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Erythropoiesis-stimulating protein delivery in providing erythropoiesis and neuroprotection

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Erythropoietin (EPO), a glycoprotein, plays an important role in erythropoiesis and neuroprotection. EPO therapies for anemia or neurodegenerative diseases require frequent injections or high-dose systemic administration which may cause unwanted side effects. Various strategies for EPO delivery have been investigated for increasing EPO bioavailability and decreasing side effects, including nano/micro particles, PEGylation of EPO and transport-mediated delivery systems. Nano/micro particles provide EPO with long-term effect and protect EPO against proteolytic cleavage. PEGylated EPO prolong circulating time and reduce injection frequency of anemia treatment. A transport-mediated delivery system enables protein to cross biological barriers. Presently, there is no report about an effective delivery system of EPO for neuroprotection. This review focuses on EPO delivery systems for erythropoiesis or neuroprotection with prolonged duration and enhanced bioavailability.

Keywords: blood–brain (retina) barrier, drug delivery system, erythropoiesis, erythropoietin, fusion protein, glycoengineering, microencapsulation, nano/micro particles, neuroprotection, PEGylation

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1. Introduction

Human erythropoietin (EPO)'s main function is to stimulate the proliferation and differentiation of erythroid progenitor cells for maturing red blood cells. It is biosynthesized mostly in the kidney and recombinant human EPO (rhEPO) is produced from Chinese hamster ovary or baby hamster kidney cells by recombinant DNA technology [1]. Recombinant human EPO is a glycoprotein consisting of 165 amino acid residues with three N-linked carbohydrate chains (Asn24, Asn38 and Asn83) and one O-linked carbohydrate chain (Ser 126). Two internal disulfide bonds between cysteine residues 7-161 and 29-33 are necessary for achieving EPO activity. The cleavage of the disulfide bond at cys 7-161 leads to the formation of covalently bound aggregate which is inactive and immunogenic. Molecular mass of whole rhEPO is about 30.4 kD [2,3]. A variety of rhEPO including epoetin alfa, epoetin beta, epoetin delta, epoetin omega and darbepoetin alfa (DPO) are used in the clinic. Epoetin alfa, beta, delta and omega are four isoforms of rhEPO [4,5]; DPO is produced by glycoengineering, containing two additional N-linked carbohydrate chains to improve the stability and pharmacokinetics properties [6]. Marketed pharmaceutical products of rhEPO are used as injections. The formulations and pharmacokinetics of rhEPO are shown in **Table 1**. Preparations of rhEPO are mainly used for the clinical treatment of anemia associated with chronic renal failure, cancer and HIV infection [1]. Recently, EPO and EPO receptor (EPOR) have been widely investigated in the nervous

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Table 1. Route of administration, half-life and ingredients for marketed rhEPO pharmaceutical products.

Brand name (composition)	Route of administration	Half-life (hours)	Ingredients	Buffer system	pH
Epogen® (Epoetin α)	IV	4 – 13	Human albumin	Citrate and phosphate	6.9
	SC	5 – 24			–
Eprex® (Epoetin α)	IV	4 – 13	Human albumin	Citrate	–
	SC	5 – 24	Polysorbate 80, Gly	Phosphate	–
Procrit® (Epoetin α)	IV	4 – 13	Human albumin	Citrate and phosphate	6.9
	SC	5 – 24			–
Recormon® (Epoetin β)	IV	4 – 12	Urea, Gly, Leu, Ile, Thr, Glu, Phe, Polysorbate 20	Phosphate	–
	SC	13 – 28			–
Dynepo® (Epoetin δ)	IV	4 – 13	Polysorbate 20	Phosphate	–
	SC	27 – 33			–
Repotin® (Epoetin ω)	IV	4 – 13	Human albumin	Citrate	–
	SC	5 – 24			–
Aranesp® (Darbepoetin α)	IV	21	Human albumin	Phosphate	6.0
	SC	49	Polysorbate 80		6.2

IV: Intravenous; SC: Subcutaneous.

