Review

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Novel biomarker and drug delivery systems for theranostics – extracellular vesicles

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Abstract: Extracellular vesicles (EVs) are nano- and micro-sized double-layered membrane entities derived from most cell types and released into biological fluids. Biological properties (cell-uptake, biocompatibility), and chemical (composition, structure) or physical (size, density) characteristics make EVs a good candidate for drug delivery systems (DDS). Recent advances in the field of EVs (e.g., scaling-up production, purification) and developments of new imaging methods (total-body positron emission tomography [PET]) revealed benefits of radiolabeled EVs in diagnostic and interventional medicine as a potential DDs in theranostics.

Keywords: biomarkers; DDS; exosomes; J-PET; positronium.

Introduction

For the first time, vesicles formed inside a cell were observed by Gorge E. Palade and Marilyn G. Farquahar using a transmission electron microscope (TEM) in 1959 [1]. They observed protein rich, absorption droplets, formed in the glomerular epithelium, supposed to carry of ferritin molecules across the wall of glomerular capillaries. This fundamental observation by G. E. Palade laid the foundations for research into vesicle cargo transport [2]. Another important discovery made by P. Wolf suggested that the lipid-rich particles might originate from the osmophilic granules of platelets as extrusion of platelets, even in the absence of coagulation. He suggested that the liberation of this material was the basis of platelets activation known to occur with storage [3]. A recent paradigm about origin and composition of cell released vesicles states that extracellular vesicles (EVs) are double-layered membrane entities derived from most cells and released into biological fluids [4]. The average size of EVs is estimated between 30 and 500 nm and covers the range of smaller *exosomes* (30–100 nm) and bigger ectosomes (over 100 nm) [5]. This characteristics makes EVs to be an ideal medium for design a drug delivery system (DDS) [6] (Figure 1). The terms *exosomes* and *ectosomes* are widely accepted in the scientific community to distinguish *ectosomes*, as EVs generated by membrane budding and shedding, from *exosomes* created within and released from multivesicular bodies (MVB). In fact, the concept of EV terminology has been extensively discussed and reviewed to designate the families of studied extracellular vesicles [7]. The biggest EVs, around 1 μm and more, comprises the population of apoptotic bodies [8], which control downstream cell disassembly, and, in our opinion, they do not have properties assigning them for DDS development.

Extracellular vesicles as biomarkers

Following the observations by Wolf, platelets are the main contributors in the membrane vesicle formation [3]. However, platelets are the source of the bigger EV-family members, called ectosomes or microparticles (MP) [9, 10]. The formation of EVs in platelets occurs due to platelet activation after stimulation with the agonists: thrombin, collagen, and the thrombin receptor agonist peptide SFLLRN or with calcium ionophore A23187 [11, 12] (Figure 2). Platelet MPs

