosol infection mouse model [34].

OMV-derived vaccines are another promising source of vaccines as they have been shown to be efficient in several studies, they offer exceptional pharmaceutical stability and can be obtained at large scale with high yield adapting bacterial fermentation production [35, 36]. They may be engineered to possess the desired number and type of antigens. Moreover, due to their particulate nature they are efficiently taken up by T cells and the innate immune system. Thus OMVs act as their

own adjuvant and do not require additives which overall reduces formulation complexity and increases ease of production [14, 37, 38]. Pathogen OMVs, for example from *Haemophilus influenza, Pseudomonas aeruginosa, Vibrio cholera, Bordetella pertussis* and others mediated protection against infections in several mouse models [22, 39] which indicates their huge therapeutic potential. The most advanced approach which is now approved for clinical trials (NCT01214850) is based on the combination of three major antigens from *Neisseria meningitides* with bacterial OMVs and has shown safety and efficiency in humans [40, 41].

EVs as therapeutic entities

EVs have been successfully employed for selective and targeted therapy of cancer and inflammatory diseases [10, 42, 43], although for some approaches ambiguous results have been reported [44]. It is now believed that EVs are natural intercellular "communicators" and convey specific target cell interaction. They offer additional promising features such as reduced immunogenicity and complement activation, physiological composition and advantageous size distribution to overall take advantage of active and passive targeting mechanisms [1, 4, 45] Nevertheless, EVs have not extensively been studied as potential therapeutic entities in infectious diseases. Mammalian EVs play an important role in the inherent host immune defence against pathogens. Recent studies suggested that EVs from stimulated monocytes inhibit the growth of *S. aureus in vitro via* aggregation of bacteria [46]. In this work, it was shown that neutrophil-derived microvesicles may restrict bacterial dissemination by their β2 integrin-dependent binding onto the bacterial surface. In a follow-up study it was revealed that neutrophil-EVs that were released upon specific bacterial activation were similar in size but significantly different in morphology, composition and anti-infective activity compared to spontaneously released EVs or those released upon cell death [47]. In other studies, human tracheobronchial cells produced EV-like particles that were active against human influenza virus due to the presence of virus-binding sialic acid in the vesicle membrane [48]. Both examples indicate that mammalian EVs may work as innate immune mechanism against infections, but the effect could be limited to specific vesicle populations. Pivotal for the development of EVs as drug carriers is their controlled clinical grade production and optimised isolation techniques [49]. Current approaches apply preparation of EVs from monocyte-derived dendritic cells [50], immortalisation of human embryonic stem cell-derived mesenchymal stem cells [51] or cardiosphere-derived cells [52], and indicate that significant efforts are underway.

Bacteria are also known to use OMVs as efficient defence strategy against other bacterial species during competition for environmental niches. Vesicles, for example from *Myxobacteria* or *Pseudomonas species* contain hydrolytic factors as secondary products aimed at killing prey microbes [53, 54]. By priming the producing bacteria with antibiotics, it was shown that these autolytic OMVs additionally contained the drug and showed synergistic bactericidal effects [54]. Recently, an alternative strategy for antibacterial delivery was assessed by preparing unconventional liposomes

from the membranes of prokaryotes and loaded with antibiotics [55]. These bacterio-mimetic liposomes reduced the minimal inhibitory concentration to a certain extend but the effect was dependent on the bacterial source. Finally, bacterial OMVs were also engineered for other therapeutic applications, for example as well-tolerated drug carrier with low endotoxicity [56]. These vesicles were derived from genetically modified bacteria with low immunogenicity, loaded with RNA-based drugs and equipped with human epidermal growth factor receptor 2 (HER2)-specific affibodies and were successfully employed in a cancer mouse model.

