Anti-polyethylene glycol antibodies alter the protein corona deposited on nanoparticles and the physiological pathways regulating their fate *in vivo*

Philippe Grenier[1], Iara Maíra de Oliveira Viana[1,2], Eliana Martins Lima[2], Nicolas Bertrand[1]

1. Faculty of Pharmacy, CHU de Quebec Research Center, Université Laval, 2705 Laurier Blvd, Québec, Canada G1V 4G2
2. Laboratory of Pharmaceutical Technology, Federal University of Goiás, Goiânia 74605-220 Goiás, Brazil

Correspondence to: Nicolas Bertrand, nicolas.bertrand@pha.ulaval.ca

2705 Laurier Blvd, Room T4-13, Quebec City, QC, Canada, G1V 4G2.

Abstract: Multiple studies highlight the strong prevalence of anti-poly(ethylene glycol) (anti-PEG) antibodies in the general human population. As we develop therapeutic modalities using this polymer, it is increasingly relevant to assess the importance of anti-PEG antibodies on biological performances. Here, we show that the anti-PEG Immunoglobulin M (IgM) raised in mice following the injection of polymeric nanoparticles could have significant neutralizing effects on subsequent doses of PEGylated nanosystems *in vivo*. The circulation times of PEGylated nanoparticles and liposomes were strongly reduced in animals with circulating anti-PEG IgMs, irrespective of the PEG density or the surface properties of the system. In comparison, despite that anti-PEG IgMs could bind free methoxy-terminated PEG and PEGylated bovine serum albumin, the circulation kinetics of these systems remained unaltered in the presence of antibodies. The binding of IgMs to the PEGylated surface of nanoparticles alters the nature of the proteins adsorbed in the surrounding corona, notably due to the activation of the complement cascade. These changes are responsible for the observed differences in circulation times. In comparison, the PEG-BSA is unable to activate complement, even in the presence of anti-PEG IgMs. These results inform on how anti-PEG antibodies can affect the fate of PEGylated nanomaterials and highlight how the architecture of nanoparticles impacts the deposition of the protein corona.

Keywords (5-7) Anti-drug antibodies (ADA), poly(ethylene glycol)-b-poly(lactic co glycolic acid) (PEG-PLGA), nanoparticles, drug delivery, pharmacokinetics, biodistribution, liposomes.

Upon entering the bloodstream and other physiological environments, the surface of nanomaterials absorbs a complex corona of proteins that influences how they are subsequently perceived by biological systems [1, 2]. Understanding which proteins are involved in this corona and how they can alter the interactions of nanomaterials with living systems is an important prerogative to design more effective nanoparticles for biomedical applications [3]. Various sophisticated studies have described how the deposition of proteins on nanoparticles can affect *in vitro* interactions with macrophages [4, 5] or cancer cells [6]. Nevertheless, understanding how the protein corona affects the fate of nanoparticles in living organisms with functional circulatory, metabolic and immune systems is of paramount importance for the development of safe and effective nanomedicines.

In addition to model polystyrene or inorganic nanoparticles that can inform on fundamental concepts [5, 6], mechanistic studies must also include materials which are used clinically or are being developed for translational applications. In general, these materials need to be engineered to limit aggregation and excessive deposition of proteins and be biodegradable. In previous work, our efforts focused on the fate of polymeric nanoparticles with different surface coatings after one intravenous

injection to naive animals [7]. This work used knockout animals to show that interactions of nanoparticles with apolipoproteins had a stronger effect on their blood circulation than interactions with the complement system [7]. Specifically, adsorption of apolipoprotein E (ApoE) could prevent rapid opsonisation of some nanoparticles upon entry in the bloodstream, while also contributing to the slow removal of nanoparticles from the circulation via interactions with the receptor for Low Density Lipoproteins (LDLR). In comparison, the circulation times of nanoparticles in C3 knockout mice, that is transgenic mice that are unable to activate the complement cascade, were very similar to those observed in wildtype animals. Finally, like other studies [8-11], our work highlighted the importance of coating nanoparticles with sufficient density of poly(ethylene glycol) (PEG) to minimize their clearance from the blood.

Due to its high flexibility, its solubility in aqueous and organic solvents, as well as its accredited biocompatibility, PEG has become a hallmark of many pharmaceutical products [12]. While low molecular weight PEGs can be employed as solvents or viscosity enhancing agents, larger macromolecules are commonly used to decrease the immunogenicity of biologics [13], or alter their pharmacokinetics [14]. In nanomedicine, surface decoration of nanoparticles with PEG has been employed for more than 25 years to minimize interactions with the immune system and prolong circulation times [9, 11, 15]. Surface functionalization of nanomaterials with PEG appears to increase the biocompatibility of nanoparticles prepared from metal [4], mesoporous silica [16], hydrophobic polymers [7] or lipids [8], as well as carbon nanotubes [17]. PEG is therefore a common ingredient of many drug delivery systems intended for clinical translation.

When PEG was initially introduced to reduce the clearance of biologics [18] and liposomes [11], the non-immunogenic nature of the polymer was often highlighted as one of its main characteristics. Although PEG remains a well-tolerated synthetic polymer, it is now appreciated that it interacts with the immune system in unexpected ways [19]. In an elegant study, Lai and his group [20] compared the plasma of contemporary healthy individuals to historical plasma samples from a biobank spanning 4 decades and covering multiple demographics. They showed that low levels of anti-PEG antibodies are detectable in the plasma of 47 to 72% of patients, irrespective of the collection date. Parallel studies in healthy donors without prior exposure to PEGylated therapeutics living in Austria, the US and Taiwan also show that anti-PEG immunoglobulins are present in 20 to 44% of blood samples [21, 22]. Recently, a genome-wide association study conducted in patients from Taiwan identified the immunoglobulin heavy chain locus as a site of genetic variation that could predispose to the development of anti-PEG IgMs [23]. The existence of anti-PEG antibodies in the blood of patients is therefore a prevalent phenotype that needs to be accounted for when designing novel therapies.

Although the exact origin of anti-PEG antibodies in humans remains unknown for the moment, two independent groups reported that repeated injections of PEGylated liposomes could trigger an accelerated clearance effect in animals [24, 25]. In other words, the circulation times of PEGylated liposomes were significantly shorter in animals when they had already received the same formulation, 5 to 7 days before. Pivotal studies from these groups and others have shown B lymphocytes in the spleen can recognize PEGylated liposomes and induce the production of anti-PEG antibodies, which are subsequently responsible for the enhanced clearance [26-28]. Beyond liposomes, other systems can also trigger the production of anti-PEG antibodies, for example PEG-PLGA nanoparticles [29] and lipid complexes of nucleic acids [30].

Because nanomedicines can contribute to the production of anti-PEG immunoglobulins, it is important to understand how these antibodies affect the fate of PEGylated therapeutics. Lately, a survey conducted among clinicians routinely prescribing PEGylated therapeutics showed that, although most

physicians were aware of the possible existence of anti-drug antibodies, only 22% of them knew that such antibodies could be directed against the polymer [31]. In the clinics, neutralizing antibodies developing in patients treated with some PEGylated biologics are known to decrease their efficacy, for example with PEG-asparaginase and PEG-uricase [32, 33]. In these studies, the reactivity toward PEG of (at least part of) the neutralizing IgG and IgM antibodies was established using distinct PEGylated molecules: PEG hydrogels, other PEG-conjugates, and free PEG. Besides efficacy, pre-existing antibodies directed toward PEG could be accountable for some of the allergic and adverse reactions seen upon dosing of PEGylated pharmaceuticals in patients [34].

