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Exosomes as drug delivery vehicles for Parkinson's disease therapy

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ABSTRACT

Exosomes are naturally occurring nanosized vesicles that have attracted considerable attention as drug delivery vehicles in the past few years. Exosomes are comprised of natural lipid bilayers with the abundance of adhesive proteins that readily interact with cellular membranes. We posit that exosomes secreted by monocytes and macrophages can provide an unprecedented opportunity to avoid entrapment in mononuclear phagocytes (as a part of the host immune system), and at the same time enhance delivery of incorporated drugs to target cells ultimately increasing drug therapeutic efficacy. In light of this, we developed a new exosomal-based delivery system for a potent antioxidant, catalase, to treat Parkinson's disease (PD). Catalase was loaded into exosomes *ex vivo* using different methods: the incubation at room temperature, permeabilization with saponin, freeze–thaw cycles, sonication, or extrusion. The size of the obtained catalase-loaded exosomes (exoCAT) was in the range of 100–200 nm. A reformation of exosomes upon sonication and extrusion, or permeabilization with saponin resulted in high loading efficiency, sustained release, and catalase preservation against proteases degradation. Exosomes were readily taken up by neuronal cells *in vitro*. A considerable amount of exosomes was detected in PD mouse brain following intranasal administration. ExoCAT provided significant neuroprotective effects in *in vitro* and *in vivo* models of PD. Overall, exosome-based catalase formulations have a potential to be a versatile strategy to treat inflammatory and neurodegenerative disorders.

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1. Introduction

Parkinson's disease (PD) is one of the fastest growing neurological disorders in the developed world. The expected increase in lifespan of the population will further lead to a rise in age-related diseases, including neurodegenerative disorders. PD is known to be associated with brain inflammation, microglia activation and secretory neurotoxic activities, including reactive oxygen species (ROS) [1–4]. Samples from PD brains have shown reduced levels of redox enzymes, catalase and superoxide dismutase, and other antioxidants [5–7]. This may cause oxidative stress and neurodegeneration in PD patients. In this regard, catalase is one of the most potent in nature antioxidants that deactivates one million free radicals per second per molecule catalase in a cycle of catalytic reaction. As such, a successful brain delivery of catalase may be instrumental for PD therapy.

Regrettably, the blood–brain barrier (BBB) severely limits transport of this large protein. In fact, 98% of all potent drugs that may improve therapy of various diseases of the central nervous system (CNS) are not in clinic because of their inability to cross the BBB [8]. Different drug nanoformulations have been developed to overcome the BBB [9,10]. Unfortunately, the opsonization of drug-loaded nanoparticles in the bloodstream caused two main problems of drug nanoformulations: nanotoxicity and rapid drug clearance by mononuclear phagocyte system (MPS) [11]. To solve this problem, a polyethyleneglycol (PEG) corona was introduced to perpetuate a stealth effect. However, although the PEGylation decreased drug uptake by MPS, it concurrently reduced the interaction with target and barrier cells, thus, decreasing drug biodistribution, particularly, in the brain [12–14]. In addition, the development of immune response to PEG corona significantly increased clearance of PEGylated drug nanocarriers [15–17]. For example, PEGylated liposomes were reported to lose their long-circulating property on the second week following systemic administration in mice [17]. This may become a real problem in case of chronic disease conditions, such as PD that requires a prolonged drug treatment. Furthermore, it was reported that 22%–25% of healthy blood donors already have pre-existing PEG antibodies due to the everyday exposure to PEG in cosmetics, food, *etc.* [18,19]. In this respect, several reports indicated that exosomes may have an immune privileged status that can efficiently decrease drug clearance [20–22]. Thus, exosome-based nanocarriers may

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function as an "invisibility cloak" for incorporated therapeutic agents; they can diminish clearance by MPS, and at the same time increase drug transport to the brain.

Regarding their nature, exosomes are nanosized vesicles secreted by a variety of cells [23], in particular, cells of the immune system: dendritic cells [24], macrophages [25], B cells [26], and T cells [27]. Exosomes were initially thought to be a mechanism for removing unneeded proteins. Recent studies revealed, they are actually specialized in long-distance intercellular communications facilitating transfer of proteins [28], functional mRNAs and microRNAs for subsequent protein expression in target cells [29,30]. To shuttle their cargo, exosomes can attach by a range of surface adhesion proteins and specific vector ligands (tetraspanins, integrins, CD11b and CD18 receptors), and deliver their payload to target cells [24,31]. Noteworthy, exosomes possess an intrinsic ability to cross biological barriers. Thus, tumor-derived exosomes and microvesicles originated in the brain of glioma-bearing mice, and human glioblastoma patients were detected in the blood circulation indicating their ability to cross the BBB [32].