Furthermore, OMVs may also exhibit positive effects on the host immune tolerance; in particular probiotic-derived vesicles regulate immune reactions. It was shown that OMVs from commensal bacteria have fundamental roles in supporting the maturation of the immune system [22]. Vesicles from the probiotic gut commensal *Bacteroides fragiles* induce elevated numbers of regulatory T cells, increased anti-inflammatory IL-10 levels and were thus beneficial in a model of experimental colitis [57]. Other OMVs possess beneficial effects by improving provision of gut nutrients and removal of metabolites that are potentially carcinogenic [58]. Finally, it was shown that OMVs may also provide protective effects against allergic and chronic asthma. In this study, *Helicobacter pylori* virulence entities associated with the bacteria's OMVs were critically responsible for inducing tolerance of DCs *in vivo* [59]. These examples clearly indicate the biomedical potential of OMVs for immune regulation and anti-inflammatory outcome.

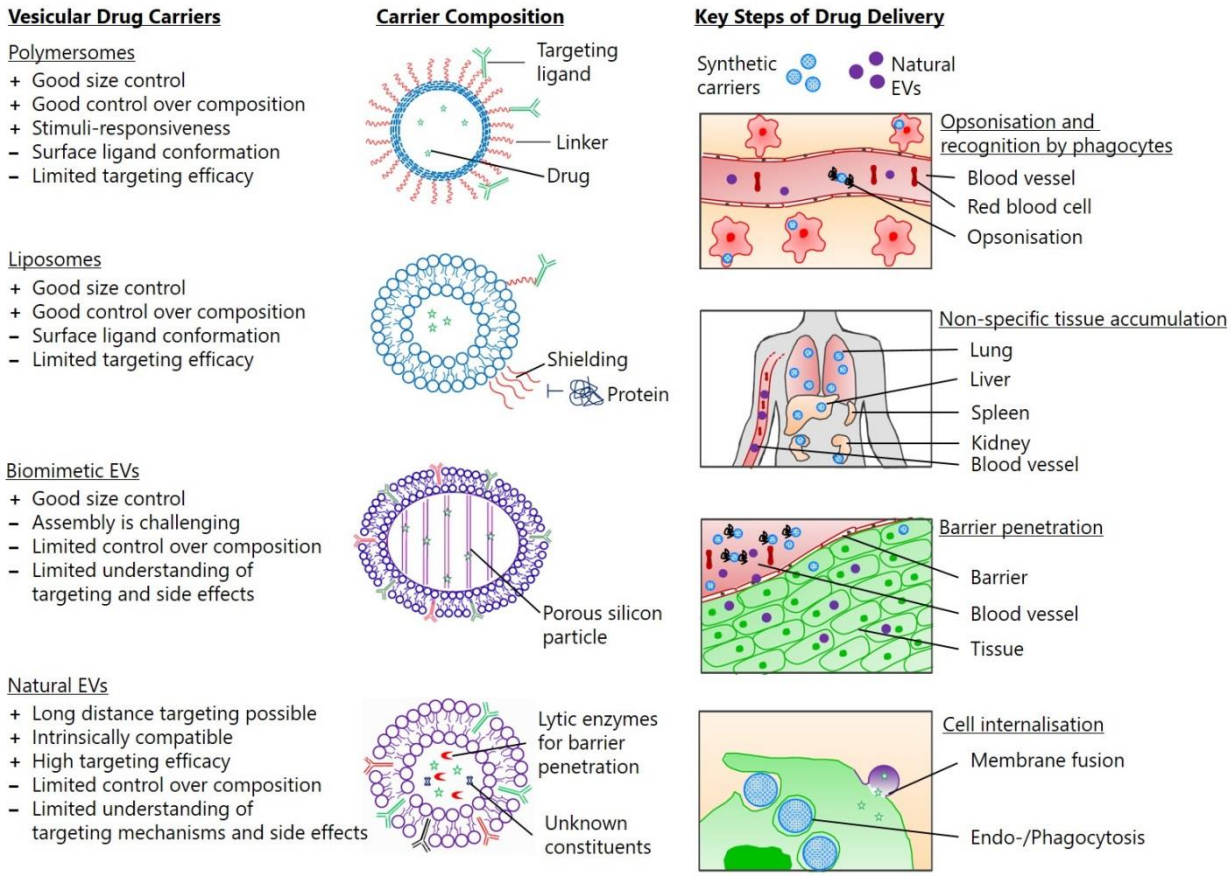

Figure 1. *Overview of current delivery systems for (antibiotic) drugs and their comparison to naturally-derived EVs. Natural EVs feature important properties, such as inherent long distance targeting abilities and intrinsic*

biocompatibility that make them auspicious drug carriers. Moreover, EVs may not easily be opsonised and detected by the immune system upon administration to the human body. Their unspecific accumulation in undesired tissue is thought to be less prominent and they may be able to penetrate biological and cellular barriers through additional mechanisms that are difficult to reproduce synthetically.