The impact of anti-PEG antibodies on the therapeutic effects of nanomedicines remains unclear (for review see [35]). In patients, irrespective of the presence of anti-PEG antibodies, repeated cycles of PEGylated liposomal doxorubicin can result in gradually increasing blood exposure (as measured by the area under the plasma concentration *vs*. time curve (AUC)) [36]. This effect could be ascribed to a progressive deleterious effect of doxorubicin on the macrophages of the mononuclear phagocyte system. Similarly, studies in animals suggest that nanoparticles containing anticancer payloads could prevent the production of anti-PEG antibodies, also due to the cytotoxic effects of the drugs on B cells [37]. Since most PEGylated nanomedicines currently on the market are in the oncology space, it might be difficult to study the production of anti-PEG antibodies using clinically-approved drugs. In parallel, no data is currently available regarding how the presence of pre-existing anti-PEG antibodies affects the blood circulation of commercially-available PEGylated liposomes. The Phase 1 studies of two recently-approved PEGylated systems, liposomes of irinotecan (MM-398/PEP02, Onivyde®), and cytarabine:daunorubicin (CPX-351, Vyxeos®), did not assess the status of enrolled patients with regard to anti-PEG antibodies [38, 39]. To expand nanomedicines beyond applications in cancer and assess the implications of anti-PEG antibodies when developing new therapeutic modalities, further studies in the absence of cytotoxics are therefore necessary.

This work aims at better understanding the biological consequences of circulating anti-PEG antibodies with respect to interactions between PEGylated nanoparticles and blood proteins. Herein, we injected clinically-relevant nanoparticles to induce an immune response in animals and looked at how the phenotypic changes affected the fate of subsequent doses of nanoparticles and other PEGylated systems. We found that the architecture of the nanoparticles, where PEG chains are neatly patterned at a solid interface, influences the binding of anti-PEG IgM in comparison to PEGylated protein conjugates. Furthermore, in animals with circulating anti-PEG antibodies, changes in the protein corona surrounding nanoparticles, notably activation of the complement cascade, can explain their increased clearance rates *in vivo*.

Results and discussion

Characterizing phenotypic changes after a first injection of PEGylated nanoparticles.

To raise an anti-PEG immune response, empty nanoparticles were intravenously injected to healthy Balb/c mice. These nanoparticles were prepared from methoxy-poly(ethylene glycol 5,000)-b-poly(lactic-co-glycolic acid) (PEG_{5k}-PLGA), and had a diameter of 100 nm, a total PEG content of 20 wt%, and a PEG density of 40 PEG chains per 100 nm^2. These nanoparticles are known to be long-circulating [7] and, apart from their lack of pharmacologically active payload, are similar to some drug delivery systems presently investigated in the clinic [40]. **Figure 1A** shows that injection of 500 µg of nanoparticles (*i.e.*, 100 µg of PEG) can trigger an augmentation of circulating levels of anti-PEG antibodies, within 5 to 7 days. In comparison, equivalent doses of monomethoxy PEG 5,000 (PEG_{5k}) triggered only a minimal immune response. This suggests that the patterning of PEG on the surface of nanoparticles makes it more immunogenic than freely soluble polymer chains. These findings are similar to what was previously

observed with liposomes [41]. An isotyping kit able to recognize immunoglobulins M, A, and G (1 to 3), was used to assess whether the immune response triggered by nanoparticles raised anti-PEG immunoglobulin isotypes beyond IgM. This experiment confirmed that the anti-PEG immune response was mainly an IgM response (**Figure 1B**). In comparison with the injection of some other PEGylated systems which could also raise a weak concomitant IgG response [35], sensitization of healthy mice with PLGA-PEG nanoparticles represents a reproducible way of studying the effect of anti-PEG IgMs. In comparison, injections of solutions of free $mPEG_{5K}$ at equivalent doses (100 µg/animal) showed minimal increases in levels of anti-PEG IgM (**Supplementary figure 1**).

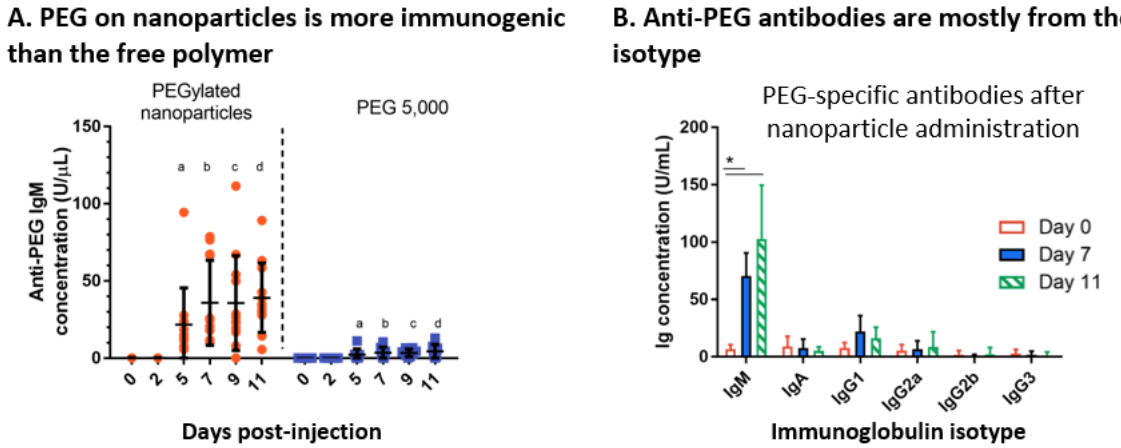

Figure 1 **PEGylated nanoparticles show a stronger production of anti-PEG immunoglobulin M (IgM), without affecting the overall concentrations of other antibodies. A.** The titers of anti-PEG IgM increase 5 to 7 days after an injection of PEGylated nanoparticles, whereas the injection of methoxy-terminated PEG 5,000 is less immunogenic. Values represent individual animals (n = 9-12), a, b, c, d $p < 0.05$ between each other. **B.** The PEG-specific antibodies are mostly of the IgM isotype. Values represent means ± SD (n = 7-8), * $p < 0.05$.

Impact of anti-PEG antibodies on the pharmacokinetics of PEGylated architectures

The antibody response triggered by the intravenous injection of PEGylated drug delivery systems can facilitate the removal of subsequent doses from the blood, a neutralizing phenomenon coined the "accelerated blood clearance" effect [35]. In most studies, the same material is administered twice: to induce the immune response, and to observe the neutralizing effect. These experiments show the role of antibodies upon re-exposure to the same material, but few assessed their ability to cross-react with other PEGylated molecules. In **Figure 2**, seven days after raising an immune response as described above (PEG_{5k}-PLGA nanoparticles, 100 nm in diameter, 40 PEG chains per 100 nm^2), we looked at the circulation kinetics of different PEGylated architectures, all injected at 500 µg per animal, that is approximately 20 mg/kg. The physicochemical characteristics of the systems studied here are presented in **Supplementary Table 1**, while the anti-PEG IgM titers and details of the pharmacokinetic experiments are presented in **Supplementary Table 2**.

For comparison, **Figure 2A** shows the neutralizing effect of anti-PEG antibodies when PEG-PLGA nanoparticles with high PEG densities, *i.e.*, those used to raise an immune response, are reinjected. The neutralizing effect is visible from the increase in clearance in pre-sensitized mice compared to naive animals: a 2.9-fold decrease in the blood exposure over 6 hours ($AUC_{0\text{-}6h}$) is observed in IgM-positive *vs*. IgM-negative mice. Interestingly, the neutralizing effect of IgMs was significantly reduced when higher

doses of polymeric nanoparticles were injected, suggesting a possible saturation of the neutralizing effect (**Supplementary Figure 2**).

Accelerated clearance is also observable when the second dose of polymeric nanoparticles has approximately one third the PEG coverage of those used for sensitization (**Figure 2B**). In naive animals, these nanoparticles are cleared from the bloodstream intrinsically faster than those with higher PEG densities [7]. Nevertheless, despite differences in PEGylation and circulation times, the $AUC_{0\text{-}6h}$ observed in IgM-positive mice is also decreased by 2.7-fold, compared to reference animals.

