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Mini review

Accelerated blood clearance (ABC) phenomenon upon repeated injection of PEGylated liposomes

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Abstract

We and a Dutch group reported that "empty" PEGylated liposomes (approximately 100 nm) lose their long-circulating characteristic when they are administrated twice in the same animal with certain intervals (referred to as the accelerated blood clearance (ABC) phenomenon). Very recently, we showed that anti-PEG IgM, induced by the first dose of "empty" PEGylated liposomes, is responsible for inducing the phenomenon, based on the observation that IgM thus produced selectively binds to the surface of subsequently injected PEGylated liposomes, leading to substantial complement activation. It is generally believed that nanocarriers coated with a polymer, such as PEG, have no or lower immunogenicity. However, the results indicated evidence that unexpected immune responses occur even to such polymer-coated liposomes. Such immunogenicity of "empty" liposomes presents a serious concern in the development of liposomal formulations and their use in the clinic. In addition, through series of our studies, it was demonstrated that the magnitude of the ABC phenomenon depends on the physicochemical property of injected liposomes as a first dose, time interval between injection, lipid dose and drug-encapsulation.

Keywords: IgM; Polyethylene glycol (PEG); Accelerated blood clearance (ABC) phenomenon; Liposomes; Repeated injection; Complement activation

1. Introduction

A long-circulating liposome (PEGylated liposome) which is sterically stabilized with surface-coupled polyethylene glycol (PEG) and has approximately 100 nm in a mean diameter can enhance their lifetime and that of entrapped therapeutic agents in the blood circulation (Klibanov et al., 1990; Allen et al., 1991b; Papahadjopoulos et al., 1991). It is hypothesized that the presence of PEG on the liposome attracts a water shell to the liposomal surface, providing a steric barrier against opsonins and/or recognition by cells of mononuclear phagocyte system (Lasic et al., 1991; Senior et al., 1991; Torchilin et al., 1994). This, in turn, results in a decrease in the elimination rate of liposomes from the blood stream.

In clinical setting, repeated injection of PEGylated liposomes must be required in the case of treatment with therapeutic formulations. The use of a repeated injection regimen for the treatment of several diseases has been described extensively for PEGylated liposomal preparations that contain chemotherapeutic drugs. However, detailed studies about the *in vivo* pharmacokinetics of PEGylated liposomes on the repeated injection scheme were lacking.

We and a Dutch group have found that an intravenous injection of PEGylated liposomes causes a second dose of PEGylated liposomes, injected a few days later, to lose their long-circulating characteristics and accumulate extensively in liver, despite the presence of PEG on their surface (Dams et al., 2000; Laverman et al., 2001; Ishida et al., 2002, 2003a,b, 2004, 2005, 2006a,b,c, 2007; Wang et al., 2005, 2007). This phenomenon is referred to as the "accelerated blood clearance (ABC) phenomenon" (Dams et al., 2000) and observed in mice, rats and a rhesus monkey. It is the aim of this article to review the unexpected alteration of pharmacokinetic behavior of PEGylated liposome on repeated injection (the ABC phenomenon) and discuss the underlying mechanism to induce such phenomenon.

PEG is a neutral, crystalline, thermoplastic polymer with a high solubility in both water and organic solvent (Lee et al., 1995; Elbert and Hubbell, 1996). When considering a lipo-

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some containing PEGylated lipids, it is believed that the PEG will extend away from the liposome into the solvent (aqueous media), not into the lipid bilayer (Carignano and Szleifer, 2000). Since PEG is not attracted to the lipid bilayer, the polymer provides kinetic rather than thermodynamic protection of the surface (Carignano and Szleifer, 2000; Satulovsky et al., 2000). Two regimens for PEGs attached to a surface depending on the grafted density of the polymers have been proposed: Mushroom regime and Brush regime (de Gennes, 1980; Needham et al., 1997). If the graft density is low the PEG is considered to be in the mushroom regimen. On the other hand, if the density is high the PEG is considered to be in the brush regimen. The degree of surface coverage is determined by the molecular weight of the PEG as well as the graft density. Compared to the counterparts without PEG modification, PEGylated liposomes showed extended circulation times in all mammalian species including mice, rats, dogs, and humans (Blume and Cevc, 1990; Klibanov et al., 1990; Allen et al., 1991b; Maruyama et al., 1992; Woodle and Lasic, 1992; Gabizon et al., 1993; Woodle, 1998). The long-circulating property of PEGylated liposomes has been generally attributed to suppression of protein adsorption onto the liposome surface and suppression of interaction with macrophages due to the hydrophilic mobile steric barrier provided by the PEG brushes (Allen et al., 1991a; Senior et al., 1991; Chonn et al., 1992; Needham et al., 1992; Woodle and Lasic, 1992; Torchilin et al., 1994; Nikolova and Jones, 1996).