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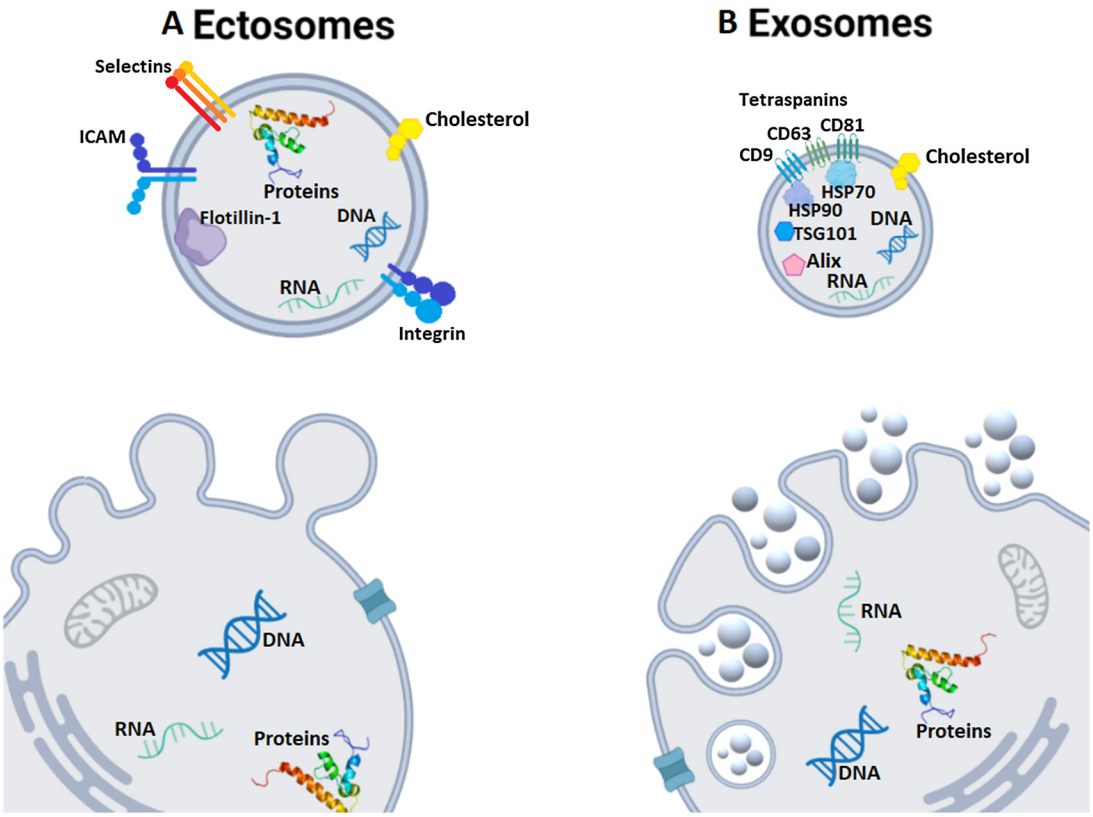

Figure 1: Schematic representation of extracellular vesicle (EV) formation and characteristics. (A) Ectosomes are shed from the cell plasma membrane. Examples of ectosomes cargo are mostly cell adhesion receptors like: integrins, selectins, ICAM and flotillin-1. (B) Exosomes are created inside the cell in multivesicular body (MVB) and released to extracellular space upon fusion of MVB with the cell membrane and its subsequent opening of their lumen. Some proteins that are mostly identified are accepted as specific exosome markers, such as tetraspanins (CD9, CD63, CD81), heat shock proteins (HSP70, HSP90) and MVB biogenesis proteins (Alix, TSG101) [6]. Tetraspanins belong to the family of membrane proteins with four transmembrane domains that play a role in adhesion and signaling.

(PMPs) have proven to be biomarkers in cardiovascular diseases by indicating emergency conditions as myocardial infarction (MI) [13], stroke [14], or those long-term complications of diabetes [15, 16] and atherosclerosis [17].

The significance of PMP in patients with diabetes mellitus to determine their clinical outcome and complications including diabetic nephropathy, has been first proposed as a new blood biomarker [16]. Recently, we may

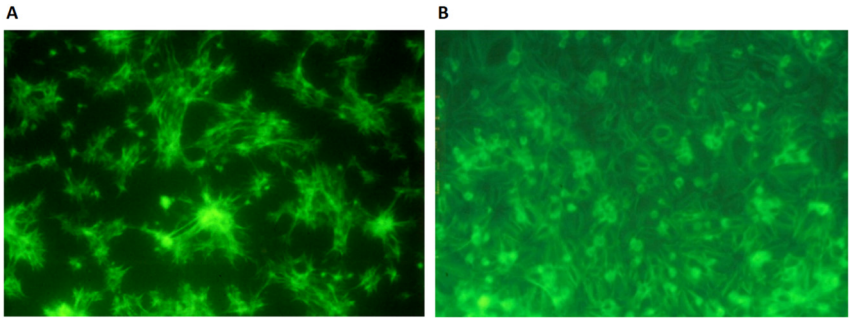

Figure 2: Fluorescent images of human phalloidin-stained platelets after activation with 1 IU of thrombin. (A) Platelets obtained from plasma of a healthy volunteer and (B) the same volunteer after taking 150 mg of acetyl-salicylic acid (ASA). (A) In this fluorescent image, the visibility and contrast of the spindle-shaped platelets are prevalent. Activated by thrombin, platelets aggregate and secrete vesicles. (B) Acetylsalicylic acid inhibits platelet aggregation. Weak image contrast indicates that platelets are not being activated (2002, from the archives of E.Ł. Stępień). The scale of Figures A and B is the same.