Liposomes of phospholipids with a diameter of 100 nm were also injected to naive and sensitized animals. In naive animals, PEGylated liposomes remain in the bloodstream longer than their non-PEGylated counterpart (1.6-fold increase in AUC over 6 h) (**Figure 2C-D**), but this variation is somewhat smaller than those reported by others [11]. These differences can be ascribed to the shorter timeframe of the study, but another phenomenon might be at play. T.M Allen and C. Hansen have observed that doses of non-PEGylated liposomes above~500 μg started to saturate the mononuclear phagocyte system and would show decreased blood clearance [42]. Comparatively, they found that increasing the doses of PEGylated liposomes did not prompt such an effect. In the present study, partial saturation of the clearance of non-PEGylated liposomes might explain their longer than usual circulation times. Nevertheless, in immunized mice, cross-reactivity of neutralizing antibodies was observed when PEG was present. The $AUC_{0\text{-}6h}$ of PEGylated liposomes decreased approximately 1.5-fold in sensitized animals compared to naive mice (**Figure 2C**), while remaining the same for non-PEGylated liposomes (**Figure 2D**). Given the important differences in the surface of polymer nanoparticles and liposomes, these results confirm that the neutralizing effect is likely due to the recognition of PEG by the antibodies identified above. In the liposomes used herein, the phosphatidylcholine surface is zwitterionic and PEG chains are tethered to negatively-charged phospholipids; this structure is chemically very different from the PEG-PLGA copolymers.

Of note, the amplitude of the neutralizing effect observed with PEGylated liposomes seems lower than what is observed with PEG-PLGA nanoparticles (1.5- vs. 2.9-fold). Nevertheless, this decrease cannot be directly ascribed to variations in the affinity of IgMs for PEGylated liposomes. A dose of 500 μg per animal was chosen to normalize comparisons between systems. However, the lower volumetric mass density of liposomes would result in larger numbers of injected particles. This higher number and increased interface could result in saturation of the neutralizing effect as observed with higher doses of PEG-PLGA nanoparticles.

The previous experiments with four different systems confirm that anti-PEG antibodies have a neutralizing effect on various nanomaterials where PEG is patterned on the surface. Long blood circulation times or identical PEGylation architectures are therefore not necessary for IgM to have a neutralizing effect. Nevertheless, circulating anti-PEG IgMs do not affect the clearance of all PEGylated architectures in the same fashion. In **Figure 2E**, injection of a solution of free methoxy-terminated PEG 10,000 in naive and sensitized animals shows identical circulation profiles: the free polymer is rapidly cleared from the bloodstream irrespective of the presence of anti-PEG antibodies. Due to the very rapid clearance of the polymer, likely due to glomerular filtration [43], the lack of neutralizing effect by the antibodies could result from the limited possible interactions between PEG and the IgMs, in the bloodstream. Therefore, PEGylated bovine serum albumin (PEG-BSA), a model protein, was also injected to animals with and without circulating anti-PEG IgMs (**Figure 2F**). The intrinsic clearance of this protein and PEG-PLGA nanoparticles in naive animals are comparable. The [^{14}C]-labeled PEG-BSA prepared for the pharmacokinetic experiments have approximately 17-20 PEG chains are tethered per molecule of BSA (**Supplementary Information** and **Supplementary Figure 3**). Here, despite the possibility for longer interactions between the PEGylated protein and the antibodies in the bloodstream, the pharmacokinetic in naive and sensitized mice are identical.

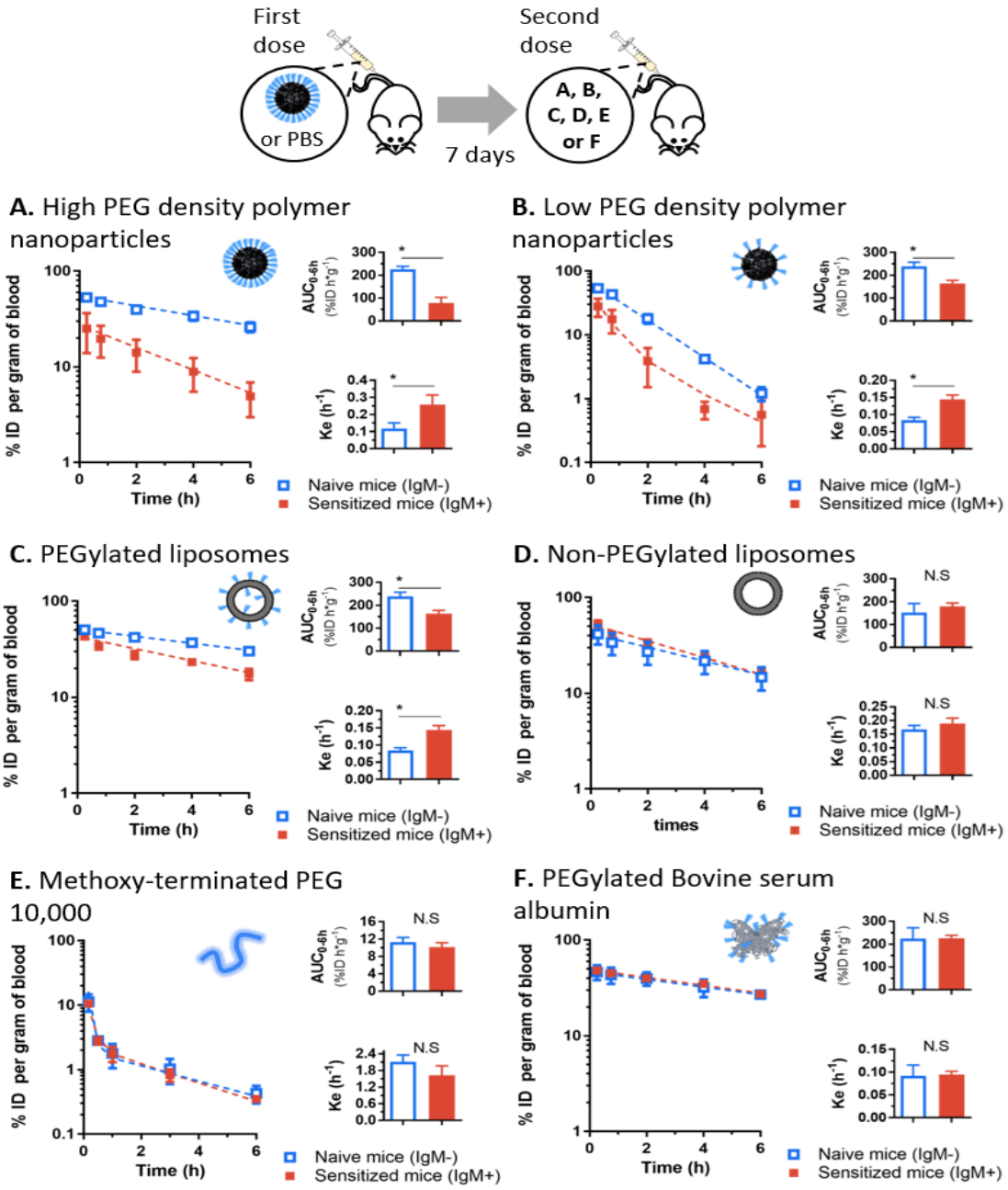

Figure 2 Mice with circulating anti-PEG IgMs clear some PEGylated nanosystems more rapidly than naive mice, but not free polymer and PEG-BSA. PEGylated nanoparticles identical to those used to raise the immune response are cleared faster from the systemic circulation in sensitized mice (IgM+) compared to naive mice (IgM-) **(A).** Similarly, antibodies also have a neutralizing effect on nanoparticles with lower PEG density, even if their intrinsic circulation times and PEGylation architecture are different **(B)**. For liposomes prepared with phospholipids, surface PEG seems essential to observe the neutralizing effect of the antibodies **(C and D)**. The circulation kinetics of free methoxy-terminated PEG 10,000 **(E)** and PEG-BSA **(F)** is identical, irrespective of the presence of anti-PEG IgMs. Data represents means ± SD (n =4-5), * $p < 0.05$.