PEGylated liposomes have been under clinical investigations for many years. The only commercially available PEGylated liposome preparation containing anticancer reagent is Caelyx or Doxil, a preparation of PEGylated liposomes that contain doxorubicin (DXR). Initially, the indication of this preparation was the treatment of AIDS-related Kaposi's sarcoma (Grunaug et al., 1998; Krown et al., 2004). Recent studies have reported the use of Doxil/Caelyx for the treatment of several other malignant diseases like non-small cell lung cancer, breast cancer, ovarian cancer, prostate cancer, endometrial cancer, hepatocellular carcinoma and soft-tissue sarcoma (Muggia et al., 1997; Koukourakis et al., 1999; Chidiac et al., 2000; Halm et al., 2000; Hubert et al., 2000; Lyass et al., 2000; Markman et al., 2000; Escobar et al., 2003; Orditura et al., 2004; Coleman et al., 2006). Most recently, several combination regimens incorporating Doxil/Caelyx and free forms of anticancer drugs, such as capecitabine, carboplatin, cyclophosphamide, gemcitabine, mitomycin C, paclitaxel, topotecan, temozolomide, and vinorelbine, are continuously evaluated in clinical trials (Gnad-Vogt et al., 2005; Katsaros et al., 2005; Mirchandani et al., 2005; Overmoyer et al., 2005; Poh et al., 2005; Bourgeois et al., 2006; Caraglia et al., 2006; Verhaar-Langereis et al., 2006).

2. Accelerated blood clearance of PEGylated liposomes upon repeated injection

In a clinical setting, repeated injections of PEGylated liposomal formulations should be applied, e.g., in case of multiple courses of chemotherapy. However, very few studies have focused on the pharmacokinetics of the liposomes upon repeated injections.

Goins et al. (1998) reported similar pharmacokinetics of serial injections of PEGylated liposomes in rabbits given 6 weeks apart. They observed only slight increased accumulation of injected liposome in liver and decreased accumulation in spleen, but no changes in circulation time. Oussoren and Storm (1998) also reported unchanged pharmacokinetics when they injected four doses of PEGylated liposomes to rats at 24 or 48 h intervals. In contrast to these studies, we and other groups found remarkable pharmacokinetic changes with repeated injections of PEGylated liposomes.

Dams et al. (2000) first showed that prior dose of "empty" PEGylated liposomes influences pharmacokinetics and biodistribution of second dose of the liposome in rats and a rhesus monkey, when those liposomes were administrated for example with a week interval. The circulation time of second injected PEGylated liposomes dramatically decreased, accompanied by highly increased uptake in the liver and in the spleen. We then observed similar phenomenon in rats and mice (Ishida et al., 2003a,b, 2006c). Our detail investigations demonstrated that the enhance blood clearance was reached at maximum level 4–7 days after first dose in rats (Fig. 1) and 10 days in mice.

3. Estimated mechanism of inducing the ABC phenomenon

The mechanisms underlying the induction of the ABC phenomenon have been investigated. Dams et al. (2000) demonstrated that the enhanced blood clearance of the PEGylated liposomes was also observed in rats after transfusion of serum obtained from rats that had received PEGylated liposomes 1 week earlier. This indicates that soluble serum factor(s) are involved in such phenomenon. This observation has been confirmed by us (Ishida et al., 2003a). In addition, Dams et al. (2000) demonstrated that the serum factor is a heat-labile molecule which co-eluted on a size extrusion column with a 150 kDa protein and are not IgG and IgM. In contrast to the report of Dams et al., we recently showed that upon repeated injection, "empty" PEGylated liposomes produced an abundance of anti-PEG IgM (Ishida et al., 2006b,c; Wang et al., 2007). Finally, we proposed the following mechanism for the induction of the ABC phenomenon (Fig. 2): anti-PEG IgM, produced from spleen in response to an injected dose of PEGylated liposomes, selectively binds to the PEG on a second dose of these liposomes, injected several days later, and subsequently activates the complement system. This, in turn, leads to opsonization of a second dose of PEGylatde liposomes by C3 fragments and, as a consequence, to enhanced uptake of PEGylated liposomes by the Kupffer cells in liver.