propose the novel approach utilizing urine EVs (UEVs) and their molecular signatures revealed by Raman spectroscopy [18, 19], proteomics [20, 21], mirRNomics [22] or cytomics [23, 24]. EVs are also very promising biomarkers in cancer, not only in the urine, were UEVs are most relevant in prostate or bladder cancer [25, 26], but also in other types of cancers including hepatoma [27, 28], pancreatic [29] or lung cancer [30] as diagnostics augmentation.

Platelet-derived microvesicles (PMVs) shed from platelet surface and constitute the majority of circulating microvesicles. PMVs are assigned to have procoagulant, proinflammatory and proatherosclerotic effects associated to chronic platelet activation [31]. Formation of PMVs is timely related to the translocation of aminophospholipids into an external membrane layer and cytoskeleton reorganization. These processes are mediated by increased levels of calcium ions (Ca^{2+}). Compared with platelets, the procoagulant activity of PMV lasts longer and occurs even far from the site of platelet activation. The biological effects of PMVs include more than just blood coagulation: (1) PMVs stimulate platelet formation; (2) help leukocytes bind to activated endothelium; (3) provide the basis for pro-inflammatory and proatherosclerotic responses [32, 33]. These effects were explained by a sequence of pathways, e.g., p-selectin. This is a cell adhesion molecule on the surface of activated vascular endothelial cells that is responsible for the interaction of the inner layer of the blood vessel with activated thrombocytes [34, 35].

Extracellular vesicles as therapeutic agent and target

Pathological conditions like inflammation, hypercoagulation, diabetes or neoplasia accelerate *in vivo* EV formation. The mechanism of increased vesicle secretion is not fully understood, but at least one cause needs to be considered: hyperactivation – the presence of uncontrolled or subthreshold stimuli. For example, in endothelial cells, hyperhomocysteinemia [36, 37], hyperglycemia [38–40], hypercholesterolaemia [41] or hypoxia [42, 43] induce intense EV formation. In cancer patients, there are about 10^{10} EVs per mL in the circulation, twice as many as in a healthy population [44].

Currently many preclinical studies investigating the effectiveness of EV as biomarkers and therapeutic agents have been conducted and specific clinical trials have been established. At the ClinicalTrials website by US National Institutes of Health (NIH) information about major clinical applications for EVs is available [45]. We found 113 studies having "exosomes" phrase, 41 trials were related to "extracellular vesicles," 17 trials additionally included "biomarker," 10 among them included "therapeutic tools." On the other hand, 55 trials are related to the use of "exosomes" as biomarkers and 72 as therapeutics. Search was performed without filters.

An interesting example is the one-arm Pilot Clinical Study which took place at Ruijin Hospital Shanghai Jiao Tong University School of Medicine (China) that concerned inhalation of mesenchymal stem cells (MSC) exosomes for treating severe novel coronavirus pneumonia (NCT04276987). Twenty-four participants between 18 and 75 years old were receiving conventional treatment and five aerosol inhalations of MSCs-derived exosomes (2.0 × 10E8 nano vesicles/3 mL) for 5 days [46].

One more completed interventional clinical trial was conducted by A. Gąsecka et al. from Medical University of Warsaw, Poland (NCT02931045) including 60 patients, aged over 18 years with MI to compare the effectiveness of ticagrelor and clopidogrel treatment. Patients received oral ticagrelor 180 mg once followed by 90 mg twice daily (maintenance dose) or clopidogrel 300 mg/600 mg once followed by 75 mg once daily. Inhibition of P2Y1 and P2Y12 receptors decreases platelet aggregation and involves the release of different subpopulations of PEVs, ticagrelor reduces the release of procoagulant PEVs from stimulated platelets, this may impact the clinical benefit in patients treated with this drug [47].