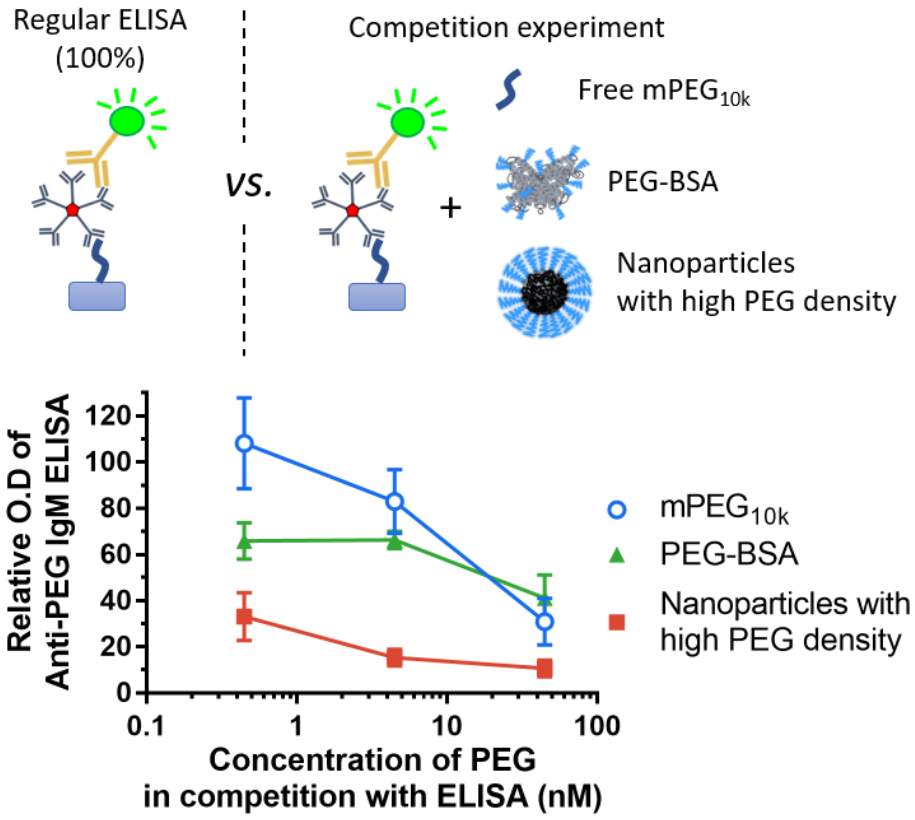

Figure 3 Competition experiments between immobilized PEG-BSA (commercial ELISA) and free $mPEG_{10k}$, PEG-BSA or PEGylated nanoparticles show that nanoparticles have better affinity for anti-PEG IgM than the solutions of polymer and protein conjugate. An ELISA against anti-PEG IgM was reproduced on the same plasma samples with competing $mPEG_{10k}$, BSA-PEG or PEGylated nanoparticles. Results were normalized by the optical density obtained in the absence of any competing PEG. At high concentrations of competing PEG (45 nM), all sources of PEG decrease the interactions of anti-PEG IgM with the bottom of the ELISA plate. At lower concentrations (4.5 nM, and below), PEGylated nanoparticles still prevent the binding of IgM to the coated antigen, whereas competition from $mPEG_{10k}$ and PEG-BSA decreases significantly. Values represent means ± SD (n = 3).

In vitro characterization of PEG-BSA conjugate **(Supplementary Figure 3)**, but also the results of IgM quantification (where immobilized monoPEGylated-BSA is the reference epitope of the commercial anti-PEG ELISA kit), show that the antibodies present in the serum can bind PEGylated BSA. However, in these assays, both conjugates are coated at the bottom of polystyrene plates. A competition experiment was conducted to detect differences in the ability of anti-PEG IgMs to bind these systems (**Figure 3**). In this experiment, the antibodies from the serum of sensitized animals can bind either immobilized mono-PEGylated BSA at the bottom of the ELISA plate or free mPEG, PEG-BSA or PEG-PLGA nanoparticles in the solution. **Figure 3** shows that at high concentrations (45 nM of competing PEG), free $mPEG_{10k}$ and PEG-BSA can compete with the immobilized polymer chains, confirming that PEG is truly an epitope of the polyclonal IgMs. However, at low concentrations, the PEG on the surface of nanoparticles is much more effective at preventing IgM from binding to the ELISA wells: at 0.45 and 4.5 nM of PEG, respectively, the ELISA signal of the serum samples was decreased by 60 and 80% of their initial values in the presence of PEG-PLGA nanoparticles. Comparatively, at these concentrations, PEG-BSA abrogated only 40% of the signal, and mPEG had very little effect. This suggests that the multivalent and ordered architecture of the

PEG coating on the surface of nanoparticles can be recognized by IgMs more efficiently than free polymer chains or PEG-BSA, presumably due to the ordered architecture of PEG chains on nanoparticles.

The impact of circulating anti-PEG IgMs on the protein corona surrounding nanoparticles.

Current understanding of nano-bio interfaces suggests that the protein corona on nanoparticles impacts on their biological fate [44]. The proteins on nanoparticles can be separated between 'soft' and 'hard' coronas: corresponding to proteins which interact 'loosely' or 'strongly' with the nanoparticle surface, respectively [1]. The hard corona formed in the sera of naive and sensitized mice was compared by label-free, shotgun proteomics. The relative abundance of proteins on the surface of nanoparticles was compared by spectral count (% NSpCk, equivalent to the fraction of all proteins found in the corona) [45]. Despite large differences in pharmacokinetics between naive animals and those with circulating anti-PEG IgMs, **Figure 4A** shows subtle changes in the protein corona. In sensitized animals, the amounts of immunoglobulins and apolipoproteins on the surface of the nanoparticles appear to be increased, while the proteins from the complement cascade are somewhat decreased. Ninety-percent of the 20 most abundant proteins are present in both conditions, the top-6 proteins being the same in naive and sensitized sera. These six proteins represent almost 50% of all proteins adsorbed on the surface of nanoparticles (**Supplementary Figure 4**).

To deepen the interpretation of the results, proteins were categorized according to their biological functions (see **Supplementary Table 3** for categorization of individual proteins). Comparing with the corona obtained in naive mice, changes were perceivable in the pattern of immunoglobulins deposited on the surface of nanoparticles from the serum of sensitized animals (**Figure 4B**). In these animals, the deposition of IgM-specific constant chains augments slightly (1.05-fold, around 4-5% of all proteins), while the involvement of J chains to the protein corona increases by 2.3-fold (reaching >2% of all proteins in IgM-positive mice). J chains are 15 kDa polypeptides responsible of the polymerization of secreted antibodies IgA (dimer) and IgM (pentamer) [46]. Given the very minor deposition of IgA chains on nanoparticles in both conditions (less than 0.1%), this increased proportion of J chains in the protein corona can be indirectly ascribed to a higher deposition of IgMs.

Apolipoproteins are another class of proteins whose deposition on the surface of nanoparticles seems impacted by the presence of anti-PEG IgMs (**Figure 4C**). This class together represents a large fraction of the protein corona of nanoparticles: around 25 and 30% of total proteins for naive and sensitized mice, respectively. Noticeably higher amounts of apolipoproteins C-IV and ApoE are adsorbed on nanoparticles when they are incubated in the serum of sensitized animals (4.4- and 1.5-fold increases, respectively). In these conditions, these two proteins represent together almost 15% of all proteins on the nanoparticles, compared to approximately 9% of the corona obtained in naive mice. This might be of particular relevance given the implication of these proteins on the clearance of nanoparticles from the bloodstream *in vivo* [7].