Furthermore, our recent study revealed that the ABC phenomenon is not induced in splenectomized rats, which goes along with a dramatic reduction of serum IgM level (Ishida et al., 2006b). This indicates that the spleen plays an important role in promoting the formation of anti-PEG IgM. In addition, we showed that the immune reaction in the spleen against the "empty" PEGylated liposomes extends over a period of at least 2–3 days following the first injection. This pattern of production of IgM is similar to that of splenic marzinal zone (MZ)

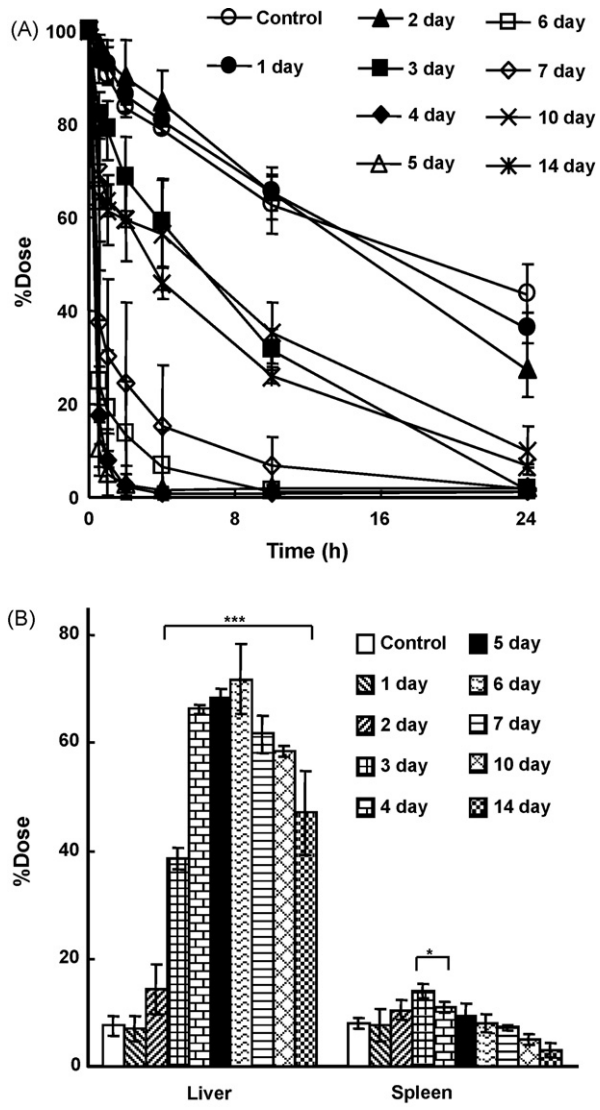

Fig. 1. Accelerated blood clearance and enhanced organ uptake of second dose of PEGylated liposomes. Rats were pretreated with PEGylated liposomes (lipid dose, 0.001 μmol/kg). (A) Blood clearance of second dose of radio-labeled PEGylated liposomes (5 μmol/kg). (B) Hepatic and splenic accumulation at 24 h following the injection. Each value represents the mean ± S.D. ($n = 3$). p-Values apply to differences between the control and treated rats. $^{*}p < 0.05$, $^{***}p < 0.005$, cited from (Ishida et al., 2006c) with permission from Elsevier.

B cells that are responsible for the first line of defense and are able to produce large amounts of neutralizing antibodies in a short period (3–4 days) (Martin et al., 2001; Zandvoort and Timens, 2002). PEGylated liposomes with long-circulating properties reportedly tend to accumulate in the spleen rather than in the liver. The PEG polymer could be characterized by a highly repetitive structure bearing similarity to a type-2 T-cell independent (TI-2) antigen. Therefore, the following hypothesis leading to the production of anti-PEG IgM is proposed. Once the "empty" PEGylated liposomes reach the spleen, they bind and crosslink to surface immunoglobulins on PEG (or PEGylated liposome)-reactive B cells in that organ and consequently trigger the production of an anti-PEG IgM that is independent of T-cell help.

