

Drug delivery for bioactive polysaccharides to improve their drug-like properties and curative efficacy

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ABSTRACT

Over several decades, natural polysaccharides (PSs) have been actively exploited for their wide bioactivities. So far, many PS-related reviews have been published; however, none focused on the delivery of bioactive PSs as therapeutic molecules. Herein, we summarized and discussed general pharmacokinetic properties of PSs and drug delivery systems (DDSs) developed for them, together with the challenges and prospects. Overall, most bioactive PSs suffer from undesirable pharmacokinetic attributes, which negatively affect their efficacy and clinical use. Various DDSs therefore have been being utilized to improve the drug-like properties and curative efficacy of bioactive PSs by means of improving oral absorption, controlling the release, enhancing the *in vivo* retention ability, targeting the delivery, exerting synergistic effects, and so on. Specifically, nano-sized insoluble DDSs were mainly applied to improve the oral absorption and target delivery of PSs, among which liposome was especially suitable for immunoregulatory and/or anti-ischemic PSs due to its synergistic effects in immunoregulation and biomembrane repair. Chemical conjugation of PSs was mainly utilized to improve their oral absorption and/or prolong their blood residence. With formulation flexibility, *in situ* forming systems alone or in combination with drug conjugation could be used to achieve day(s)- or month(s)-long sustained delivery of PSs per dosing.

ARTICLE HISTORY

Received 12 September 2017
Revised 17 October 2017
Accepted 20 October 2017

KEYWORDS





Polysaccharide; nano-sized carrier; conjugation; *in situ* forming system; pharmacokinetics

1. Introduction

Polysaccharides (PSs) are not only one of the four basic substances that constitute lives, but also one of the most abundant natural products. They have been found to have a wide spectrum of bioactivities, like immunomodulation, anti-tumor, anti-virus, anti-ischemia, anti-oxidation, anti-inflammation, etc. (Chen et al., 2016; Dong et al., 2016). Moreover, naturally occurring PSs are often stable *in vitro*, biocompatible and biodegradable *in vivo*, and nontoxic with a wide range of doses. These are all favorable characteristics for use in pharmacy.

Though playing an important role in various biological processes, PS-based drugs cover only a limited area in clinical treatment (Ernst & Magnani, 2009). This is mainly due to the following facts. First, natural PSs are now still mainly produced by extraction and purification, which makes their availability quite poor (costly and time consuming). Second, most PSs prepared have a board molecular weight (MW) distribution. This, together with their much more complex structure than other macromolecules like protein and DNA, results in a serious lack of knowledge on their structure–function relationship. Third, with some exceptions (e.g. heparin), many bioactive PSs exert their effects mildly and often with a lag

time, which might make developers and practitioners ignore their safe, holistic, and/or synergistic curative characteristics. Last, most PSs show poor drug-like properties. Specifically, on the one hand, PSs belong to Class III of the Biopharmaceutics Classification System, which means that the oral absorption of PSs will be poor and erratic. On the other hand, neutral PSs generally show low protein binding in blood and low uptake and clearance by the reticuloendothelial system due to their hydrophilic and uncharged nature. Therefore, they will be eliminated rapidly via the kidneys if their hydrodynamic size is smaller than the glomerular filtration threshold (~10 nm) (Venturoli & Rippe, 2005). Together, these poor pharmacokinetic behaviors severely limit the administration effectiveness and compliance of PSs. Though the development and clinical application of PS-based drugs suffer from many obstacles not limited to the above, their medicinal value and importance are beyond doubt. It is believed that they might become a next major breakthrough of new drug discovery in near future in terms of (i) increasingly depleting of the other kinds of leads and (ii) the essential biological role and relatively untapped status quo of PSs (Ernst & Magnani, 2009).

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For many years, PS-related research has always been a focus in the pharmaceutical field. According to the Scopus database, there are 1390, 1473, and 1378 research article titles containing the word 'polysaccharide' in year 2014, 2015, and 2016, respectively. However, the majority of research is related to the application of PSs as excipients/adjuvants rather than therapeutic molecules. They were used either in ordinary dosage forms as matrix materials, binders, disintegrants, suspending agents, and emulsifiers (to name a few) or in advanced drug delivery systems (DDSs) for the hydrophilic modification of nanocarrier surfaces, the development of polymeric drugs, the achievement of targeted drug delivery, and so on. Generally, excipients are required to be biologically inert; however, in some cases, bioactive PSs were developed as an excipient with few, if any, considerations to their bioactivities. For example, prebiotics PSs like inulin were developed as the matrix of colon-targeted DDSs. After repeated administration, such PSs could change gut flora, induce some biological effects, and/or have an influence on the oral absorption of other drugs. In another study, fucoidan, which has a variety of bioactivities like anti-tumor, anti-virus, anti-thrombus, and anti-inflammation (Kusaykin et al., 2008), was used as a reducing and capping agent to prepare doxorubicin-loaded gold nanoparticles (NPs) for photoacoustic imaging and drug delivery (Manivasagan et al., 2016). Similarly, selenium NPs (~25 nm) were decorated by the water-soluble sulfated *Ganoderma lucidum* PS for anti-inflammation (Wang et al., 2014). In some other cases, bioactive PSs were intentionally developed as both an excipient and a synergistic agent of other drugs. For example, pH-sensitive lappaconitine-loaded low-MW heparin (LMWH) not only, due to the sulfur group, enhanced the dissolution of lappaconitine (a hydrophobic alkaloid), but also had a synergistic effect on the analgesic effect of lappaconitine (Sun et al., 2016b). Therefore, special care must be paid to understanding and evaluation of both such bioactive PS-related researches.

So far, there are many PS-related reviews published. For example, 366 reviews were displayed under the search strategy 'TITLE (polysaccharide) AND PUBYEAR >2013' in the Scopus database on October 17 2017. Some reviews summarized the preparation, chemical structure, and bioactivities of PSs, and some introduced their excipient applications. However, none focused on the delivery of bioactive PSs as therapeutic molecules. That is exactly why this review was proposed, which first summarized and discussed the general pharmacokinetic properties of PSs and then various oral and injectable DDSs for improving drug-like properties and curative efficacy of PSs.