Exosomes were exploited as drug delivery vehicles in several investigations [33–37]. In particular, exosomes loaded with an anti-inflammatory low molecular drug, curcumin, were shown to protect mice from lipopolysaccharides-induced brain inflammation [36]. Moreover, exosomes were harnessed for systemic delivery of exogenous siRNA across the biological barriers [20,33,37–39]. The incorporation of the therapeutic agents into exosomes increased the circulation time, preserved drug therapeutic activity, and improved the brain delivery. The similar methodology was applied to polymer nanoparticles that were covered with erythrocytes membranes [40]. Sheltering of nanoparticles with cellular membranes/lipids produced a potent stealth effect increasing their time circulation in the blood. Furthermore, cellular membranes of monocytes and macrophages were utilized to manufacture nanovesicles by their serial extrusion through filters with diminishing pore sizes [41]. According to this report, cell-derived nanovesicles loaded with anticancer agent, doxorubicin (Dox) significantly reduced tumor growth in mice without adverse effects that were reported for equipotent free Dox. Noteworthy, the effects of Dox-loaded exosomes were superior than the commercially available Dox-loaded liposomes, Doxil; the liposomal formulation was inefficient in reducing tumor growth in this model [41]. Noteworthy, exosomes can exert unique biological activity reflective of their origin. According to a recent study, exosomes released from mesenchymal stem cells produced significant cardio-protective paracrine effects against myocardial ischemia/reperfusion injury even without any drug loaded [42]. Overall, these reports indicate that exosomes may function as exceptional vehicles for the delivery of therapeutic agents.

We demonstrated earlier that macrophages preloaded with nanoformulated catalase, or transfected with catalase-encoded plasmid DNA can release exosomes with incorporated catalase, and facilitate drug transfer to target cells of neurovascular unit: neurons, astrocytes, and brain microvessel endothelial cells [43–45]. This process resulted in profound therapeutic effects in mouse models of PD [44,46]. We report here the development of a new exosomal-based formulation of catalase (exoCAT) that was obtained by drug loading into naïve exosomes *ex vivo*. Different approaches for drug incorporation were validated: incubation at room temperature (RT) with or without saponin permeabilization, freeze–thaw cycles, sonication, or extrusion of exosomes in the presence of catalase. The obtained exoCAT formulations were evaluated for catalase loading efficiency, release and antioxidant activity. Selected optimal exoCAT efficiently decreased oxidative stress and increased neuronal survival in *in vitro* and *in vivo* models of PD. We hypothesized that the encapsulation of catalase into exosomes may preserve catalase enzymatic activity, prolong blood circulation time, and reduce immunogenicity, thereby improve drug therapeutic efficacy. Importantly, this technology may be more generally applicable to the treatment of many CNS diseases, in particular neurodegenerative disorders [47–49] such as PD, Alzheimer's disease, infectious diseases (meningitis, encephalitis, and HIV-associated neurocognitive disorders [50,51]), stroke [52,53], and lysosomal storage diseases [54,55].

2. Materials and methods

2.1. Reagents

Catalase from bovine liver was purchased from Calbiochem (San Diego, CA). Lipophilic fluorescent dyes, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate (DIL), and 2-(5-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2H-indol-2-ylidene)-1,3-pentadienyl)-3,3-dimethyl-1-octadecyl-perchlorate (DID) were purchased from Invitrogen (Carlsbad, CA, USA). 6-hydroxydopamine (6-OHDA), lipopolysaccharides (LPS), rhodamine isothiocyanate (RITC), and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Interferon gamma (INT-γ) was purchased from Peprotech Inc. (RockyHill, NJ, USA).

2.2. Cells

A mouse macrophage cell line (Raw 264.7) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA, cat # TIB-71), and cultured in Dulbecco's Modified Eagle's Media (DMEM) (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). Neuronal PC12 rat adrenal pheochromocytoma cell line was obtained from ATCC, and cultured in Dulbecco's Modified Eagle Medium (Hyclone, South Logan, UT, USA) supplemented with 10% FBS, and 1% (v/v) of both penicillin and streptomycin. The cells were grown in an incubator with optimal culture conditions of 37 °C and 5% CO_2, and the medium was routinely replaced every 2–3 days.

Bone marrow derived macrophages (BMM) were obtained by differentiation of bone marrow stem cells extracted from murine femurs (C57BL/6, female mice) as described in [56]. The cells were cultured for 10 days in media supplemented with 1000 U/mL macrophage colony-stimulating factor (MCSF). The purity of monocyte culture was determined by flow cytometry using FACS Calibur (BD Biosciences, San Jose, CA, USA). Mouse primary cultured cortical neurons and dopaminergic (DA) neurons from *substantia nigra pars compacta* (*SNpc*) were isolated from mouse pups cortex and midbrain as described [57].

2.3. Isolation of exosomes

Concomitant media from Raw 264.7 macrophages grown on 75T flasks (20×10^6 cells/flask) were collected, and exosomes were isolated using gradient centrifugation [24]. In brief, the culture supernatants were cleared of cell debris and large vesicles by sequential centrifugation at 300 *g* for 10 min, 1000 *g* for 20 min, and 10,000 *g* for 30 min, followed by filtration using 0.2 μm syringe filters. Then, the cleared sample was spun at 100,000 *g* for 1 h to pellet the exosomes, and supernatant was collected. The collected exosomes (10^{11}–10^{12} exosomes/flask) were washed twice with phosphate buffer solution (PBS). To avoid contamination by the FBS-derived exosomes, FBS was spun at 100,000 *g* for 2 h to remove exosomes before the experiment. The recovery of exosomes was estimated by measuring the protein concentration using the Bradford assay and by Nanoparticle Tracking Analysis (NTA). The obtained exosomal fraction was re-suspended in PBS (500 μL, 1 mg/mL total protein), and characterized for size and polydispersity.

2.4. Loading of exosomes

Four approaches for catalase incorporation into exosomes were evaluated: the incubation at RT with or without saponin (Method I), freeze–thaw cycles (Method II), sonication (Method III), and extrusion (Method VI). For Method I, naive exosomes released from Raw 264.7 macrophages were diluted in PBS to a concentration of 0.15 mg/mL of