Another example is the ExoDx Prostate IntelliScore (EPI) trial (Exosome Diagnostics, Waltham, MA, USA) to investigate the non-invasive test for discriminating benign/low-grade prostate cancer (PC) from high-grade prostate cancer (HGPC) detection, as the decision-making test to continue cancer treatment with prostate biopsy. The idea of this test covers gene expression analysis in UEVs [48]. Based on these examples, we can conclude that EVs by their heterogeneity reflect meaningfully a clinical state and can be used for clinical application.

Extracellular vesicles as drug delivery systems

Since the discovery of EVs, they have been envisioned as natural delivery vehicles in molecular therapy that are able to reach and accumulate at their target site without damaging other organs. The heterogeneity of the EV structure makes them appropriate for the loading of various cargo, including chemotherapeutics, non-coding

RNAs, synthetic nanoparticles, and oncolytic viruses [49]. EVs are encapsulated by a functionalized and glycosylated cellular membrane [50] which gives specific EV targeting skills both *in vitro* and *in vivo*. However, EV-based DDS have several limitations which must be considered: (1) some plasma proteins have similar density as exosomes (Figure 3); (2) pro-coagulant properties; (3) drug-efflux activity.

Exosomes and ectosomes may share similar size and density, making differentiation of EV sub-classes highly challenging. Additionally, other plasma components, such as lipoproteins: high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), proteins: albumin, β-lactoglobulin, thyroglobulin and several viruses, have similar sizes and densities with EVs. It confounds selective size- or density-based EV isolation [58].

EVs are composed from negatively charged phospholipids that are dominantly exposed on ectosome surface (Figure 1). These characteristics assign EVs pro-coagulant properties observed mainly in platelet or mesenchymal stem cell derived EVs [59]. The other limitation refers to the presence of drug-efflux pumps in EVs membrane and is highly relevant in the context of multidrug resistance (MDR). First, P-glycoprotein (P-gp) and ATP-binding cassette transporter G2 (ABCG2) present in EVs may mediate the active sequestration of drugs in those vesicles. Second, recipient cells may get the drug-efflux pumps that are shed from donor cells [60].

Scaling of EV production for theranostic purpose

Despite the wide use of EV in the diagnosis and therapy of various diseases, the problem of efficient methods of EV isolation remains significant. The small size of EVs and their heterogeneity are the major problems in isolation (Figure 3). The methods currently used for the isolation of EVs are sucrose or iodixanol density gradient ultracentrifugation, hydrostatic dialysis (HFD), micro- and ultrafiltration (UF), low vacuum filtration (LVF) [61], size-exclusive chromatography (SEC), EV precipitation with polyethylene glycol or commercial reagents (e.g., protamine) and ultracentrifugation (UC) [62]. The current gold standard is being a method based on UC [63]. UC is limited by long duration, presence of contaminants in the EV preparations and the need for expensive equipment. The advantages of ultracentrifugation involve applicability for EV isolation from large volumes of biological samples, which does not require a set of reagents [64]. In case of LVF, EVs are not contaminated with abundant proteins, and the bulk volume of liquid samples can be