2. General pharmacokinetic properties of PSs

2.1. Oral administration

Most PSs are poorly absorbed in the gastrointestinal tract due to their hydrophilic and macromolecular nature. Generally, only a very small part of the dose administered (less than 2%) (Mehvar & Shepard, 1992; Lin et al., 2010b) is absorbed mainly by fluid-phase pinocytosis and passive diffusion through aqueous pores (whose mean radius is about

4 Å) in the intestinal epithelium. Even if the hydrodynamic volume of a PS is significantly larger than the aqueous diffusion path, diffusive absorption will still more or less happen, thanks to the 'snake-like' movement of its flexible chain. In general, the oral absorption of a PS is negatively influenced by its MW. Namely, the absorption reduces with the increasing of MW. For example, compared to a low-MW chitosan (3.8 kDa), the oral absorption of a high-MW one (230 kDa) was observed to decrease more than 25 times in rats (Chae et al., 2005). In addition, some pieces of evidence indicate the presence of specific receptor-mediated mechanisms for the intestinal absorption of high-MW dextrans/glucans (Koyama et al., 1996; Rice et al., 2005; Tomita et al., 2009). Following oral administration of 1 mg, glucans were found (i) to be bound and internalized by intestinal epithelial cells and gut-associated lymphoid tissue (GALT) cells, (ii) to modulate the expression of some pattern recognition receptors (e.g. Dectin-1 and toll-like receptor 2) in GALT cells, and (iii) to achieve anti-infection bioactivity (Rice et al., 2005). Overall, oral bioavailabilities of two neutral glucans 'laminarin (4.9%) and scleroglucan (4.0%)' were higher than that of glucan phosphate, a polyelectrolyte (0.5%) (Rice et al., 2005). *In situ* and *in vitro* rat studies also indicated that the intestinal transport of (1→3)- β -D-glucan (laminaran) involved specific membrane transporters (Tomita et al., 2009). In few cases, exceptionally high oral bioavailabilities (several tens of percent) have been reported for some charged PSs. For example, a 35.9% bioavailability was reported on an acidic PS (17–30 kDa) from a traditional Chinese medicine 'Liuwei Dihuang decoction' after oral administration of 100 mg/kg to mice (Zheng et al., 2000). Since an HPGPC (high performance gel permeation chromatography) method with postcolumn fluorescence derivatization was used, the PS itself rather than its degradation products was determined. In another study, a 9.54% bioavailability was reported on a marine sulfated PS (11 kDa) after oral administration of 50 mg/kg to rats, which was also determined by the same HPGPC methodology (Li et al., 2013).

Being different from starch and other types of macromolecular drugs like proteins and polypeptides, most bioactive PSs are stable in the stomach and small intestine. Without being markedly absorbed and metabolized, most of the dose given orally will reach the large intestine and be metabolized into oligosaccharides and/or short-chain fatty acids by bacterial flora residing there. Some PSs exactly exert their efficacy by this way (Shi et al., 2015a; Shoaib et al., 2016; Zhou et al., 2016). For example, after oral administration, ginseng PSs recovered the disturbed gut flora caused by over-fatigue and acute cold stress, especially improving the growth of *Lactobacillus* spp. and *Bacteroides* spp. (two major metabolic bacteria of ginsenosides). This resulted in the improved intestinal metabolism and absorption of some ginsenosides in rats (Zhou et al., 2016). Similarly, oral administration of Radix Ophiopogonis PS (ROPS, a graminan-type fructan) reinstated the abnormal gut flora, decreased the ratio of Firmicutes to Bacteroidetes, and thus, produced potent hypoglycemic and anti-obesity effects in obese mice induced by a high-fat diet (Shi et al., 2015a). No such effects were observed after intravenous injection of ROPS (our unpublished data). On the

other hand, orally administered hyaluronan (300 kDa) was indicated to be degraded into oligosaccharides by large-intestinal flora, which were subsequently absorbed and distributed into various tissues, including the skin (Kimura et al., 2016).

2.2. Injection administration

Following intravenous administration, the plasma level of PSs generally exhibits an apparent biexponential decrease (Kandrotas, 1992; Mehvar & Shepard, 1992; Huang et al., 1996; Héroult et al., 2002; Karoli et al., 2005; Lin et al., 2005). Most calculated pharmacokinetic parameters appear to be more or less dependent on MW, among which the renal clearance and the distribution volume are often affected the most and least, respectively. A sigmoidal-shaped relationship between the apparent MW and the half-life of neutral PSs can be expected in terms of their low protein binding and simple elimination route (i.e. mainly being excreted by the kidneys). That is to say, neutral PSs whose hydrodynamic size is smaller than the sieving threshold of the glomerular capillary wall (~10 nm) (Venturoli & Rippe, 2005) are generally excreted by the kidneys quickly and unchanged. Sharp prolongation in half-life will happen when the PS size increases to around the sieving threshold, followed by a level-off with the PS size further increasing to a degree. For dextrans, the threshold MW (i.e. the apparent MW corresponding to the mean point of the sigmoid) was about 50 kDa (Seymour, 1992). Note that different PSs with the same MW often exhibit more or less different hydrodynamic sizes due to different hydrophilicity, branch number and length, and charge number and density. On the other hand, negatively charged PSs like heparin are extensively bound to plasma proteins, taken up by the reticuloendothelial system, and excreted in the urine (mainly as metabolites or unchanged depending on the dose) (Kandrotas, 1992). The distribution volume of PSs is generally around the plasma volume, indicating their very limited tissue distribution. This is mainly due to their hydrophilic and macromolecular nature which makes them difficult to pass through the blood vessel. For the same reason, once being distributed, they will tend to be detained in the tissues. Liver, spleen, and kidney tissues are the ones where high accumulation of PSs often happens. For example, the tissue/plasma AUC (area under curve) ratios of dextrans were found to be ≤ 29 and ≤ 10 for the rat liver and spleen, respectively. Moreover, dextran levels in them still kept high after 96 h but already fell below the detection limit in plasma at ~12 h (Mehvar et al., 1994). On the other hand, the tissue where PSs are distributed least is generally the brain due to the blood-brain barrier and a lack of specific transportation ways. Like renal clearance, tissue distribution of PSs shows significant MW dependency. In addition, as a PS is actually a mixture of macromolecules with different MWs, the initial fast decrease in plasma levels after intravenous injection is more or less attributed to the fast elimination and distribution of the relatively smaller MW part.