When looking with more scrutiny into the proteins from the complement cascade, qualitative changes are observed (**Figure 4D**). Proteins from the classical activation pathway are 1.3-fold more abundant in the protein corona when nanoparticles are incubated in IgM-positive *vs*. control serum. This augmentation appears to combine with a decrease in the deposition of proteins from the lectin and alternative activation pathways (2- and 1.5-fold reduction, respectively), but importantly with a 2.4-fold reduction in the amount of complement inhibitors found on the surface of the nanoparticles.

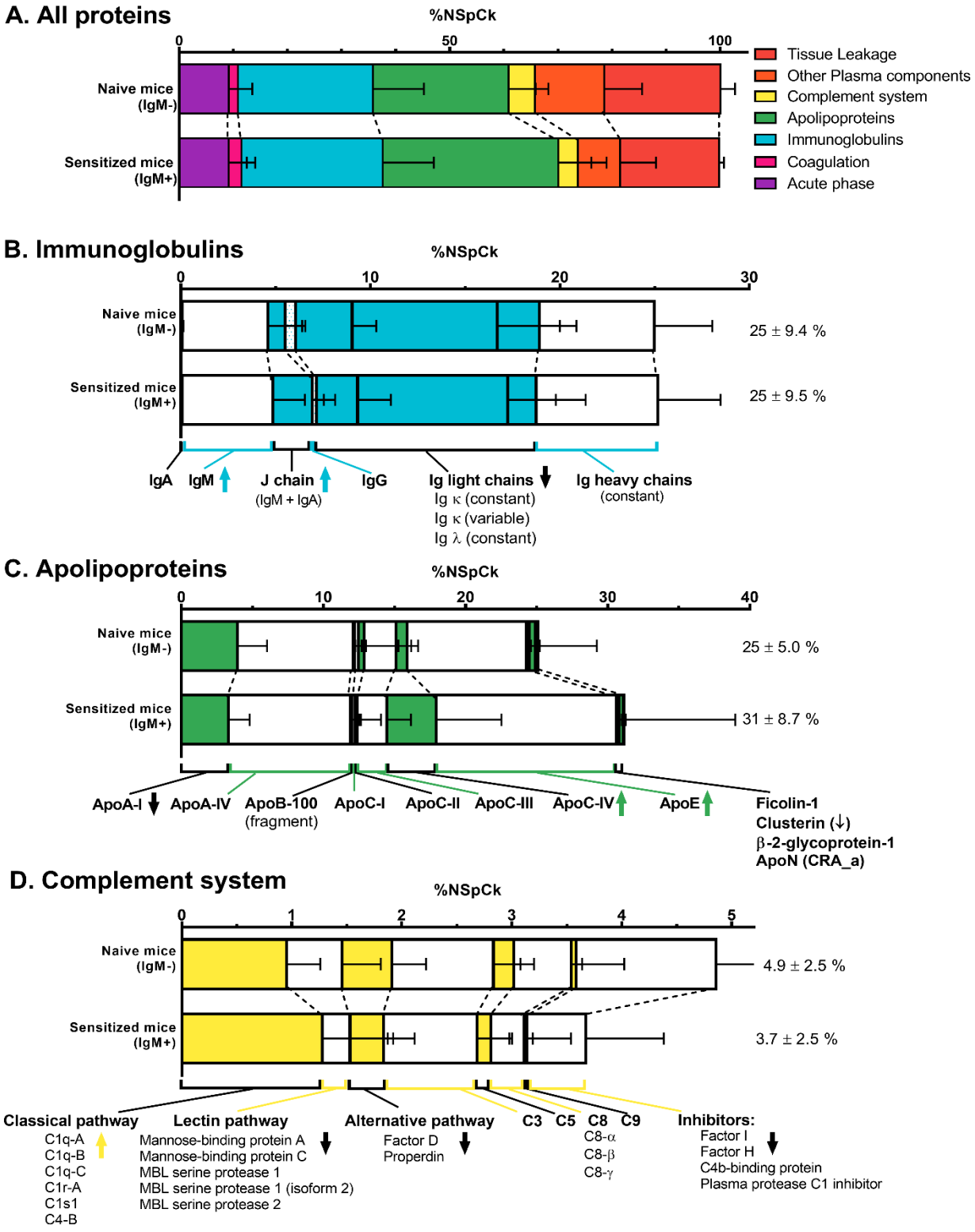

Figure 4 **For a given nanoparticle, the presence of anti-PEG IgMs influences the protein corona deposited on nanoparticles. A**. Subtle differences are perceivable in the protein corona found on nanoparticles incubated in the plasma of naive and sensitized mice. **B**. Higher amounts of IgM constant chains and J chains, a component of IgAs and IgMs, are deposited on the surface of nanoparticles in sensitized animals. **C**. The presence of anti-PEG IgMs also alters the deposition on apolipoproteins (Apo E, Apo C-IV and Apo A-IV). **D**. The serum of sensitized mice (IgM+) favors the deposition of higher proportions of proteins involved in the classical activation pathway of the complement system and a reduced contribution of complement inhibitors. Values represents means ± SD (n = 5).

In naive animals, PEG-PLGA nanoparticles are known to be modest activators of the complement system. We have previously shown that this modest activation is not sufficient to explain the perceptible differences in the blood clearance of nanoparticles with low and high PEG densities.[7] However, the current proteomics experiments showed decreased deposition of complement inhibitors on nanoparticles incubated in the sera of sensitized mice. As this technique can hardly detect the cleavage of C3 fragments and the other zymogens of the complement system, we decided to look at the complement activation of PEG-PLGA nanoparticles *in vitro*, measuring the production of C5a (a by-product of the activation of the cascade) by ELISA.

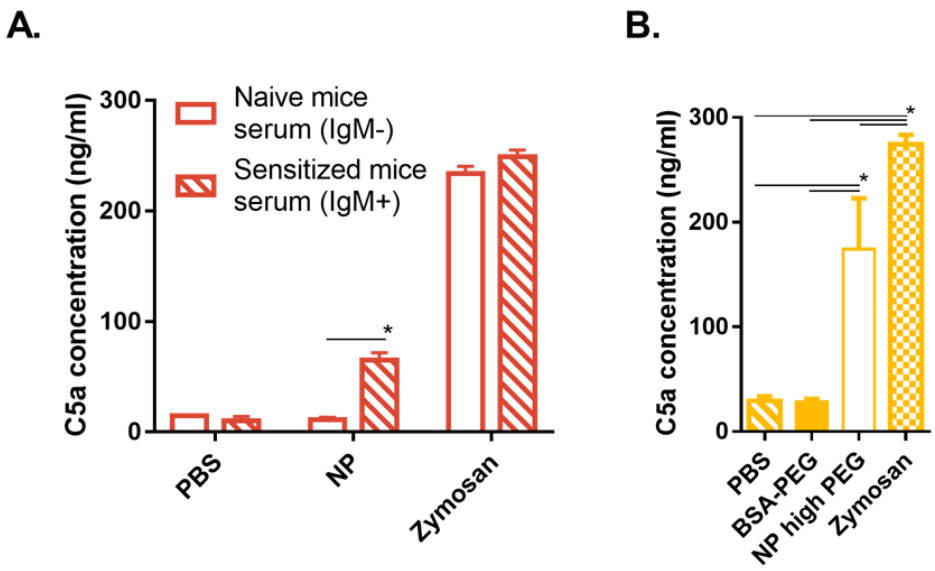

Figure 5 The presence of anti-PEG IgMs potentiates the activation of the complement cascade by PEGylated nanoparticles, but not by PEG-BSA. A. In serum from sensitized mice, the activation of the complement by PEGylated nanoparticles is more pronounced than in the serum of naive animals. **B**. At concentration where both colloids bind IgMs, complement activation is stronger with nanoparticles than with PEG-BSA. Values represent means ± SD (n = 3), * $p < 0.05$.