Are EV-based approaches seminal alternatives in infection research?

The rise of severe pathogen infections is becoming a leading cause for morbidity and mortality worldwide. For example, in Europe alone up to 25,000 people die each year from infections with resistant bacteria [60]. Alarmingly, the number of effective anti-infective treatments constantly decreases, which in turn further amplifies the need to improve the potency of our existing therapy arsenal and develop new sensitive detection techniques [61]. Incorporation of antibiotics, for example into liposomes, has been investigated as a highly promising strategy to overcome microbial drug resistance [62]. Liposomes loaded with antibiotics show improved drug delivery to the site of infection, protect antibiotics from degradation and reduce their side effects [63, 64]; with a few commercial applications *e.g.,* Ambisome®. Natural EVs have been shown to be promising targets for sensitive and early biosensing of infections [25]. Interestingly, it has been discovered that some mammalian and bacterial EVs exhibit inherent antibacterial activity [46, 48] serving as potential anti-infective therapeutics [54] or constitute a model for new biomimetic systems [65]. In other fields of drug delivery, EVs exhibited a natural composition and therefrom resulting better biocompatibility [12, 66, 67], better physical and physiological stability and superior loading efficiency compared to synthetic nanocarriers [66, 68], and they have shown great promise as selective and targeted biogenic carriers that are biocompatible and well-tolerated [42, 43, 69]. EVs are designed to find their way to target tissue in a highly specific manner that is incredibly difficult to reproduce in synthetic systems (***Figure 1***). Engineered carriers always rely on a compromise between efficient protein shielding to avoid opsonisation and recognition by immune cells (camouflaging) on the one hand, and sufficient density of the targeting moieties (*e.g.*, antibodies) on the other hand. Additionally, targeting moieties are required to be in the right conformation to allow efficient ligand binding and targeting. Particularly the conformation of integrins, however, is difficult to achieve in synthetic systems. Recent approaches have therefore employed natural membranes on porous silicon particle templates and more recently also template-free leukosomes to achieve targeting based on cell-mimetic drug carriers [70]. The authors have demonstrated that biomimetic proteolipid vesicles show remarkable plasma circulation times and targeting efficacies [71]. While this approach has been shown to work very well, the use of cell-derived membrane compounds carries risks similar to the use of EVs (limited control over composition and residual risk of introducing virulence factors) and hence the added value remains questionable. Moreover, natural EVs may carry interesting additional characteristics for barrier penetration (*e.g.,* lytic enzymes, etc.) and tissue homing that cannot be reproduced synthetically (***Figure 1***). EVs may thus allow Trojan-horse-based delivery of anti-infective drugs at unprecedented efficacy and specificity. Provided that EVs do not contain any unwanted immune-active constituents,

side effects may be drastically reduced compared to traditional drug therapy systems. The use of EVs derived from autologous cells may be a first step towards a personalised medicine with very little side effects and high therapeutic efficiency and specificity. While bacterial OMVs have been used as safe and versatile vaccines [38] and as well-tolerated delivery system [56, 57], many questions concerning the EVs' suitability for clinical applications remain, for example safety of cell sources and correct conformation of EV-derived antigens. Building upon biological proof-of-principle studies, further fundamental and translational research, and efforts on harmonisation of regulatory aspects will be needed in the near future [5, 72]. Another important issue to be addressed in the future is their reliable and reproducible isolation and purification, and their robust analytics. Ongoing biotechnological developments combined with promising results in clinical trials will pave the way towards the future use of EVs in infection therapy. Despite current obstacles in the production of EVs for drug delivery applications, we feel that the unique tissue specificity along with the multistage drug delivery properties constitute features that cannot easily be achieved by synthetic nanocarriers, and hence give significant momentum to continuing investigations and development of EV-based strategies.

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