Many bioactive PSs, e.g. phosphosulfomannans (Karoli et al., 2005), ROPS (Lin et al., 2010b), heparins (Kandrotas,

1992; Héroult et al., 2002), and dermatan sulfate (Huang et al., 1996) (to name a few), suffer from the mentioned-above undesirable pharmacokinetic attributes, which negatively affect their efficacy and clinical use. Therefore, it is urgent and needed to develop effective delivery systems to improve their pharmacological action and/or to reduce the administration frequency for ultimately improved patient benefits.

3. Nano-sized insoluble DDSs

There are several purposes to loading bioactive macromolecules within nano-sized DDSs, including (i) protection of them from degradation *in vitro* and *in vivo*, (ii) targeted delivery of them to acting sites, (iii) achievement of sustained release for prolonged therapeutic effects, and (iv) improvement of oral absorption. So far, a number of such investigations have been performed on protein, polypeptide, RNA, and DNA, but much fewer on bioactive PSs (Davis et al., 2008; Petros & DeSimone, 2010; Suk et al., 2016). As most bioactive PSs are stable enough *in vitro* and *in vivo*, such a delivery way is mainly applied to achieve the last three aims for them. In addition, liposomes are often chosen for some bioactive PSs to achieve a synergistic effect (Table 1).

3.1. Liposomes

Liposomes are spheroidal phospholipid vesicles with one or more bilayer membrane structures. They have received great attention during the past four decades as carriers of various drugs for a wide spectrum of applications (Torchilin, 2005; Agrawal et al., 2017; Dou et al., 2017). Liposomes are one of the most promising DDSs for hydrophilic drugs in terms of their unique interior aqueous compartment structure, biocompatibility, high safety, formulation flexibility, wide applications in target delivery, and synergistic effects in immunoregulation and biomembrane repair (Khaw et al., 1995; Khaw et al., 2007; Takahama et al., 2013).

3.1.1. Synergistic immunomodulatory effects

At present, liposomes are one of the most commonly used DDSs for bioactive PSs. Many PS immunomodulators had been loaded into liposomes with the aim of achieving a synergistic immunomodulatory effect. PS-loaded liposomes introduced in this part were all prepared via the reverse-phase evaporation method.

For example, *Ganoderma lucidum* polysaccharide liposomes (GLPS-Ls) were prepared with GLPS (a neutral heteroglycan), soybean phospholipid, tween-80, and cholesterol (unknown:11:1.05:1, mass ratio). The entrapment rate and average particle diameter for GLPS-Ls were $71.43\% \pm 0.49\%$ and 164.3 ± 9.2 nm, respectively (Liu et al., 2015). *In vitro*, GLPS-Ls could enhance the activation of peritoneal macrophages (enhanced NO production, inducible nitric oxide synthase activity, phagocytic activity, and cytokine levels) compared to GLPS and blank liposomes (Liu et al., 2016a). Ovalbumin was further encapsulated in GLPS-Ls using a

Table 1. Drug delivery systems for bioactive polysaccharides to improve their drug-like properties and curative efficacy.

Name	Key properties	DDSs and achieved improvements
<i>Ganoderma lucidum</i> PS	A neutral heteroglycan composed of D-glucose, D-galactose, D-mannose, D-xylose, L-fucose, and L-rhamnose (5.35:2.67:1:1.19:0.38:0.37, molar ratio); MW, 37 kDa (Bao et al., 2002)	Liposome (<i>in vitro</i> ; SC): improved immunoregulatory activities and adjuvanticity (Liu et al., 2015, 2016a,b)
Radix <i>Ophiopogonis</i> PS	A graminan-type fructan (a Fruf (2 → 1) backbone with a Fruf (2 → 6) Fruf (2 → branch per average 2.8 of main chain residues); MW, 4.8 kDa (PDI, 1.41) (Xu et al., 2005) IV: $t_{1/2}$, ~0.5 h; MRT, 0.65 h. Oral BA: 1.7% (rats) (Lin et al., 2010b)	Liposome (<i>in vitro</i> ; SC or IM): improved immunoregulatory activities and adjuvanticity (Fan et al., 2014, 2015a, 2016a,b; Sun et al., 2016a) PEG-coated liposome (IV): 45-fold prolonged $t_{1/2}$; improved targeting delivery (Wang et al., 2015) Mono-PEGylation (IV): 32- to 100-fold prolonged $t_{1/2}$; improved targeting delivery, therapeutic effect, and administration compliance (Lin et al., 2011a,b; Sun et al., 2013) ISFS (SC): day(s)- to week(s)-long sustained delivery; improved administration compliance (Shi et al., 2015b; Wang et al., 2017) ISFS + MonoPEGylatin (SC): week(s)- to month(s)-long sustained delivery; improved administration compliance (Shi et al., 2014)
Astragalus PS	A (1 → 4)-linked dextran backbone with a (1 → 6)-linked branch every 10 residues; MW, 20.7 kDa (Niu et al., 2011)	Liposome (<i>in vitro</i> ; SC): improved immunoregulatory activities and adjuvanticity (Fan et al., 2011, 2013a) ISFS (poloxamer-based): week-long sustained release; improved administration compliance (Yu et al., 2017)
<i>Lycium barbarum</i> PSs	A neutral heteroglycan composed of glucose, xylose, galactose, rhamnose, and mannose (49.8:31.3:9.3:7.5:6.4, molar ratio); MW, 24–241 kDa (Amagase et al., 2009; Wu et al., 2010)	Liposome (<i>in vitro</i> ; SC): improved immunoregulatory activities and adjuvanticity (Bo et al., 2015, 2016)
<i>Rehmannia glutinosa</i> PS	An acidic PS composed of L-arabinose, D-galactose, L-rhamnose, and D-galacturonic acid (10:10:1:1 or 14:7:3:8, molar ratio); MW, 35.7 kDa (Tomoda et al., 1994; Tan et al., 2012)	Liposome (<i>in vitro</i> ; SC): improved immunoregulatory activities and adjuvanticity (Huang et al., 2014, 2016)
Epimedium PS	An acidic PS composed of xylose, galactose, rhamnose, arabinose, and galacturonic acid (6.4:2.3:1.8:6.9:82.6, weight ratio); MW, 1.46 kDa (Li, 2005)	Liposome (<i>in vitro</i>): improved immunoregulatory activities; synergistic effects with propolis flavone (Fan et al., 2013b, 2015b)
Propylene glycol alginate sodium sulfate (Li et al., 2013)	A marine sulfated PS composed of β -D-mannuronic acid and α -L-guluronic acid; MW, 11 kDa (PDI, 1.6) IV: $t_{1/2}$, 3.11 h; MRT, 2.59 h. Oral BA: 9.54% (rats)	PLGA nanoparticles: increased oral BA, 18.2% (rats); prolonged MRT, 84.7 h
Heparin	A highly sulfated glycosaminoglycans; MW, ~15 kDa Injection: $t_{1/2}$, ~1.5 h (Kandrotas, 1992). Oral BA: hardly absorbed (Chen et al., 2009)	Positively charged nanoparticles (chitosan with a low MW of 30 kDa): increased oral BA, 20.5% (rats) (Chen et al., 2009)
Low-MW heparin	A highly sulfated glycosaminoglycans; MW, less than 8 kDa Injection: $t_{1/2}$, 0.57–4 h (Samama & Gerotziafas, 2000; Hwang et al., 2012). Oral BA: hardly absorbed (Dong et al., 2007; Hwang et al., 2012; Alam et al., 2014); 1.6% (monkeys) and 7.4% (rats) (Alhilal et al., 2014)	Positively charged nanoparticles (poly(ϵ -caprolactone)/Eudragit® RS,1:1): increased oral BA, 51–59% (rabbits); prolonged anticoagulant effect, 8–11.5 h (Hoffart et al., 2006) Conjugation (DA): increased oral BA, ~16% (mice) (Dong et al., 2007) Conjugation (dimeric DA): increased oral BA, 19.3% (rats) (Hwang et al., 2012) Conjugation (T-DA): increased oral BA, 19.9% (monkeys) and 33.5% (rats) (Alhilal et al., 2014) Conjugation (T-DA)+complexation (DCEA): increased oral BA, 34.3% (rats); prolonged MRT, 7.5 h; improved anti-angiogenic efficacy (Alam et al., 2014)
Chinese yam PS	A neutral PS composed of glucose and galactose (1.52:1, molar ratio); MW, 16.6 kDa (Yang et al., 2015)	PLGA nanoparticles: slowed release and improved immunomodulatory activity <i>in vitro</i> (Luo et al., 2016)
Antrodia camphorate PS	A (1 → 3)- β -D-glucan; MW, >200 kDa (Kong et al., 2013)	Positively charged nanoparticles (chitosan with a MW of 200 kDa): improved anti-tumor efficacy <i>in vitro</i> (Chang et al., 2015)
Low MW chondroitin sulfate (Xiao et al., 2014)	A sulfated glycosaminoglycans; MW, 4.1 kDa Oral administration: $t_{1/2}$, 5.9 h; AUC _{0–24 hr} , 55.2 mg/(L h) (rats)	Conjugation (α -linolenic acid): prolonged $t_{1/2}$, 11.2–18.8 h; increased oral AUC _{0–24 hr} , 80.1–172.4 mg/(L h) (rats)