system (**Table 2**). Neuroprotective functions of EPO associated with antiapoptosis, antioxidation, neurotrophic action and angiogenesis can be applied to several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and glaucoma [7,8]. Patients may be intravenously or subcutaneously injected 12 times monthly, which causes a high risk for infective dermatitis at the injection site, reducing the patient's acceptance. Although EPO following systemic administration can pass through blood–brain and blood–retina barriers or endothelial cells tight junction, high-dose EPO (5000 U/kg) is necessary for achieving neuroprotection. Long-term and high-dose systemic administration may result in unwanted side effects [9-11]. Thus, development of EPO preparations with long-lasting action and improvement of EPO availability for target cells are highly encouraged. This review proposes the following strategies: i) modification of EPO by chemical conjugates or fusion protein or glycoengineering to prolong EPO *in vivo* activity; ii) micro/nano particles base delivery systems increasing EPO duration and delivery efficiency; iii) use of surface antigen to recognize specific cells; and iv) transport systems to deliver EPO across biological barriers, increasing EPO to target sites efficacy.

2. Micro/nano particles – base delivery systems of EPO

Encapsulating protein into nano/micro particles is an important strategy to extend protein activity duration and reduce administration frequency following a single injection. Recently, long-term release microparticle products have been marketed to effectively deliver drugs, such as leuprorelin (Leuplin Depot®, Takeda Chemical Industries Ltd., Tokyo, Japan), lanreotide (Somatuline Depot®, Ipsen Pharma Biotech, Signes, France) and recombinant human growth hormone (rhGH; Nutropin Depot®, Genentech Inc., South San Francisco, CA, USA). **Table 3** summarizes the preparation methods and biomaterials used on EPO-loaded nano/micro particle properties. Synthetic polymers, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(DL-lactide-co-glycolide) (PLG) are widely used to prepare nano/micro particles. The advantages of these materials are that they are not only biodegradable, biocompatible and non-toxic, but also diversify materials chosen for formulation. PLG rhEPO-loaded microparticles can be prepared by w/o/w solvent evaporation method associated with two significant defects, non-continuous release and rhEPO degradation [12-14]. Linear triblock copolymers consisting of hydrophobic and hydrophilic blocks have been used as substitute for PLG to prepare rhEPO-loaded microparticles. This resulted in continuously releasing rhEPO due to combining different polymers to give a more suitable erosion time [15-16]. Adding protein stabilizers to the formulation to protect the active protein, such as bovine serum albumin, lactide monomers or 2-hydroxypropyl-p-cyclodextrin, can reduce the formation of rhEPO aggregates and burst release [13,14]. Using hydrophilic polysaccharide and sodium hyaluronate as protective materials averts water/organic solvent interfaces, which lower the amount of denatured rhEPO for 3 days of continuous release [12,17]. To solve the problem of EPO instability in particles, human serum albumin (HSA) is selected as a stabilizer, due to HSA having much less

Table 2. Summary of EPO used in neuroprotective treatment.

Model	Administration, dose and time	Treatment course	Result	Ref.
EPO systemic administration				
Spontaneous glaucoma mice	3,000 – 12,000 U/kg, QW	6 months	Reduced loss of retinal ganglion cells	[10]
Acute retinal ischemic rat	5,000 U/kg	Once	Promoted retinal neuron survival and restored function	[66]
Transgenic SOD G93A mice (ALS model)	1,000 U/kg, BIW	2 months	Delayed the onset of motor deterioration	[67]
EAE rodents	5,000 U/kg, QD	1 – 2 weeks	Improved neurological function on EAE test	[68,69]
Middle cerebral artery occlusion rat	5,000 – 10,000 U/kg, QD	1 – 7 days	Reduced cell apoptosis and improved neurological function on foot fault and corner test	[55,68,70]
EPO local administration				
Optic nerve axotomy rat	Intravitreal injections, 2 U/eye, Q3D	Four times	Rescued retinal ganglion cells apoptosis	[71]
MPTP-induced parkinsonism mice	Intracerebral injections, 16 U/mice	Once	Restored locomotor activity	[72]
6-OHDA-induced parkinsonism rat	Intrastriatal injections, 20 U/rat	Once	Protected tyrosine hydroxylase neurons and improved rotational asymmetry and forelimb use	[73]

ALS: Amyotropic lateral sclerosis; BIW: Twice weekly; EAE: Experimental autoimmune encephalomyelitis; EPO: Erythropoietin; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA: 6-hydroxydopamine; Q3D: Once every 3 days; QD: Once daily; QW: Once weekly; SOD: Superoxide dismutase.