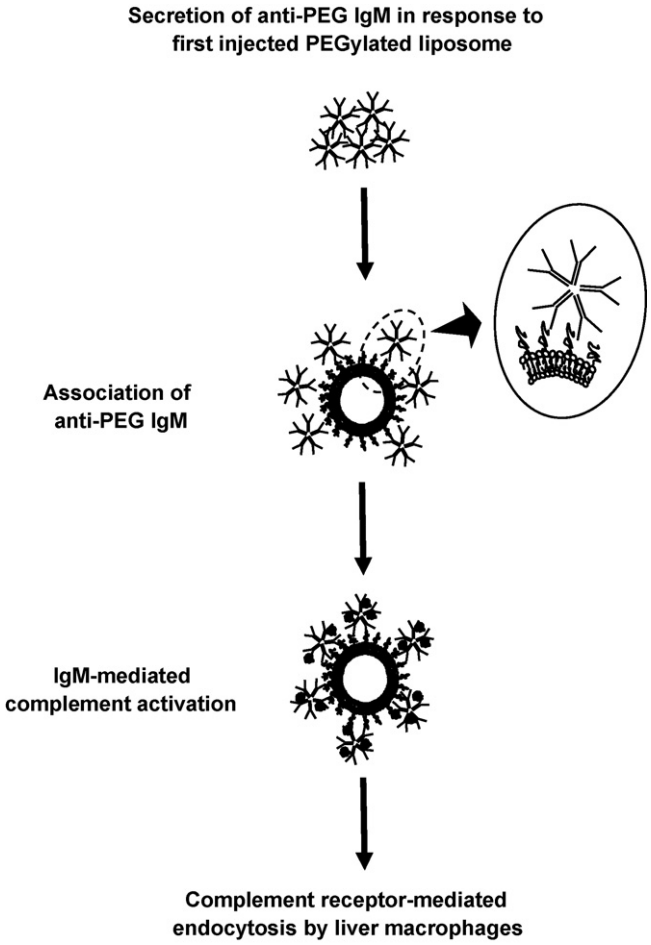

Fig. 2. Representation of the sequence of events leading from anti-PEG IgM induction to accelerated clearance of PEGylated liposomes. Cited from (Ishida et al., 2006c) with permission from Elsevier.

4. Effect of physicochemical properties of liposome on inducing the ABC phenomenon

Earlier reports (Dams et al., 2000; Laverman et al., 2001; Ishida et al., 2003a,b, 2006a,c; Wang et al., 2005) demonstrated that various factors, such as dose and physicochemical properties of the initially injected liposome, the interval between injections remarkably influence the magnitude of the induced ABC phenomenon. This could be explained by assuming that all these factors affect the anti-PEG IgM responses against the injected "empty" PEGylated liposomes.

4.1. Effect of dose

We demonstrated that there was a strong inverse relationship between the dose of initially injected PEGylated liposomes and the extent to which the ABC phenomenon was induced: the higher the dose (more than 1 μmol phospholipids/kg) the smaller the phenomenon (Fig. 3) (Ishida et al., 2005). The higher lipid doses (>1 μmol phospholipids/kg) may lead the B cells to become apoptotic and thus attenuate the phenomenon. At the recommended dose intensity of Doxil for patients (Lyass et al.,