PS: polysaccharide; MW: molecular weight; PDI: polymer dispersity index; SC: subcutaneous injection; IV: intravenous injection; MI: intramuscular injection; $t_{1/2}$: plasma half-life; MRT: mean residence time; BA: bioavailability; AUC: area under the curve; PEG: polyethylene glycol; PLGA: poly(D,L-lactide-co-glycolide) copolymer; DA: deoxycholic acid; T-DA: tetrameric DA; DCEA: deoxycholyethylamine; ISFS: *in situ* forming system.

repeating freeze–thaw method. The vaccine-containing GLPS-Ls could induce more powerful antigen-specific immune responses (higher antigen-specific IgG antibodies, better proliferation and higher cytokine secretion of splenocytes, and more significant activation of T cells) than each single-component formulation in mice (Liu et al., 2016b).

ROPS (a neutral fructan) was also encapsulated in liposomes (ROPS:soybean phosphatide:cholesterol, 0.84:8:1, mass ratio; entrapment rate, 64.9%; average particle size, 245 nm; zeta potential, –4.56 mV; PDI, 0.326) to improve its antioxidative activity and specific and nonspecific immunoregulatory activities (Fan et al., 2014, 2015a, 2016b, Sun

et al., 2016a). Overall, ROPS-loaded liposomes (ROPS-Ls) showed significantly better efficacy than free ROPS. Typically, compared to ROPS, ROPS-Ls could not only markedly enhance the phagocytic index, splenocyte proliferation, proportion of CD4⁺ and CD8⁺ T lymphocytes, and antigen-specific antibody titers in mice immunized with ovalbumin, but also improve the protective rate against Newcastle disease virus in chickens after subcutaneous or intramuscular injection of ROPS-Ls (containing 2.0 or 4.0 mg ROPS) (Fan et al., 2015a, 2016b). The same ROPS-Ls, after subcutaneously injected, also achieved a better adjuvant activity for improving cellular and humoral immunity than free ROPS in mice inoculated with inactivated porcine parvovirus (Fan et al., 2016a).

Astragalus polysaccharide liposomes (APS-Ls) were prepared with APS (a neutral dextran), soybean phospholipid, and cholesterol (0.8:8:1, mass ratio) (entrapment rate, 47.50% ± 0.15%; loading rate, 5.40% ± 0.17%; particle size, <150 nm) (Fan et al., 2011). APS-Ls could more significantly promote the splenocyte proliferation than APS and blank liposomes *in vitro*. Moreover, APS-Ls could more significantly enhance the splenocyte proliferation, ovalbumin-specific antibody responses, and IFN- γ and IL-6 secretion than aluminum hydroxide in mice immunized with ovalbumin. Overall, APS-Ls could significantly improve the adjuvanticity and bioactivity of APS (Fan et al., 2013a).

Lycium barbarum polysaccharide liposomes (LBPS-Ls) were prepared with LBPS (a neutral heteroglycan), soybean phospholipid, cholesterol, and Tween-80 (1:25:10.4:unknown, mass ratio) (entrapment rate, 86.37% ± 0.63%; average particle size, 121 nm; PDI, 0.237) (Bo et al., 2015, 2016). As a whole, encapsulating LBPS in liposomes increased its immunoactivity. LBPS-Ls could significantly increase the splenocyte proliferation, the ratio of CD4⁺ to CD8⁺ T cells, and the cytokine secretion of macrophages *in vitro*. On the other hand, LBPS-Ls also showed the immunological adjuvant activity for porcine circovirus type 2 (PCV2) vaccine in mice (increasing the PCV2-specific IgG antibody responses and enhancing the secretion of Th1 cytokines 'IFN- γ and TNF- α ' and Th2 cytokine 'IL-4') (Bo et al., 2016).