Table 3. Various EPO-loaded nano/micro particles preparation methods and characteristics.

Drugs	Preparation method	Biomaterial	Particle size	Residual solvents	Denatured protein	LE (%)	Release time	Ref.
RHEPO	w/o/w	PLG	25 μm	250 ppm DCM[b]	3%	71	24 h	[13,14]
		PLG/BSA as stabilizer	28 μm	–	1%	86		[13]
		PLG/additional 5% lactide	43 μm	75 ppm DCM	–	96		[14]
	w/o/w	PLG-PEO-PLG (linear)/BSA as excipient	45 μm	–	5%	86	14d	[15]
	w/o/w	PLG-PEO-PLG (star-branched)	73 μm	–	10%	40	24 h	[16]
	W_{cores}/O/W[c]	PLG/HSA and PK as cores	6 μm	–	4% (1st day) – 39% (27 days)*	68	27d	[20]
	Spray-drying (w/o)	PLG/additional 5% lactide	8 μm	25 ppm DCM	–	98	24 h	[14]
	Spray-drying	Hyaluronic acid (MW 1000 k)	4 μm	–	nd	–	72 h	[17]
	Spray-freeze drying	PLG/mannitol and glycine as excipients	–	–	1%	10	21d	[21]
	Reverse-phase evaporation	DPPC and SS[e]	150 nm	–	82%‡	17	–	[25]
DPO	Spray-drying (s/o)	PLG/trehalose as excipient	69 μm	200 ppm DCM	0.4%	103	28d	[22,24]
	Spray-freeze drying (s/o)	PLG/trehalose as excipient	29 μm	–	1.1%	95	28d	[24]

**In vitro* release condition.

‡percentages of inactive rhEPO.

BSA: Bovine serum albumin; LE: Loading efficiency; DCM: Dichloromethane; DPPC: ; DPO: Darbepoetin alfa; EPO: Erythropoietin; DPPC: Dipalmitoylphosphatidylcholine; HSS: Human serum albumin; PEO: Polyethylenoxide; PK: Polylysine; PLG: Poly(DL-lactide-co-glycolide); rhEPO: Recombinant human EPO; s/o: Solid in organic phase; SS: Soybean-derived sterol mixture; W_{cores}: In w_1 phase; rhEPO is pre-encapsulated in cores; w/o/w: Water in oil in water.

immunogenicity than other excipients [18]. To prepare rhEPO microparticles, rhEPO is initially encapsulated in cores with nano-scale by poly(L-lysine) and HSA to improve rhEPO stability both at the preparation and release stages and to increase the loading efficiency of integral rhEPO [19,20]. Because minimization of water content is a potential approach to maintaining protein stability, spray-drying or spray-freeze drying is an alternative method to microencapsulate protein drugs and will rapidly reduce the water content in the preparation process. Comparing spray-drying w/o emulsion method and w/o/w solvent evaporation method to prepare rhEPO-loaded PLG microparticles, the spray-drying w/o method resulted in lower denatured rhEPO, higher loading efficiency and lower residual solvent than the w/o/w solvent evaporation method [13,14,21]. Using spray-drying and spray-freeze drying methods to manufacture DPO microparticles, the particle size (69 vs 29 μm), yield (34 – 57 vs 73 – 82%), protein integrity (99.6 vs 98.9%) and bioavailability (21 vs 28%) have been recorded [22-24]. Liposome is another useful carrier for rhEPO, which evades clearance by the reticuloendothelial system. rhEPO-loaded liposome (EPO-Lip) exhibits more suitable pharmacokinetics and pharmacodynamic effects than free rhEPO, which result in increased AUC, low clearance rate, less steady-state volume distribution value, high counts and percentage in reticulocyte but the inactive form of rhEPO is high, more than 82%. The inactive rhEPO may result from the degradation in liposome preparation and blood circulation [25]. Advantages in EPO micro/nano particles base delivery systems for injection include prolongation of drug duration and reduction of administration frequency. The greatest challenge is to maintain the EPO stability until it arrives at the target sites.