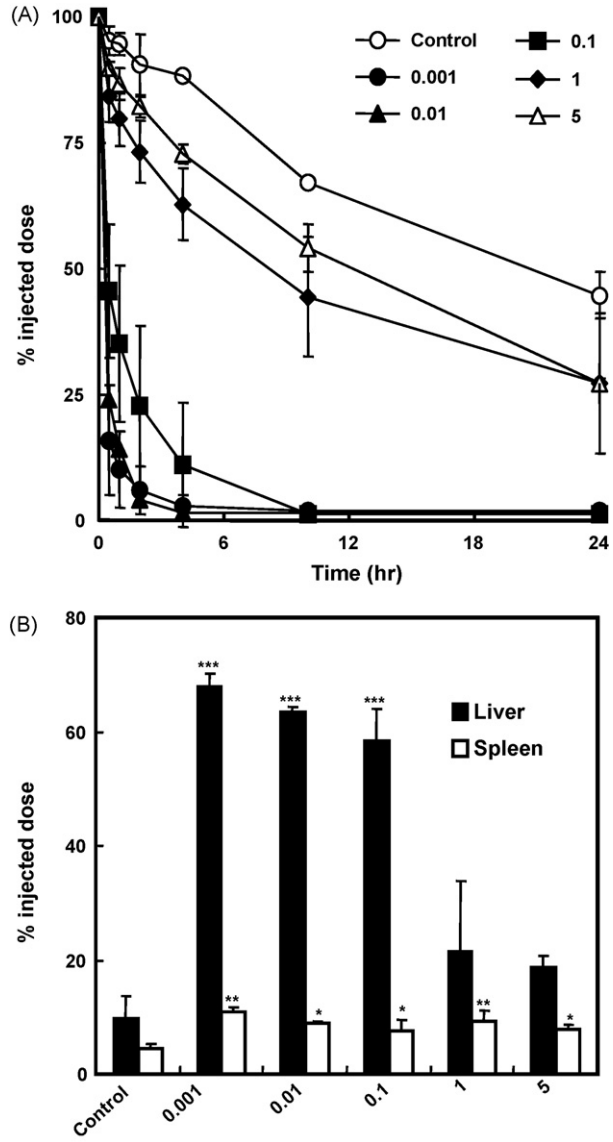

Fig. 3. Effect of lipid dose of first injection on the induction of the ABC phenomenon by PEGylated liposomes. Rats were pretreated with PEGylated liposomes at a dose of 0.001, 0.01, 0.1, 1 or 5 μmol/kg of body weight. Rats pretreated with HEPES buffered saline (pH 7.4), instead of PEGylated liposomes, were served as controls. At day 5 post-first injection, radio-labeled PEGylated liposomes (5 μmol/kg) were intravenously injected. (A) Blood clearance profile of the radio-labeled PEGylated liposomes. (B) Hepatic and splenic accumulation of the radio-labeled PEGylated liposomes at 24 h following the injection. Each value represents the mean ± S.D. of three separate experiments. *p*-Values apply to differences between the control and treated mice. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, cited from (Ishida et al., 2005) with permission from Elsevier.

2000), a higher lipid dose of PEGylated liposomes (15 μmol phospholipids/kg or even more) is generally administered. Thus, the higher lipid doses used in the clinic may present a major cause of no induction of the ABC phenomenon.

4.2. Effect of PEG-surface density and PEG-chain length

PEGylated liposomes containing 5 mol% of $mPEG_{2000}$-DSPE significantly induced the ABC phenomenon at a lower lipid dose (0.001 μmol phospholipids/kg) in rats, while conventional liposomes without PEG-coating did not (Ishida et al., 2005). Increasing the PEG density at liposomal surface beyond 5 mol% attenuated rather than induced induction of the phenomenon, but elongation of the PEG chain length up to M.W. 5000 had no effect (Ishida et al., 2005). Interestingly, conventional liposomes induced rapid blood clearance of second injected PEGylated liposomes at a higher lipid dose (more than 5 μmol phospholipids/kg) (Ishida et al., 2005; Wang et al., 2005, 2007), which is consistent with the observation of Dams et al. (2000). But, in contrast to PEGylated liposomes, conventional liposomes did not cause a drastic phenomenon at lower lipid dose (less than 0.1 μmol phospholipids/kg). It seems that the lipid dose in a first injection affects the magnitude of the phenomenon induced in a physicochemical property of liposomes-dependent manner.

4.3. Effect of size and surface charge

At a dose of 0.1 μmol phospholipids/kg, conventional liposomes (without PEG modification) of 110 nm did not cause a drastic ABC phenomenon, irrespective of the surface charge (Wang et al., 2005). When for the first injection smaller-size liposomes (approximately 60 nm) were used, either charged or PEG-modified, but not neutral, the phenomenon was clearly manifest (Wang et al., 2005). Apparently, the induction and magnitude of the phenomenon is not only determined by the PEG modification but also by the size and surface charge of the first injected liposomes.