The adjuvanticity of *Rehmannia glutinosa* polysaccharide liposomes (RGPS-Ls) was also confirmed in mice inoculated with inactivated PCV2 antigen after subcutaneous administration of 0.25 mL of the vaccine formulation containing RGPS-Ls (2 mg RGP and 20 mg lipid) (Huang et al., 2016). RGPS-Ls were prepared with RGPS (an acidic heteroglycan), soybean phospholipid, cholesterol, and Tween-80 (5.5:40:5:4, mass ratio) (entrapment rate, 72.75% ± 0.32%; average particle size, 171 nm; PDI, ~0.38) (Huang et al., 2014).

Liposomes could also significantly enhance the immunomodulatory effect of the combined immunopotentiator of epimedium polysaccharide (EPS, an acidic heteroglycan) and propolis flavone (PF). EPS-PF liposomes (EPS-PF-Ls) were prepared with EPS-PF, soybean phospholipid, and cholesterol (84:6:1, mass ratio) (entrapment rates of EPS and PF, 84.9% and 71.4%; average particle size, 150–200 nm) (Fan et al., 2013b). *In vitro* studies indicated that EPS-PF-Ls could not only remarkably improve the expression and secretion of NO, induced nitric oxide synthase, chemokines, cytokines,

and costimulatory molecules, but also increase the phagocytic and pinocytic activity of Kupffer cells. Moreover, EPS-PF-Ls significantly enhanced the ability of Kupffer cells on stimulating the proliferation and antigen-presenting ability of splenic lymphocytes compared to free EPS (Fan et al., 2015b).

3.1.2. Others

It has been a long time to investigate anti-ischemic activities of natural PSs. So far, there are no less than 24 kinds of natural PSs that were found to be effective on the treatment of a variety of tissue ischemia (Dong et al., 2016). With a 'plug and seal' effect to help the repair process of biomembranes (Khaw et al., 1995; Khaw et al., 2007; Takahama et al., 2013), liposomes are promising as a carrier for anti-ischemic and/or cell-protective PSs to achieve a synergistic effect. On the other hand, long-circulating liposomes (LC-Ls) that combat immediate uptake and clearance by the reticuloendothelial system (Levchenko et al., 2012; Paulis et al., 2012; Takahama et al., 2013) could be used to improve the PS-loading stability and to promote the selective delivery of PSs. For example, ROPS-loaded LC-Ls were prepared with ROPS, fully hydrogenated soybean phosphatidylcholine, cholesterol, and distearyl phosphatidylethanolamine-PEG2000 (1.46:4.68:3:0.37, molar ratio) using the reverse-phase evaporation method (ROPS loading, 77.73 ± 1.45 mg/mmol; zeta potential, -1.41 ± 0.45 mV; average particle size, 171.7 ± 7.7 nm; PDI, 0.114 ± 0.071) (Wang et al., 2015). Such LC-Ls showed improved stability in the rat plasma with the cumulative releases of ROPS being only about 7.4%, 12.5%, and 17.9% at 3, 8, and 24 h, respectively. On the other hand, compared to ROPS, such LC-Ls showed an increase of 45 times in plasma half-life (17.6 h) in rats after intravenous injection. Moreover, ROPS loaded in such LC-Ls achieved not only 2.4–3.4 times higher targeting efficacy but also 2.9–8.6 times higher exposure in infarcted rat myocardia than mono-modified ROPS with 40-kDa polyethylene glycol (PEG), although their plasma pharmacokinetic behaviors were comparable. Thus, loading in LC-Ls appears to be more suitable than mono-PEGylation for passively targeting ROPS to ischemic myocardia (Wang et al., 2015).

3.2. Nanoparticles

3.2.1. Oral administration

To improve the oral availability of propylene glycol alginate sodium sulfate (PSS, a marine sulfated PS), PSS-loaded poly(D,L-lactide-co-glycolide) copolymer (PLGA) NPs (PSS-NPs) were prepared by a double (W₁/O/W₂) emulsion and solvent evaporation method (Li et al., 2011). The average size of PSS-NPs was 182 nm (PDI, 0.012), the entrapment rate was 75.8%, and the drug loading rate was 10.8%. Following oral administration to rats, PSS-NPs not only extended the residence time of PSS in the blood, but also significantly improved the relative bioavailability (190.1%) compared to the PSS solution (Li et al., 2013). The contributing factors might include absorption in the form of NPs, concentrated PSS levels in absorption sites, and/or sustained release of PSS from the

NPs. Similar results were observed on LMWH (tinzaparin)-loaded NPs (408 ± 16 nm; entrapment rate, $31.0\% \pm 2.3\%$) prepared with a blend of poly(ϵ -caprolactone) and Eudragit[®] RS (a polycationic polymethacrylate) (1:1, w/w) by a double emulsion method (Hoffart et al., 2006). The *in vitro* release profile of tinzaparin (a highly sulfated glycosaminoglycan) from the NPs was characterized by a 32% initial burst release followed by a release plateau after 24 h (40%) due to the strong electrostatic interaction between tinzaparin and Eudragit[®] RS. In fasted rabbits, the oral absolute bioavailabilities were 51% and 59% for two doses studied (200 and 600 anti-Xa U/kg), respectively, and the anticoagulant action was delayed by 3–4 h, but extended up to 8–11.5 h. In terms of the relatively large particle size and the polycationic attribute, such NPs are believed not to be absorbed considerably, and the improved oral absorption of tinzaparin therefore should be mainly due to its close release to the intestinal wall as a result of the intimate and strong contact of the NPs with the oppositely charged mucus (Lamprecht et al., 2006). In addition, the polycationic polymer 'Eudragit[®] RS' might act like chitosan to cause a structural reorganization of tight junction-associated proteins and, thus, promote the paracellular transport. In another study, heparin (a highly sulfated glycosaminoglycan) was loaded in chitosan NPs (80–280 nm) with an almost 100% entrapment rate by using an excessive amount of positively charged chitosan to bind heparin (Chen et al., 2009). After oral administration of the NPs to rats, the absolute bioavailability of heparin was about 20.5%; meanwhile, the absorption of chitosan into the general circulation was found to be insignificant. Thus, the mechanism of oral absorption enhancement should be the same to that for tinzaparin mentioned above.