Targeting delivery is an approach to amplify protein efficiency and enlarge biological effect. Attempting high specificity binding of aptamer or antibody to target cell antigen makes targeting drug delivery possible. Antibodies are well-known for specifically binding to their target antigen. Aptamer is an oligonucleic acid with a complex three-dimensional structure engineered through systematic evolution of ligands by exponential enrichment (SELEX), which has high specificity for its target molecule, low immunogenicity and good stability in organic solvents and high temperature. [26]. Cell surface antigen can be used to recognize and select specific cell populations. In *in vitro* studies, liposome with specific antibody conjugate has higher binding affinity to tumor cells, which increases the cytotoxicity of doxorubicin up to 90% [27]. In *in vitro* studies, PLA nanoparticles with aptamer conjugation increase 77-fold binding affinity [28]. In *in vivo* studies, the 2′-fluoro-pyrimidine modified RNA aptamer-mediated targeting delivery system effectively enhances the efficacy of anticancer drug to reduce xenograft tumor volume in nude mice, decrease body weight loss and maintain 100% survival rate [29,30]. Various antigens specifically appear on EPOR existent cells, including glycophorin A on erythroid progenitor cells [31], thy1 and neurofilament-L on retinal ganglion cells [32], and tyrosine hydroxylase on dopaminergic neurons [33]. Therefore, the approach for attaching specific aptamer or antibody on the drug carrier surface can be used to design particle-based EPO delivery systems to aim EPO to the target site.

3. Modification of EPO by chemical conjugates, fusion protein or glycoengineering

3.1 Chemical conjugates

Chemical modification is an important approach to change protein properties. Proteins bring together large molecules to improve stability and reduce the renal clearance by decreasing the glomerular filtration. This concept is also applied to modify protein molecules for extending half-life. The modification of molecules through the process of connecting a hydrophilic polymer, poly(ethylene glycol) (PEG) is called 'PEGylation'. PEGylation changes the stability, immunogenicity and pharmacokinetics properties of protein by reducing the renal clearance and also enhancing proteolytic resistance and eschewing recognition of immune cells. In the clinical setting, peginterferon α-2a (Pegasys®, Hoffmann-La Roche Inc., Nutley, NJ, USA) and peg interferon α-2b (Peg-Intron®, Schering-Plough, Innishanon, Ireland) have been used for the treatment of hepatitis C; peginterferons have longer half-life than the interferon without PEGylation. PEG with a molecular mass of about 30 kD are covalently attached to amino groups of lysine or N-terminus of rhEPO by forming amide linkages [34-36]. PEG–EPO (methoxy polyethylene glycol-epoetin beta; Mircera®, Hoffmann-La Roche Inc., Nutley, NJ, USA) is approved by the US FDA for the treatment of anemia associated with chronic renal failure to maintain stable hemoglobin levels by monthly administration. The half-life of PEG–EPO is longer (i.v.: 134 h; subcutaneous: 139 h) than epoetin and DPO. The absolute bioavailability for subcutaneous PEG–EPO, epoetin and darbepoetin alfa are 52, 32 and 37%, respectively. The clearance of i.v. PEG–EPO (0.49 ml/h/kg) is approximately one-third of darbepoetin alfa and 1/16 of epoetin. These results suggest that PEG–EPO prolongs the duration of EPO in the circulation [37,38]. PEGylation technique is also applied to modify nanoparticles surface. PEGylated liposome can avoid high-speed clearance by reticuloendothelial system (RES). Doxorubicin-loaded PEGylated DPPC/SS-liposome has longer circulation time than unmodified liposome [39]. PEGylation is an ideal approach to improve the pharmacokinetics and stability of protein itself and prolong the circulation time of a drug-loaded carrier.