Figure 5A shows that the production of C5a by nanoparticles with high PEG density is increased upon incubation in the serum of sensitized animals, compared to naive mice. IgMs are known to participate in complement activation *via* the classical pathway [47]. In combination with the proteomics experiment showing lower amounts of inhibitors and increased deposition of proteins from the classical activation pathway, this strongly suggests that the binding of anti-PEG IgMs contributes to the activation of the complement cascade by PEGylated nanoparticles. These results are in accordance with those of Kiwada and his group [48] which show that anti-PEG antibodies can increase complement activation triggered by PEGylated liposomes. In a parallel experiment, the complement activation triggered by PEGylated nanoparticles in the serum of sensitized animals was compared to that triggered by incubation with PEG-BSA. **Figure 5B** shows that, even in the presence of anti-PEG IgMs, incubation of serum with PEG-BSA does not trigger the activation of the complement. To ensure that variations in complement activation are not ascribed to differences in the ability to bind IgMs, this experiment was conducted at 5 mg/mL of PEG-BSA (and PEG-PLGA nanoparticles), a concentration where the conjugate can bind anti-PEG IgMs. These results therefore suggest that the binding of anti-PEG IgMs by the architecture of the PEG-PLGA nanoparticles triggers the activation of the complement system, whereas the binding of IgMs to PEG-BSA does not.

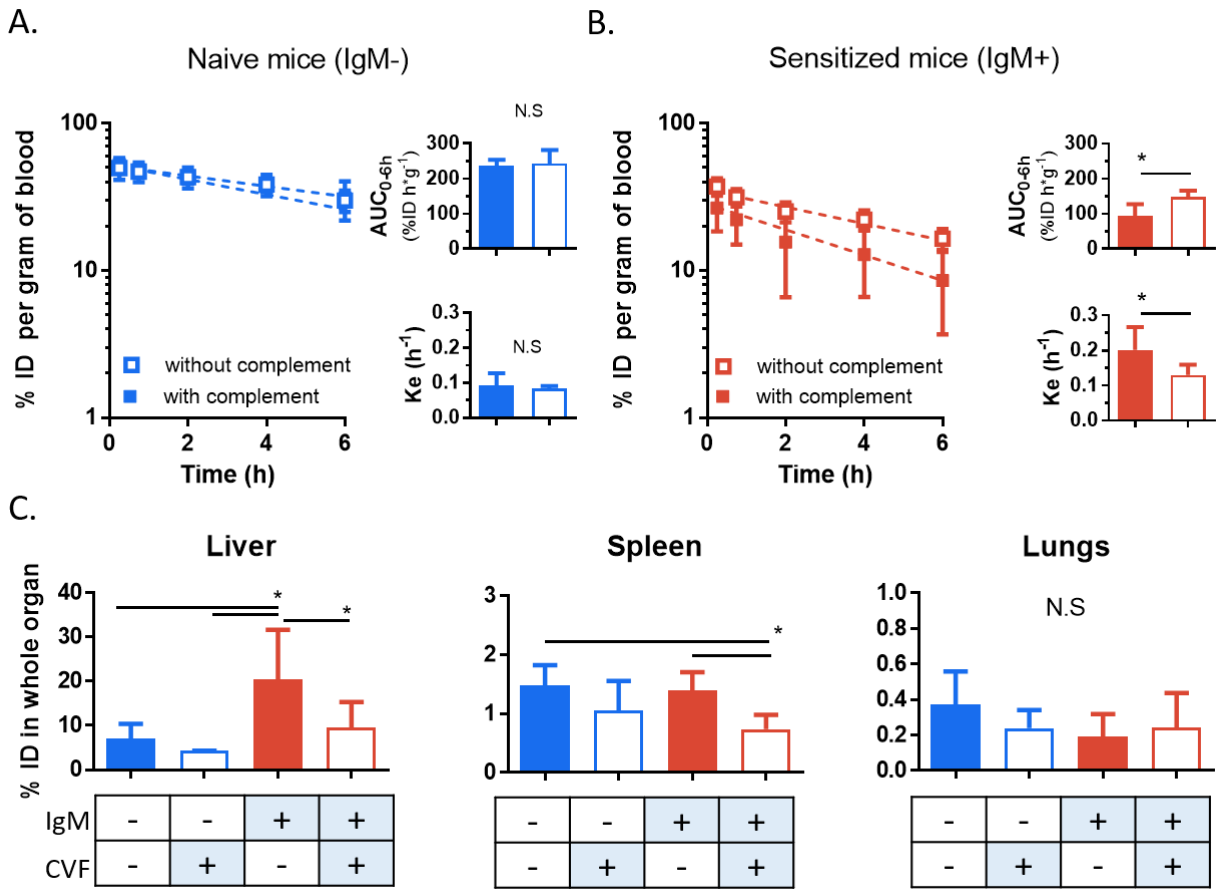

Figure 6 The complement cascade is implicated in the clearance of nanoparticles in the presence of anti-PEG IgMs, but not in naive animals. A. The circulation times of nanoparticles with high PEG coverage are similar in mice, irrespective of their ability to activate complement. Animals without complement activity received intraperitoneal injections of CVF, 24 hours before the pharmacokinetic study. **B.** In sensitized mice (IgM+), the abrogation of the complement significantly impacts the ability of IgMs to clear the nanoparticles. **C.** The distribution of nanoparticles in the organs of the mononuclear phagocyte system is impacted by the presence of anti-PEG IgMs, and the ability of animals to activate the complement cascade (i.e., without and with CVF, respectively). Data represents means ± SD, * $p < 0.05$ (n = 5-8).

Previously, Shimizu *et al*. [49] had shown *in vitro* that the complement system could be implicated in the internalization of immune complexes of anti-PEG IgMs and PEGylated liposomes by splenic B cells. We therefore evaluated the circulation kinetics of PEG-PLGA nanoparticles in mice with and without the ability to activate the complement system. Here, we used intraperitoneal injections of cobra venom factor (CVF) to abrogate the complement response *in vivo* [50]; animals that receive CVF lose all ability to activate complement when stimulated with Zymosan A (**Supplementary Figure 5**). In naive mice, the circulation kinetics of the nanoparticles are similar, irrespective of the ability to activate the complement cascade (**Figure 6A**). These results are in accordance with those previously obtained in C3 knockout mice with similar nanoparticles [7]. However, in mice that have been sensitized by nanoparticles (IgM-positive), abrogation of the complement cascade results in increased circulation times (**Figure 6B**). Although the circulation times in IgM-positive animals dosed with CVF are still below those observed in naive mice ($AUC_{0\text{-}6h}$ is 60% of baseline values), this suggests that the activation of the complement is in part responsible for the accelerated clearance of anti-PEG IgMs. Besides pharmacokinetics, the distribution in organs also highlights that abrogation of the complement cascade can decrease by 2-fold the deposition of nanoparticles to the liver (**Figure 6C**). Altogether, these results demonstrate that, despite that it does not appear to be implicated in the biological fate of nanoparticles in naive mice, the complement cascade becomes an important component to explain the accelerated clearance in the presence of anti-PEG IgMs.

Conclusions

The reasons behind the increasing prevalence of anti-PEG antibodies in the human population are not fully understood yet. Nevertheless, with 25-40% of patients having anti-PEG antibodies in their bloodstream, these proteins cannot be neglected when designing new PEGylated platforms. Herein, we focused our study on anti-PEG IgMs, an antibody isotype found in the serum of patients, and which is usually present during early immune responses. The production of these antibodies was triggered in healthy wildtype mice by the intravenous injection of long-circulating PEG-PLGA nanoparticles. These nanoparticles appear to raise higher levels of anti-PEG IgM than free polymer chains. This is of importance because, despite efforts in designing other polymer coatings to passivate the surface of nanomaterials, PEG remains a very common hallmark of nanoparticles intended for biomedical applications.