3.2.2. Others

Chinese yam polysaccharide (CYPs, a neutral PS), having anti-tumor, immunomodulation, and hypoglycemic activities, was encapsulated in PLGA-based biodegradable NPs to overcome its negative attributes hampering its clinical application (e.g. short half-life) (Luo et al., 2016). By process optimization, NPs with the encapsulation rate of CYPs being 65.6% and average particle size being approximately 200 nm were prepared. *In vitro* tests revealed the achievement of slowed release and improved immunomodulatory activities of CYPs by nano-encapsulation. However, several questions need to be further answered. The first is to what degree the administration way and the subsequent *in vivo* course of the NPs affect the enhanced effects observed *in vitro*. The second is if the achieved release behavior is enough for the expected *in vivo* improvement in performance. The third is what kind of relationship it is between drug loading and drug release. In another study, *Antrodia camphorata* polysaccharide (ACPS, a neutral β -D-glucan) was encapsulated in chitosan-silica NPs (average particle size, 210 ± 13.3 nm; entrapment rate, 85.7%) and silica NPs (294 ± 25.7 nm; 76.4%), respectively (Kong et al., 2013). Both of the ACPS-loaded NPs, especially the former, showed certain preferable effects on damaging the membrane of hepatocarcinoma cells and inducing cell death compared to the equivalent ACPS solution. For example,

the former NPs could induce a similar level of apoptosis at a lower ACPS dosage ($13.2 \mu\text{g}/\text{mL}$) than the latter NPs ($21.2 \mu\text{g}/\text{mL}$) and the ACP solution ($25 \mu\text{g}/\text{mL}$) (Chang et al., 2015). Since the cytotoxicity of blank NPs on the cells was insignificant, nano-encapsulation of ACPS should be the main contributor to the improvement. These results provide a basis for further investigating the *in vivo* behavior and efficacy of these NPs.

We once tried to control the release of ROPS by loading it within either hydrophilic gelatin-based microparticles or hydrophobic PLGA-based NPs, but only weak sustained-release effects were observed (our unpublished data). This, along with the above reports, indicates that it is difficult to solve the initial burst release issue and slow down the main release phase of neutral PS-loaded NPs effectively in terms of both (i) the hydrophilicity and high dose of PSs and (ii) the large specific surface area of NPs.

4. Conjugates

Like small molecular drugs and proteinic drugs (Kang et al., 2009; Pasut & Veronese, 2009; Böttger et al., 2016), bioactive PSs could be conjugated with functional excipients to improve their delivery. Being similar to loading parent PSs to nano-sized insoluble DDSs, such conjugations mainly aimed to achieve targeted delivery, prolonged *in vivo* retention and therapeutic effects, and improved oral absorption of PSs (Table 1).

4.1. Oral administration

Several PSs, typically LMWH (a highly sulfated glycosaminoglycan), were chemically conjugated to improve their oral absorption. For example, the conjugates with bile acids could be absorbed via the apical sodium-dependent bile acid transporter (ASBT) located mainly in the ileum (Balakrishnan & Polli, 2006). When the chemical conjugate of LMWH and deoxycholic acid (DA) or parent LMWH was orally administered at a dose of 10 mg/kg in mice, the bioavailability of the former was $\sim 16\%$, while LMWH was barely absorbed (Dong et al., 2007). In another study, 6-O-desulfated LMWH was conjugated with a dimer of DA. The conjugate showed an oral bioavailability of 19.3% in rats and could significantly inhibit bone destruction and neovascularization after oral administration of 10 mg/kg to mice with arthritis induced by the murine collagen antibody (Hwang et al., 2012). Tetrameric DA-conjugated LMWH showed a further marked improvement in oral bioavailability ($19.9\% \pm 2.5\%$ and $33.5\% \pm 3.2\%$ in monkeys and rats, respectively), and notably, it prevented deep vein thrombosis effectively in rats following oral administration (Alhilal et al., 2014). Since the dissociation of tetrameric DA from the intestinal ASBT was approximately 50 times slower (because of its multipoint hydrophobic interaction with the transporter) than that of DA, the enhanced absorption should be ascribed to much higher selectivity of the conjugate toward the transporter, which facilitated its subsequent transcellular transport (Alhilal et al., 2014). In another study, a taurocholate-modified LMWH derivative was

further chemically conjugated with tetrameric DA and then physically complexed with deoxycholethylamine. The final complex showed both a remarkably promoted oral bioavailability ($34.3\% \pm 2.89\%$) in rats and significantly inhibitory effects on the angiogenesis and growth of tumor in mice. The transcellular pathway via the ASBT was also believed to be responsible for the most of the absorption (Alam et al., 2014).

Another conjugation methodology to improve oral absorption of PSs is to increase the hydrophobicity of PSs by introducing hydrophobic chains, typically long chain fatty acids. For example, low-MW chondroitin sulfates (LMWCS, sulfated glycosaminoglycans) were conjugated with α -linolenic acid, resulting in several amphiphilic conjugates, which could self-assemble to form stable micellar structures in aqueous fluid at low concentrations (Xiao et al., 2014). Moreover, the conjugates showed enhanced oral LMWCS absorption by both opening the tight junctions of intestinal cells and increasing the interaction between the intestinal cell membrane and LMWCS due to the conjugated hydrophobic α -linolenic acid moiety, rather than by disrupting the whole integrity of cell membranes. Following oral administration to rats at the equivalent 200 mg/kg dose, the $AUC_{0-24\text{ h}}$ value of one of the conjugates was 172.4 ± 20.9 mg/(L h), 3.31- and 2.12-fold higher than those of chondroitin sulfate and LMWCS, respectively ($p < .001$) (Xiao et al., 2014). This, together with an early report (Lee et al., 2001), confirms that the self-assembling micelles of amphiphilic PSs can improve the oral bioavailability of PSs.