Dimers of rhEPO are prepared by chemical crosslinking with rhEPO monomers containing free sulfhydryl groups and maleimido groups. Dimers of rhEPO have larger molecular sizes which may contribute to six times more elimination half-life (24 h) in rabbits and 26 times more biological activity in comparison with the monomers [40]. Thus, a lower dose of dimer can achieve the same

therapeutic effect as monomers because of the long circulating time. However, the immunogenicity of rhEPO dimers prepared by chemical crosslinking is still unclear and needs further investigation.

3.2 Fusion protein and glycoengineering

Fusion protein is created by expressing a hybrid gene made by combining two or more genes which alters characters of target protein and even have multiple functions. Many EPO fusion proteins have been reported, such as EPO fused individually with itself, granulocyte–macrophage colony stimulating factor (GM-CSF) and Fc. These EPO fusion proteins have longer half-life or greater biological activities in erythropoiesis by different mechanisms, such as the enhancement of binding affinity to EPOR, the extension of EPOR phosphorylated state and the increase of carbohydrate content [41-45]. Fc-EPO fusion proteins retain its biological activity to be delivered through the lung via immunoglobulin transport pathway associated with greater bioavailability (35%) for comparing rhEPO (18%) in cynomolgus monkeys [45]. Glycoengineering, a novel approach for modifying carbohydrate content of protein, may significantly increase protein potency and duration, extra N-linked carbohydrates can be added to consensus sequence (Asn-X-Ser/Thr) of protein. DPO differs in amino acid sequence from EPO especially with two additional asparagine residues (DPO: Asn 30 and Asn 88; EPO: Ala 30 and Trp 88), which provide sites for two additional N-linked carbohydrate chains with more potency (13 times) and longer half-life (3 times) in comparison with EPO [46,47].

3.3 Delivery of EPO across biological barriers

Neuroprotectants against neurodegenerative diseases must cross biological barriers, such as the blood–brain barrier (BBB) and blood–retina barrier (BRB). Large molecular weight and hydrophilic molecules cannot effectively pass through biological barriers. Transport-mediated delivery system is a non-invasive technique that is used to improve poor permeability drugs across biological barriers. Various transporters, including insulin, transferrin receptors and low density lipoprotein (LDL) receptor are expressed on biological barriers [48,49]. The murine 83-14 MAb can cross BBB via human insulin receptor mediated transport. The murine 83-14 MAb has been genetically engineered to produce a chemic antibody containing 85% human sequence, which has less immunogenic response in primates [50]. Anti-Aβ single-chain Fv antibody (anti-Aβ ScFv) and human insulin receptor monoclonal antibody (HIRMAb) fusion protein have 10 times higher brain volume distribution than anti-Aβ ScFv (100 μL/g brain : 10 μL/g brain). These results indicate that fusion protein can cross the BBB against Alzheimer's disease [51]. Brain-derived neurotrophic factor (BDNF) is a potent protein neuroprotectant that cannot pass through the BBB. OX26 attached to BDNF leads to an increase of BDNF influx in rat brain via transferrin receptor mediated transport [52]. OX26 is also attached to the nanoparticle surface to help nanoparticles to cross the BBB. Each PEGylated OX26 liposome contains 30 strands of OX26 which has greater gene uptake and expression in the brain than without OX26 conjugate [53].

4. Conclusions

EPO is used in a clinical setting to cure anemia and related diseases. Recent studies strongly indicate that EPO has potential therapeutic action in neuroprotection, and may also be applied for the treatment of moderate neurodegenerative diseases. However, large protein molecular weight and instability have significantly limited the clinical application of EPO. EPO structure modification by chemical conjugates or fusion protein technology and nano/micro particles carrier system design may achieve controlled-release EPO and optimize EPO stability. If the surface of carriers can also be modified to change original properties through PEGylation and combining antibody or aptamer, the delivery systems may further demonstrate a greater capability for prolonging protein circulating time, targeting delivery to specific sites and transporting across biological barriers. Optimum EPO developed delivery systems (**Figure 1**) will offer low dose and administration frequency, and fewer side effects for patients with EPO treatment.