In animals which have been pre-sensitized with nanoparticles, the presence of anti-PEG IgMs induces a neutralizing effect on the circulation of subsequent doses of PEGylated liposomes and polymeric nanoparticles, but not free mPEG and PEG-BSA. This appears to occur because the architecture of the nanomaterials, that is the patterning of the PEG chains on the surface, appears to facilitate the binding of immunoglobulins compared to free mPEG chains and PEG-BSA. Future work will assess whether alternative PEGylation strategies, conceivably polymer chains with comb architectures, could pave the way for better-performing nanoparticles.

Beyond favorable binding to solid interfaces, anti-PEG immunoglobulins also alter the deposition of the protein corona on nanoparticles, notably by increasing the activation of the complement cascade. The latter appears to be play an important role in the enhanced clearance observed in the presence of anti-PEG IgMs. This contrasts with results obtained in naive mice, where abrogation of the complement system did not alter the blood circulation of various polymeric nanoparticles.

These findings have implications regarding the use of nanomedicines and contribute to further our understanding of the nano-bio interactions. The present work highlights that the protein corona on the surface of nanoparticles is not something which solely depends on the physicochemical properties of the nanoparticles, but also on the phenotype of the animals, and presumably of the patients. These changes, which might appear subtle when observed only from the perspective of proteomics, result in considerable changes in the *in vivo* fate of nanoparticles, and could possibly impact their therapeutic performances. Provided that our findings in animals somehow model what happens in humans, it might be very relevant to investigate how the presence of circulating anti-PEG antibodies in patients affect the clinical outcomes of PEGylated nanomedicines.

Methods.

Preparation of PEG-PLGA nanoparticles: PEG-PLGA copolymers were synthesized as described previously [7, 51]. Briefly, the polymers were prepared with a semi batch ring opening polymerization of D,L-lactide (2-fold molar excess) and glycolide at room temperature in dichloromethane using a $mPEG_{5k}$ as a macroinitiator and 1 mol% 1,8-diazabicycloundec-7-ene as the catalyst. To ensure random polymerization, glycolide was introduced over 10 minutes as a solution of tetrahydrofuran using a syringe pump [52]. Right after the complete introduction of glycolide, the reaction was quenched by addition of benzoic acid, solvent was removed by rotary evaporation and the polymer was precipitated in cold diethylether. Molecular weights (M_n) were characterized by ^{1}H-NMR in $CDCl_3$, and the polydispersity (M_w/M_n), by gel permeation chromatography.

Nanoparticles were prepared by nanoprecipitation of acetonitrile solutions of copolymers precursors into water [53]. Briefly, the solutions of polymers were added dropwise to water under stirring. The polymer concentration, ratio, stirring speed, and the volume of water was modified to obtain different

nanoparticle architectures (PEG density and size). For our experiment, we used $PLGA_{5k}$ $PLGA_{10k}$ and $PLGA_{30k}$ to synthesize 100 nm PEGylated nanoparticle. For pharmacokinetics experiments, nanoparticles were labelled with a radioactive [^{14}C]-PLGA copolymer (M_n ~20,000, Moravek Biochemicals).

For all experiments, nanoparticles were purified and washed 5 times with ultrapure water using an ultrafiltration filter (PALL Nanosep, MCWO 100,000). The nanoparticles average diameter (Z-average) and polydispersity index (PDI), were measured before and after purification by dynamic light scattering method (DLS) at 22^{o}C with a 173-backscatter angle on a Malvern Zetasizer Nano S (Malvern Instruments, Westborough, MA). Structural characterization of the outside shell and PEG content of nanoparticles were determined by ^{1}H-NMR spectroscopy, as described previously [51]. The properties of the nanoparticles are presented in **Supplementary Table 1**.

Preparation of liposomes: Liposomes were prepared by the hydration of lipid films followed by extrusion through polycarbonate membranes [54]. Hydrogenated phosphatidylcholine (>98% pure, Coatsome NC-21E, NOF American Corporation, White Plains, NY), cholesterol (NF grade, Medisca, Montreal, Canada), and ^{14}C-cholesterol oleate (Perkin Elmer, Waltham, MA) were dissolved in dichloromethane. PEGylated liposomes were prepared by adding 5 mol% of PEG_{2k}-distearoyl-glycero-phosphoethanolamine (Sunbright® DSPE-020CN, NOF American Corporation, White Plains, NY). The organic solvent was evaporated in rotary evaporator. The thin lipid film was hydrated with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) saline buffer (20 mM HEPES, and 140 mM NaCl, pH 7.4) and then extruded through 200, 100 and 50 nm polycarbonate membranes using LipoFast® manual extruder (Avestin, Ottawa, ON, Canada). Liposomes formulations were characterized by dynamic light scattering and a colorimetric phosphorous assay to determine concentration [54].

Quantification of Anti-PEG IgM antibodies: The concentration of circulating IgM anti-PEG antibodies was assessed by ELISA (Mouse Anti-PEG IgM Mouse ELISA, PEGM-1, Life Diagnostics Inc, Chester, PA), according to the manufacturer's protocol. Briefly, plasma sample was diluted 1,000 times and incubated in ELISA plate for 1 h on an orbital micro-plate shaker at room temperature. After incubation, each well was washed 5 times with washing buffer, and HRP-conjugated anti-IgM antibodies were diluted and added as described in the manufacturer protocol. After 30 min, the plate was washed again and TMB reagent was added to each well for 20 min with gentle shaking in the dark. The color development was stopped with the addition of stop reagent. The optical density of each well was read at 450 nm with a plate reader (Tecan infinite 200 pro, Switzerland). The concentration of IgM anti-PEG was calculated by a standard curve (concentration vs. optical density) measured by the same method.

Isotyping of immunoglobulins: To assess the immunoglobulin isotype in the plasma samples of Balb/c mice a commercial clonotyping system (SBA Clonotyping system-AP, Southern Biotech, USA) was used according to the manufacturer's protocol, but using a plate coated with monoPEGylated-BSA (PBSA20-PL, from Life Diagnostics Inc, Chester, PA) Plasma samples were diluted 2,000 times with dilution buffer, and 100 µL were incubated in each well for 12 h at 4 °C. After washing the plate 3 times, 100 µL of each detection antibodies (1:500 dilution in PBS) were added, and gently shook at room temperature for 1h. The plate was washed 5 times using PBS and 100 µL of pNPP substrate solution (5 mg/mL) were added to each well. The optical density was read at 405 nm after 5 min.

The isotype of antibodies directed against PEG was assessed by replacing the polystyrene plate coated with capture antibodies aforementioned by plate coated with monoPEGylated Bovine serum albumin (BSA-PEG20k, Life diagnostics Inc, Chester, PA). The protocol described above was used to perform the rest of the assay using 120-fold dilutions of plasma.

Competitive ELISA: PEGylated nanoparticles, PEG-BSA or $mPEG_{10k}$ were incubated in diluted sensitized mice plasma sample (1000-fold) for 15 minutes at different final concentrations of PEG (44.6, 4.46 and 0.446 nM). The anti-PEG ELISA protocol was followed for the rest of the assay, as described above. Identical plasma samples without competing molecules were used as control (100%) to normalize optical density.

***In vivo* studies:** All animal studies were conducted using institutionally approved protocols at *Université Laval* (Canadian Council on Animal Care standards and Animal Research: Reporting *In Vivo* Experiments guidelines). Healthy animals were housed in a controlled environment (22 °C, 12h day/night cycle) with *ad libitum* access to food and water.

To induce the immune response, male mice (25-29 g) were intravenously injected with PBS, 500 µg of PEG-PLGA nanoparticles (5 mg/mL in PBS) or 100 µg of $mPEG_{5k}$ (1 mg/mL) by the subclavian vein, under isoflurane anesthesia (2.5%). To monitor the kinetic of immunoglobulin production (**Figure 1** and **Supplementary Figure 1**), 30-50 µL blood samples were collected by the saphenous vein in heparinized capillaries, before the injection, as well as 2, 5, 7, 9 and 11 days after. Plasma was separated by 10 min of centrifugation at 2,000 *rcf*, and frozen at – 80°C until further use.