4.2. Injection administration

PEGylated ROPSs with the long-circulating attribute and potential anti-ischemic bioactivity were prepared by reacting hydroxyl-activated ROPS with amino-terminated PEGs (Lin et al., 2010a, 2011b, Sun et al., 2013). Like other hydrophilic polymers, an S-shaped relationship was observed between the apparent MW of the conjugates and their half-life. The MW midpoint of the sigmoid was 25.4 kDa (Lin et al., 2011a), which is slightly lower than that for PEG (~ 30 kDa) (Yamaoka et al., 1994; Greenwald et al., 1996), maybe due to the less flexible and more spheroidal structure of the conjugates given by the highly branched ROPS moiety. The conjugate grafted with one 20-, 30-, or 40-kDa PEG showed an about 32, 85, or 100 times longer half-life in the rat plasma than parent ROPS (~ 0.5 h). Moreover, with 2–6 times prolonged dosing intervals, these mono-PEGylated ROPSs achieved about the same anti-myocardial ischemic effects compared to ROPS (Sun et al., 2013). Therefore, mono-PEGylation might be a promising way to reduce the injection frequency of PSs and, meanwhile, keep or improve their curative effects by a significant improvement in their pharmacokinetic behaviors. In addition, investigation on tissue distribution of mono-modified ROPS with 20-kDa PEG indicated that the conjugation reduced the distribution tendency of ROPS in the lung, brain, and kidneys by about 1.3, 1.6, and 42 times, respectively, while increased it in the liver by ~ 1.3 times (Lin et al., 2011b). Myocardial ischemia did not induce significant

changes in the conjugate distribution to tissues except for the heart. Due to the ischemia-induced EPR (enhanced permeability and retention) effect, the conjugate AUC in ischemic mouse hearts was ~ 1.6 times greater than in normal ones. Namely, PEGylation could help ROPS passively target ischemic myocardia (Lin et al., 2011b).

5. *In situ* forming systems (ISFSs)

There are many advantages exhibited by ISFSs, mainly including (i) site-specific delivery with reduced general adverse effects, (ii) easiness of preparation and administration, and (iii) reduced dosing frequency due to prolonged duration of action. At present, although ISFSs have been widely developed for the delivery of various small molecular/macromolecular hydrophilic/hydrophobic drugs at a wide spectrum of clinical conditions (Thakur et al., 2014; Juvekar & Kathpalia, 2017), there are still few reports involving their applications in bioactive PSs. In general, it is challengeable to achieve the zero-order controlled release of PSs from ISFSs in terms of hydrophilicity and the short plasma half-life of most PSs. Several tries made so far were introduced as follows (Table 1).

5.1. Day(s)-long delivery

APS (a neutral dextran), with the activities of anti-bacterium, anti-virus, and immunoregulation, was loaded in poloxamer (P)-based thermoresponsive ISFSs to achieve sustained release of the PS for reducing dosing frequency during long-term treatment (Yu et al., 2017). The formulation (18 g P407, 2 g P188, 0.15 g sodium carboxymethylcellulose, 0.85 g NaCl, and 10 g APS in 100 mL) obtained an optimal *in vitro* sustained-release profile of about 5.5 days with an initial burst release of approximately 16.3% in 12 h measured by a test tube method. Moreover, injecting the ISFS (1 g/kg APS) once to mice subcutaneously achieved comparable increases in spleen lymphocytes, immune organ indices, and serum levels of IgG, IgM, IL-2, and IL-6 compared to injecting the commercial APS injection (0.25 g/kg APS) seven times (once per day) (Yu et al., 2017). In another study, ROPS (a neutral fructan) was loaded into both hydrophilic P-based ISFSs and hydrophobic PLGA- or sucrose acetate isobutyrate (SAIB)-based ISFSs (Shi et al., 2015b). In general, hydrophilic P-based ISFSs (like 24% P407/10% P188) could only achieve day-long sustained release of ROPS in rats after subcutaneous administration at a dose of 200 mg/kg, indicating that such ISFSs could reduce daily dosing frequency and be suitable for the acute treatment of myocardial ischemia. On the other hand, two hydrophobic ISFSs '40% PLGA_{30k} in N-methyl-2-pyrrolidone (NMP)' and '30% PLGA_{50k} in NMP' exhibited low but lasting blood levels of ROPS for about four days after administered at the same dose, and thus, they might be hopeful for the long-term treatment and/or precaution of myocardial ischemia (Shi et al., 2015b). However, an obvious initial burst release of ROPS from the above hydrophobic ISFSs seems unconquerable owing to the quick exchange of hydrophilic

NMP with body fluid. This also makes longer release duration unachievable.

5.2. Week(s)- or month(s)-long delivery

To achieve such long-lasting delivery for ROPS, hydrophobic solvents were applied to the PLGA-based ISFS (Wang et al., 2017). Both significant reduction in the initial burst release (3.7–8.0 times) and remarkable prolongation of plasma exposure (from ~4 days to 10–15 days) were observed when NMP in the ISFS was replaced with a hydrophobic solvent mixture of 90% benzyl benzoate and 10% co-solvent (NMP, triacetin, or benzyl alcohol). Moreover, comparable and nearly zero-order ROPS release could be achieved by several different formulation combinations, indicating the flexibility in formulation design. Further studies indicated that one of such formulations exhibited a clear superiority over the water solution of ROPS in treating myocardial ischemia (Wang et al., 2017). Thus, ISFSs composed of hydrophobic solvent and PLGA appear to be hopeful for week(s)-long and smooth release of PSs with a short blood half-life (e.g. ROPS).

On the other hand, long-lasting delivery could be achieved by the combination of two or more delivery strategies. For example, to achieve month-long delivery of ROPS, solvent exchange-induced hydrophobic ISFSs were combined with mono-PEGylation of ROPS (Shi et al., 2014). A smooth rat blood exposure for almost a month was obtained by loading 20 kDa PEG mono-modified ROPS (whose *in vivo* mean residence time was 2.76 days following subcutaneous injection at an equivalent ROPS dose of ~50 mg/kg) in the ISFS '40% PLGA_{30k} in NMP' (Shi et al., 2014). The effect of the conjugate MW on delivery was further studied in ISFSs composed of SAIB/PLGA/NMP (3.5–5.0/1.0–3.0/3.0–4.5, mass ratio) (our unpublished data). It was found that weeks- to months-long (16–60 days) smooth drug delivery could be achieved by varying the level (10–30%) and MW (10–50 kDa) of PLGA or by employing a moderate MW conjugate (~20 kDa or ~30 kDa). With further increasing the conjugate MW to ~40 kDa, the contribution of drug elimination to its plasma retention seemed to surpass that of the ISFS and the ISFS no longer led to obvious changes in the *in vivo* behavior of the conjugate. As a whole, for most PSs that are highly hydrophilic and need to be used at a high dose per administration, more than one long-lasting delivery technologies might be needed for the achievement of month(s)-long delivery per dosing.