5. Expert opinion

Neuron damage may occur following ischemia, trauma, stress and other factors. Neuron survival, plasticity and function are very important to maintain neuron activities. Neuron survival is associated with shorter latency neuroprotective function of EPO, such as antiapoptosis and antioxidation. Neural plasticity will be affected by longer latency neuroprotective function of EPO, involving angiogenesis, neurogenesis and neurotrophic action [7,54-56]. EPO was demonstrated to improve neuron survival, plasticity and function in various neurodegenerative diseases *in vivo* (summary in **Table 2**). The Göttingen EPO–stroke study suggests systemic administration of EPO is safe and well tolerated but the dose (100,000 U/patient, human mean weight is 70 kg) is lower than most neurodegenerative diseases in animals [57]. The dose of systemic administration EPO is higher than local administration, and the effects of systemic administration EPO dose and treatment course for various neurodegenerative diseases on erythropoiesis or angiogenesis in the retina or carcinogenesis are not clear [11,58,59].

There are three main targets for involving EPO modification to achieve therapeutics in erythropoiesis and neuroprotection: i) Extending the half-life and duration of active EPO; ii) Targeting delivery of EPO to effective sites; iii) Delivery of EPO across biological barriers to obtain neuroprotection. PEGylation can prolong active protein circulating time and stability but it also increases molecular