4.4. Effect of interval between injections and consecutive injections

The interval between injections affects the magnitude of inducing the ABC phenomenon. Dams et al. (2000) showed remarkable changes on pharmacokinetics of repeated injections of PEGylated liposomes in rats and rhesus monkeys: the circulatory half-life of second dose of PEGylated liposomes dramatically decreased when given 5 days up to 4 weeks after a first injection. Our further study indicated that the magnitude of the phenomenon reaches maximum level at 5–7 days after a first injection in rats (Fig. 1) (Ishida et al., 2003a, 2005). Although the lipid dose appears to affect the magnitude of the induced ABC phenomenon, the dose does not change the period to induce the phenomenon after a first injection.

Dams et al. (2000) investigated effect of consecutive injections on biodistribution of PEGylated liposomes. The consecutive injections attenuated the accelerated blood clearance of second dose PEGylated liposomes; the fourth dose injected 3 weeks after the first injection did not show the accelerated pharmacokinetics, although the enhanced hepatic accumulation was still occurred. Our study showed that the ABC phenomenon became less pronounced, when the third dose was injected at 4, 7 or 14 day after the second injection (the second dose was given 5 week after the first injection) (Ishida et al., 2003a).

4.5. Effect of doxorubicin encapsulation

PEGylated liposome containing DXR (Doxil or Caelyx) is in clinical use. Laverman et al. (2001) studied if the commercial PEGylated liposome formulation (Doxil or Caelyx) also exhibits the ABC phenomenon upon repeated injection. They showed such formulation (Doxil or Caelyx) was not able to induce the enhanced clearance of a second formulation injection. But, 1 week after an injection with empty PEGylated liposome, the formulation (Doxil or Caelyx) was rapidly cleared from the circulation. This clearly suggests that the encapsulation of DXR prevents induction of the ABC phenomenon. They supposed that this is due to suppression of splehepatic macrophage function by DXR following first injection.

A similar study has been presented by Tardi et al. (1997). They studied the effect of encapsulated DXR on the pharmacokinetic behavior of repeated injections of ovalbumin-coated PEGylated liposomes (so-called proteoliposomes). Injection of ovalbumin-coated PEGylated liposomes elicited a strong immune response to the coated ovalbumin, leading to a rapid elimination of a subsequent liposome dose from the circulation. This immune response was prevented when the liposomes contained DXR. The authors hypothesized that the macrophages were responsible for the observed immune response.

As described in earlier section, we demonstrated that the binding of anti-PEG IgM to a second dose PEGylated liposomes and subsequent complement activation on the liposomes are a major cause of the accelerated blood clearance of a second dose of PEGylated liposomes (Ishida et al., 2006c; Wang et al., 2007). In addition, we reported that the spleen plays an important role in the production of anti-PEG IgM (Ishida et al., 2006b). These have led to the proposal, of an alternative to the theory of Laverman et al. (2001): that DXR inside the first dose of injected PEGylated liposomes reduces the production of anti-PEG IgM by interference with the proliferation of B cells in the spleen, and consequently prevents the induction of the ABC phenomenon.

In our study, we confirmed that a first injection of PEGylated liposomes containing encapsulated DXR did not lead any rapid clearance of a second dose of "empty" PEGylated liposomes (Ishida et al., 2006a). Western blot analysis and quantitative analysis revealed substantially less binding of anti-PEG IgM to PEGylated liposomes following incubation in serum from rats treated with DXR-loaded PEGylated liposomes. In addition, no complement activation was detected with serum from rats that had been treated with DXR-loaded PEGylated liposomes. Based on these findings we concluded that DXR released from liposomes accumulating in the spleen impairs the production of PEG-specific IgM as a consequence of the inhibition of B cell-proliferation and/or killing of proliferating B cells, and thus prevents the induction of the enhanced clearance of a second dose of PEGylated liposomes.

5. Conclusion

These studies demonstrated in this review suggests that any PEGylated liposomal formulation may display unexpected pharmacokinetic behavior upon repeated injection and, as a consequence, may show less therapeutic efficacy or even cause undesirable side effects in clinic. Therefore, a strategy to abrogate the immunogenicity of PEGylated liposomes without significantly compromising their *in vivo* performance would be highly desirable for the further development of promising drug delivery system. On the other hand, such immune responses may also turn out to become beneficial, if only we learn how to control the carrier-induced responses of the immune system. Therefore, studies providing further insight in the mechanisms underlying the ABC phenomenon are of great importance.

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