6. General opinions

Due to the hydrophilic nature of PSs, nano-sized insoluble DDSs are mainly used for PSs to improve their delivery rather than solubility and dissolution. The small particle size of such DDSs might (i) promote the transportation of PSs across biological barriers, (ii) prolong the blood residence time of PSs, and (iii) achieve target delivery due to modified PS biodistribution. Among various natural/artificial nano-sized structures, liposome is especially suitable for immunoregulatory PSs and anti-ischemic PSs due to its synergistic effects in

immunoregulation and biomembrane repair, respectively. Although many articles have been published on the bioactivity of immunoregulatory PS-loaded liposomes, there remains a lack of information on their *in vivo* behaviors. Since such liposomes are generally administered subcutaneously, certain sustained release of PS from liposomes should occur and contribute to the synergistic effect, which might mainly happen at the site of administration. On the other hand, anti-ischemic PS-loaded liposomes will need to be transported integrally to the ischemic site for a maximized synergistic effect if the PS needs to exert its actions at the ischemic site (e.g. protecting tissue cells against ischemia-induced damage and inducing the microvessel formation in ischemic tissues). Therefore, in this case, long enough systemic circulation and good enough *in vivo* stability are two key factors considered, and intravenous administration is preferable. When polymeric NPs are used for high-dose neutral PSs, it is difficult to solve the burst release issue effectively in terms of both the high hydrophilicity of most PSs and the high surface area/volume ratio offered by NPs. For charged PSs, electrostatic interaction can be utilized to not only significantly improve the encapsulation efficiency, but also effectively control the release behavior of PSs as well. Oral absorption enhancement induced by positively charged NPs is mainly attributed to (i) the close and concentrated PS release to the intestinal wall owing to an intimate and strong contact of such NPs with the negatively charged mucus and (ii) reversible opening of cell tight junctions which is conducive to paracellular transport.

So far, chemical conjugation of PSs is mainly utilized to (i) improve their oral absorption and/or (ii) prolong their blood residence. In the former case, conjugated is often the ligand of surface receptors of intestinal cells (e.g. monomeric or multimeric DA), and significant increase in volume is undesirable because of reduction in the efficiency of endocytosis. The grafting number of ligand per PS molecule needs to be deliberately balanced between increased endocytosis efficiency and decreased PS bioactivity, both of which are closely related to the number. In the latter case, enough increase in volume to around or above the glomerular filtration threshold is necessary, and thus, the preferred grafting moiety is highly hydrophilic polymers with enough injection safety (like PEG). Moreover, several strategies could be utilized to promote the results of such polymeric conjugation, e.g. (i) site-specific conjugation; (ii) conjugation with polymeric chains as few as possible; (iii) selectively reversible conjugation; and (iv) conjugation under conditions as mild as possible (Lin et al., 2010a). Although the first strategy has been applied to proteins with some success (Pasut & Veronese, 2007; Fontana et al., 2008), it is pretty difficult to be performed on most PSs in terms of their lack in both functional groups with specific reactivity and knowledge about their structure–function relationship. As per the second strategy, mono-conjugation is theoretically the best. However, special care must be paid to the body accumulation issue of high MW polymers.

ISFSs are superior to the above DDSs mainly at the following two aspects: (i) convenience in preparation and administration and (ii) high drug loadings. They are generally

administered subcutaneously or intramuscularly, and the drug blood level versus time profile will be determined by three process rates, i.e. release, absorption, and elimination rates. For PSs having a hydrodynamic size much smaller than the glomerular filtration threshold, their absorption and elimination rates might be markedly faster than their release rate from hydrophilic matrix-based ISFSs. If so, the profile will be mainly determined by the ISFS and day(s)-long sustained delivery could be achieved. If ISFSs with hydrophilic solvent but hydrophobic matrix are used, week(s)-long sustained delivery of PS will be achievable, but often with a high initial burst release and a low steady state level. The former is caused by the quick and substantial exchange of the hydrophilic solvent with body fluid instantly after administration, and the latter is the result of both the slow diffusion release of entrapped PS molecules from the formed hydrophobic solid depot and the rapid systemic elimination of absorbed PS molecules. Further long-term delivery via hydrophobic matrix-based ISFSs can be achieved by using hydrophobic solvent or by combining with drug conjugation. And smooth drug exposure is also achieved owing to significantly reduced initial drug release and/or significantly prolonged blood residence of the conjugate. Especially, with a reasonable combination of hydrophobic ISFSs with polymeric PSs having a MW near the glomerular filtration threshold, superior month(s)-long and smooth blood exposure of PSs could be achieved without causing the *in vivo* accumulation issue.

7. Future perspectives and concluding remarks

Although many DDS-based proprietary products containing either small molecular drugs or some macromolecular drugs (mainly proteins and polypeptides) have been successfully developed in the past several decades, it's still a relatively new field to develop DDSs for most bioactive PSs due to their poor availability and insufficient basic knowledge about their action mechanism, structure–efficacy relationship, *in vivo* course, and so on. With the effective settlement of these key obstacles, the development of PS-loaded DDSs should be able to advance rapidly as the basic ideology and methodology of DDSs are the same for all drugs. On the other hand, some specific or tailor-made DDSs might be designed and developed for PSs, with full consideration of the structural attributes and the *in vitro* and *in vivo* properties of PSs. Typically, PS is full of hydroxyl groups, which make it have a potential, whether strong or weak, to form intra- and/or inter-molecular hydrogen bonds. Branching is another important structural nature of PSs. PSs with a high branching ratio will exhibit a dense and even spheroidal configuration in water with low viscosity, and vice versa. In addition, the charging state of acidic/basic PSs could also be fully utilized to promote specific delivery.

Overall, most bioactive PSs suffer from undesirable pharmacokinetic attributes, which negatively affect their efficacy and clinical use. Therefore, it is urgent and needed to improve their delivery for patient benefits. DDSs, including liposomes, NPs, conjugates, and ISFSs, have great potential to improve drug-like properties and curative efficacy of

various bioactive PSs by means of improving oral absorption, controlling the release, enhancing the *in vivo* retention ability, targeting the delivery, exerting synergistic effects, and so on.

Disclosure statement

The authors report no conflicts of interest in this work.

Funding

This work was supported by Program for New Century Excellent Talents in University [NCET-13-0906]; the funds from Shanghai Municipal Commission of Health and Family Planning [ZY3-CCCX-3-5001] and Science and Technology Commission of Shanghai Municipality [15DZ2292000]; and the Science and Technology Development Fund of Pudong New Area [PKF-2013-003].

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