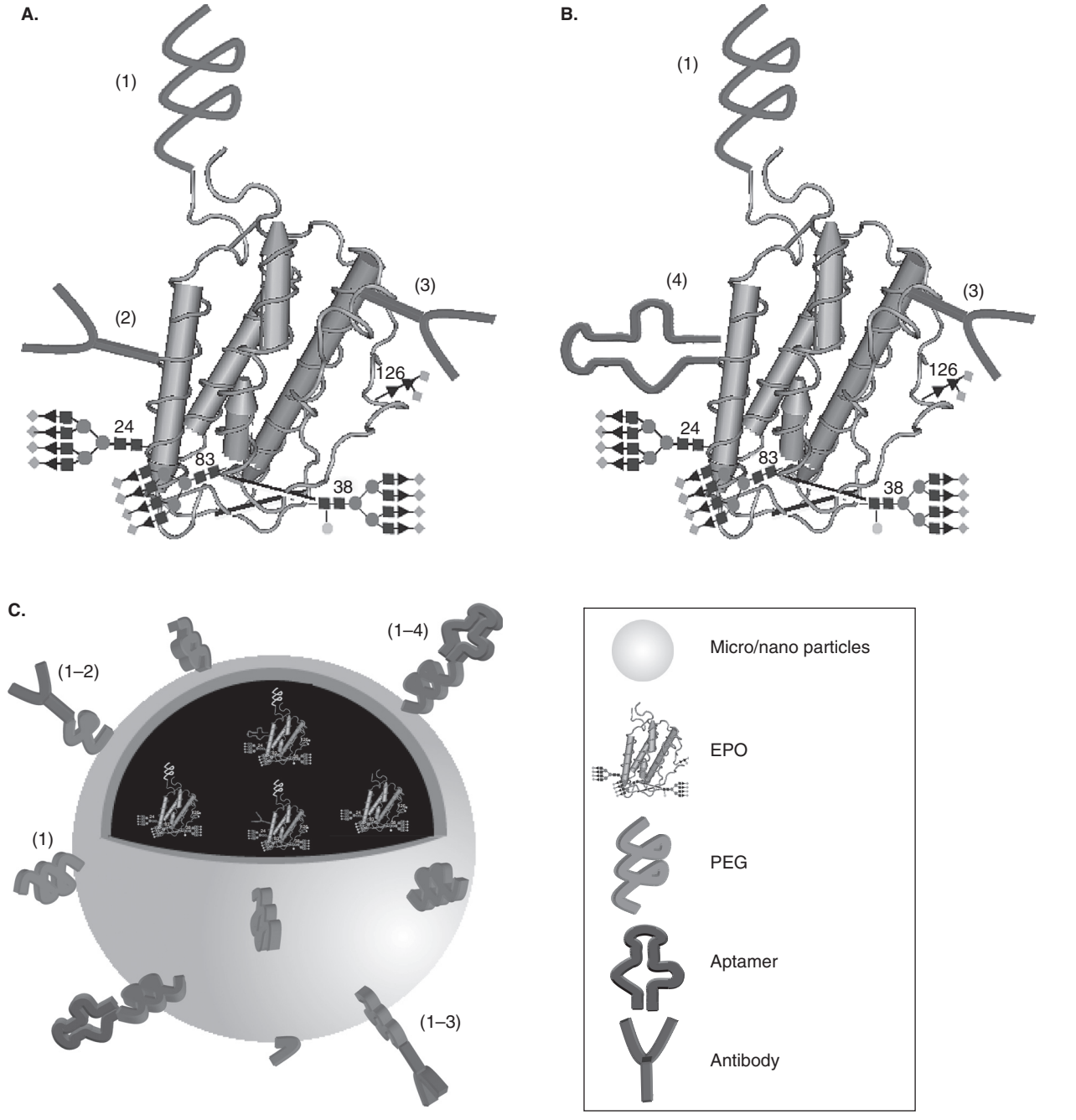

Figure 1. Delivery systems of erythropoietin. The conjugations include (1) PEG, (2) antibody for targeting delivery, (3) antibody for crossing biological barriers and (4) aptamer. (**A**) PEGylated erythropoietin (EPO)–antibody protein can be produced by fusion protein technique or covalent binding and PEGylation modification at non-active sites of EPO[3]. PEG can reduce the renal clearance of EPO and antibody can enhance EPO to cross biological barriers and targeting delivery. (**B**) PEGylated EPO–aptamer antibody can increase targeting effect of EPO. (**C**) EPO modified with none, (1), (1 and 2) and (1 and 4) can be encapsulated into micro/nanoparticles and the surface can be modified by (1), (1–2), (1–3) and (1–4) to increase the targeting effect.

weight and solubility, which results in greater difficulty in crossing biological barriers, such as BRB and BBB. The delivery systems are not very specific [60] because even the combined use of two strategies only enhances protein accumulation in organs, not in tissue or nidus. If specific antibody on the carrier is combined, targeting delivery of EPO would seem to be helpful to achieve a therapeutic level at the effective site for solving this problem. PEG–liposome with anti-gliofibrillary acidic protein can specifically bind to brain astrocytes *in vitro* but it cannot cross the BBB in animal studies [61]. A single strategy may overcome one barrier for EPO delivery, but does not reach the ultimate destination. It is preferable to use multiple strategies to achieve two or more targets. For example, PEG–liposome conjugates transport ligand and specific aptamer for prolonging circulating time, crossing biological barriers and targeting delivery (**Figure 1**). Delivery systems are prepared using multiple strategies that are difficult to optimize and evaluate. An *in vitro* model can be designed to evaluate delivery systems across biological barriers quickly. The *in vitro* model of the BBB, primary brain capillary endothelial cells and astrocytes co-cultured in cell culture inserts is well established, which has transferrin transport and uptake function for passing through biological barriers, and can be applied to evaluate biological barriers' permeability of drug delivery systems containing antibody against transferring receptor [62]. In order to evaluate neuroprotection of EPO, some target cells, retinal ganglion cells, hippocampal and cerebral cortical neurons can be cultured on the bottom of cell culture wells and determine neurons' survival in toxic conditions [63-65]. Through *in vitro* tests, the better formulations can receive further examination *in vivo*.

Many strategies are advancing EPO delivery for disease therapy. Combining these techniques, a successful EPO delivery can be achieved with an optimized delivery system, which may be developed for neuro-protection in diseases such as Alzheimer's disease, Parkinson's disease and glaucoma.

Declaration of interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

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