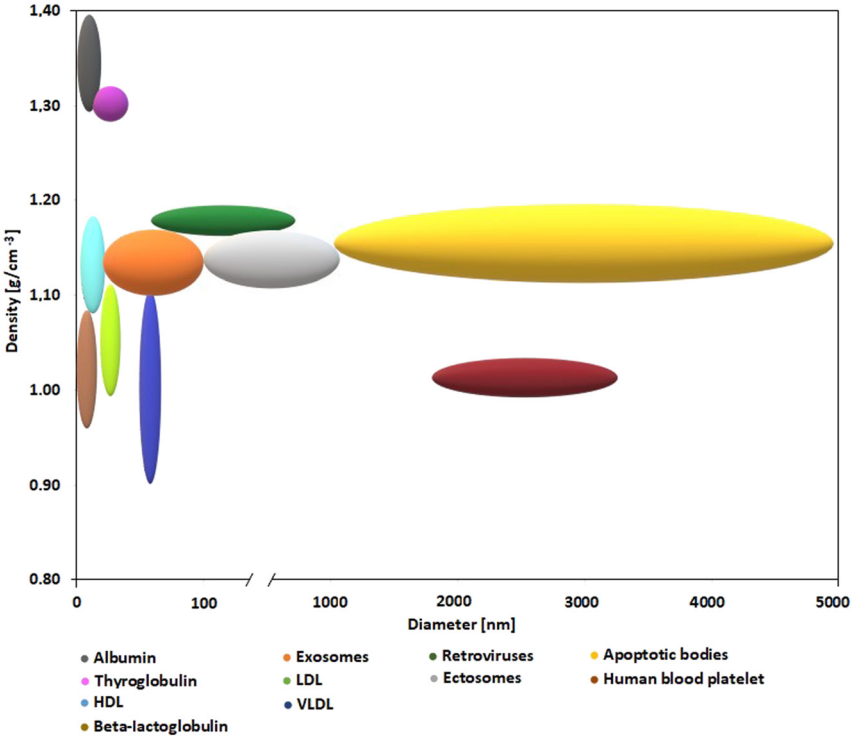

Figure 3: Dependence of density on the size of exosomes, ectosomes, apoptotic bodies [51], HDL, LDL, VLDL [52], albumin [53], β-lactoglobulin [54], thyroglobulin [55], retroviruses [56] and human blood platelet [57]. Abbreviations are explained in the text.

fractionated, what makes this method useful for urine or cell-conditioned media.

Another important issue is getting enough EVs for therapeutic purposes: the large cohorts of patients were requiring a large number of EVs. The key operations of each EV isolation are volume-reduction and purification, to avoid contamination with spurious cell fragments and extracellular proteins and protein complexes or bacteria. This is an important matter when EV isolations comes from milk, where casein micelles and polymeric structures containing casein, usually co-sediment during purification of EV [65]. This issue can be solved by divalent ions chelation to wash casein micelle.

In *in vitro* experiments, the amount of EVs depends on the scale of the cell culture. A large cell culture leads to a large volume of conditioned medium, which cause difficulties in isolation. The proposed solution is to scale-out or scale-up cell culture platforms. This includes 2D supports such as roller bottles or hyper-flasks and 3D culture systems like bioreactors [66]. Such approaches are dedicated to obtaining the maximum number of cells and EVs, also minimizing culture time and consumables. Another strategy is to increase the amount of secreted EVs by a cell. This process can be enhanced by stimulating cells through hypoxia, calcium ions, serum starvation, chemical or physical stress [67]. However, the application of cell stimulation affects their bio-physical properties, which is sometimes not required.

EV retention *in vivo* as an important issue in theranostics

Predictable kinetics is one of important attributes characterizing a biomarker. Since an injection is a common method of EV delivery to tissues, determination of the biomarker life-time in a bloodstream is crucial for the effective EV treatment. Unfortunately, EVs are quickly removed from a bloodstream by a liver or a spleen, thereby hampering delivery to distant tissues [58]. To achieve an optimal therapeutic effect of EVs, many strategies are being developed to prolong EV retention. One promising approach is using of hydrogels to maintain EV retention.

EVs derived from mesenchymal stem cells (MSC-EVs) have been recognized as a promising cell-free therapy for many diseases like acute kidney injury (AKI). Use of EVs avoids safety concerns associated with direct MSC cell engraftment. However, low stability and retention of MSC-EVs limit their therapeutic efficacy. To improve EV retention, the RGD peptide (Arg-Gly-Asp) has been proposed as a ligand which binds strongly to integrins presented on the surface of MSC-EV membranes [68]. Hydrogels containing RGD peptides could augment MSC-EV efficacy in the treatment of AKI. *In vivo* tracking of the stained EVs revealed that RGD hydrogels significantly increased stability and retention of MSC-EVs [69]. The other strategy is the use of a hydrogel containing ureido-pyrimidinone (UPy) units combined to poly-ethylene glycol chains (UPy-hydrogel). Such combination has a potential to develop a new EV-transport platform by keeping EV functionality and stability [70].

Theranostic application of EVs – the proposal

We anticipate, that advent of total-body PET scanners enabling simultaneous dynamical imaging of radiotracers' distribution in all tissues of the human body [71, 72], opens new perspectives for spatial and temporal *in vivo* tracking of radiolabeled EVs in the whole human body. In principle, EVs may be loaded with many hydrophobic theranostic radio-ligands, thus enabling imaging and therapy. Radio-imaging agents play an important role in the early diagnosis of cancer. The development of DDS for the effective transport of radio-imaging agents to a tumor site is a major topic of current radiopharmaceutical research. Additionally, the perinuclear localization of the internalized EVs shows their biological stability after their uptake to the endothelial cells [73]. Of the several imaging techniques that have been applied to track EV biodistribution in clinical intraoperative *in vitro* and *ex vivo* imaging is possible owing to the nuclear medicine techniques – positron emission tomography (PET). PET is one of the most effective methods of molecular imaging because it can detect tissue changes at the molecular level, even before the alterations turn into tumors. The introduction to PET imaging the glucose analogue labeled with Fluorine 18 (2-[18F]fluoro-2-deoxy-D-glucose, 2-[18F]FDG), administered the first time in August 1976 to human beings by Alavi A. and Reivich M. was a milestone in PET. FDG is still a dominant radiotracer in oncology, but also in cardiology and neurology [74].

To perform PET diagnostics, the patient receives an agent labeled with a radioactive isotope emitting positron (mainly 18F). In the tissue, positrons (e^+) annihilate with electrons (e^-), creating predominantly two photons, which are then registered with a PET scanner and used for image reconstruction. New concept of PET scanners (J-PET tomograph [71, 75–77]) enables detection of all events including multiphoton annihilations and prompt gammas,

not restricted to the standard double annihilation photons coincidences [78, 79]. Such approach will open a new window of theranostics, to use a multiphoton PET imaging, discussed also in this issue [80, 81]. Introducing a new biomarker, positronium imaging will bring additional advantage of PET imaging, not previously used in diagnostic medicine [82, 83]. Other nuclear medicine imaging modality is single photon emission computed tomography (SPECT). Radiolabeling EVs or liposomes empower them to track using either SPECT or PET. The main difference between these two imaging methods lies in the type of radioisotope, the signal detection and conversion into 3D images.

Two main methods are proposed for EV-radiolabeling as membrane surface labeling and intraluminal. Membrane radiolabeling includes: (1) incorporation of the radionuclide directly into the lipid membrane; (2) attaching to the membrane proteins or (3) binding *via* a chelator. Different method to radiolabel EVs is to entrap the radiotracer inside vesicle – intraluminal radiolabeling, which involves: (1) remote loading or (2) ionophore-chelator binding. EVs surface or membrane radiolabeling is currently used method [84].

Recently, mouse hepatocyte-derived EVs treated with neuraminidase (enzyme freeing by virus that digests the terminal sialic acid from glycoproteins) and ^{124}I-labelled were used for DDS development. It was observed that despite the fast retention of EVs in a liver, number of EVs was presented in different organs including lungs [85]. Another strategy of EV modification to facilitate their biodistribution is exosome PEGylation. Radiolabeled with [^{64}Cu]Cu-NOTA and PEGylated exosomes displayed a superior pharmacokinetic profile and higher accumulation in the tumor vs. traditionally reported native exosomes [86].

The field of EV research and in particular the applications of EVs as DDS has inspired scientist to propose EVs as a new medium for molecular imaging, especially for radioisotope imaging. Loading into EVs a tracer (radionuclide) will help to prove EVs targeting and delivery by following them, especially by the new PET-scanner generation, designed to study the whole, or the total (by J-PET) patient body, this new modality will help in the integration of therapeutic and diagnostics processes, to personalize treatment, and make it more effective.

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Ethical approval: This study includes two microphotographs of human platelets from the archive by Ewa Stępień.

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