The day before the pharmacokinetic experiments, 50 µL of blood was sampled from each mouse to assess the levels of anti-PEG IgM. For pharmacokinetics studies, 500 µg of [^{14}C]-labeled nanosystems (PEG-PLGA nanoparticles, liposomes, PEG-BSA or mPEG) were injected by the saphenous vein, as described above. Approximately 30-50 µL of blood was collected via the saphenous vein at various times (0.25, 0.5, 1, 2, 4 hours). Six hours post-injection, blood was collected by cardiac puncture for a terminal timepoint. Animals were euthanized by a cardiac perfusion with 3 mL of isotonic phosphate-buffered saline solution (prepared from GIBCO® DPBS 10x, ThermoFisher, Waltham, MA) and organs were collected. Biological samples were digested at 60°C (in Solvable®, Perkin Elmer, Waltham, MA), bleached with 30% hydrogen peroxide, and assessed by scintillation counting, using Hionic Fluor® (Perkin Elmer, Waltham, MA) as a scintillation cocktail.

In specified experiments (**Figure 5**), the complement cascade was depleted with two intraperitoneal injections of CVF (15 U/mouse, Quidel, San Diego, CA).

Calculation of Pharmacokinetic parameters: Pharmacokinetic parameters were calculated by non-compartmental analysis from the blood concentration (%ID per gram of blood) *versus* time. The area under the plasma concentration *vs*. time curve ($AUC_{0\text{-}6h}$) was calculated by the trapezoidal method, from 0 to 6 h. The elimination constant (k_e) was estimated by dividing the clearance (dose/$AUC_{0\text{-}inf}$) by Vd. The AUC to infinity ($AUC_{6h\text{-}inf}$) was extrapolated by dividing the last measured concentration (concentration at 6 h) by the slope of a semi-logarithmic regression of the concentrations of the last 3 timepoints.

Proteomics: The proteomics analysis was conducted to compare the protein corona on one type of nanoparticle (PEG-PLGA nanoparticles, 100 nm in diameter, 40 PEG chains per 100 nm^2) when incubated in the serum naive (IgM-) and in sensitized mice (IgM+). For the assay, 10 µL of nanoparticles (32 mg/mL) were incubated in 80µL of serum for 30 min at 37℃. Each sample was purified by 2 passages on a size exclusion chromatography column (Sephacryl S-400 HR column, 1 x 20 cm), using PBS as the mobile phase. The protein corona was precipitated with 2 volumes of cold acetonitrile and centrifuged for 30 min at 3,000 *rcf*. This method, previously published [7], was shown to precipitate all proteins in the pellet, while completely dissolving the polymer nanoparticles (**Supplementary figure 8**)

The pellet of proteins was dissolved in ammonium bicarbonate (50 mM NH_4HCO_3 with 1% sodium deoxycholate), and the concentration of proteins was assessed by the Bradford assay using a calibration

curve of BSA. All samples were diluted with 50 mM NH_4HCO_3 to obtain the same concentration of protein prior to LC-MS analysis. LC-MS analysis was conducted by the Proteomics Platform of the CHU de Québec Research Center (Quebec, Qc, Canada), as thoroughly described in the supplementary method section.

To obtain the total number of the LC-MS/MS spectra for all peptides that are attributed to a matched protein, a semi-quantitative assessment of the protein amount was conducted through the application of a spectral counting (SpC) method. The normalized SpC (NpSpC) amounts of each protein, identified in the LC-MS/MS spectra, were calculated by applying the following equation:

$$NpSpCk = \left(\frac{\left({SpC}/{(M_w)_k}\right)}{\sum_{t=1}^{n}\left({SpC}/{(M_w)_t}\right)}\right) \times 100$$

Where NpSpCk is the normalized percentage of spectral count for protein k, SpC is the spectral count identified, and Mw is the molecular weight (in kDa) of the protein k.

***In vitro* complement activation:** For *in vitro* complement activation, 40 µL of freshly-collected Balb/c mouse plasma were activated with either 20 µL of sample, for 1 h at 37 °C. Samples tested were 5 mg/mL of PEG-PLGA nanoparticles or PEG-BSA, while negative control was isotonic phosphate buffer saline (prepared from Gibco® DPBS 10x, ThermoFisher, Waltham, MA), and positive control was Zymosan A (5 mg/mL, Sigma, St-Louis, MO). The reaction was stopped by the addition of 40 µL of a solution of 25 mM ethylene diamine tetra acetic acid, and frozen at -80 °C until dosing by ELISA.

The production of complement C5a protein was measured by ELISA (C5a Mouse ELISA kit, ab193718, Abcam, Cambridge, MA,). According to manufacturer's protocol, 100 µL of a 1,000-fold dilution of plasma was added to ELISA plates and incubated at room temperature for 1 h with gentle orbital shaking. After discarding their content, wells were washed 4 times with 350 µl of washing buffer solution. A freshly prepared solution of a biotinylated complement C5a detection antibody (100 µL) was added to each well. The plate was incubated for 1 h at room temperature with gentle shaking. Each well was washed again 4 times, and 100 µL of a freshly-prepared solution of streptavidin coupled to horseradish peroxidase was incubated for 45 minutes at room temperature with gentle shaking. The solution was discarded. After washing, 100 µL of TMB One-Step Substrate Reagent was incubated for 30 minutes at room temperature with gentle shaking in the dark. The reaction was stopped by adding 50 µL of stop solution, and the optical density was read at 450 nm (Tecan infinite 200 pro, Switzerland).

Statistics: Statistics were computed with GraphPad Prism 7. Differences in group means were calculated by standard unpaired t-test or Mann-Whitney test (non-parametric) when samples failed equality of variance or normality statistical tests. Data in **figure 6** was analyzed by a one-parameter ANOVA, using Tukey as a *post-hoc* test. A value of $p < 0.05$ was considered significant.

Acknowledgments: This work was funded by the Natural Sciences and Engineering Research Council (NSERC) of Canada, and the Canadian Funds for Innovation (CFI), as well as funds obtained from the Fondation du CHU de Quebec. PG acknowledges a scholarship from the *Fondation du CHU de Quebec-Desjardins*, and the *Fonds d'Enseignement et de Recherche* of the Faculty of Pharmacy of Laval University. IMOV acknowledges a scholarship from the Doctoral Program Abroad / *CAPES Foundation within Ministry of Education of Brazil* / Process n. 88881.132236/2016-01. Dr S Bourassa, from the proteomics core of the CHU de Quebec Research Center, is acknowledged for her help with the proteomics experiments.

Conflict of Interest: The authors declare no conflicts of interest.

Contributions of authors: N.B and E.M.L. obtained funding and supervised the research. N.B designed the experiments. P.G, I.M.O.V and N.B conducted the experiments and analyzed the data. P.G and N.B wrote the paper. All authors discussed the progress of research and reviewed the manuscript.

Reference.

[1] M.P. Monopoli, C. Aberg, A. Salvati, K.A. Dawson, Biomolecular coronas provide the biological identity of nanosized materials, Nat Nanotechnol, 7 (2012) 779-786.
[2] N. Bertrand, J.C. Leroux, The journey of a drug carrier in the body: an anatomo-physiological perspective, J Control Release, 161 (2012) 152-163.
[3] P.C. Ke, S. Lin, W.J. Parak, T.P. Davis, F. Caruso, A decade of the protein corona, ACS Nano, (2017).
[4] C.D. Walkey, J.B. Olsen, H. Guo, A. Emili, W.C.W. Chan, Nanoparticle Size and Surface Chemistry Determine Serum Protein Adsorption and Macrophage Uptake, J Am Chem Soc, 134 (2012) 2139-2147.
[5] S. Schöttler, G. Becker, S. Winzen, T. Steinbach, K. Mohr, K. Landfester, V. Mailänder, F.R. Wurm, Protein adsorption is required for stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated nanocarriers, Nat Nanotechnol, 11 (2016) 372-377.
[6] A. Salvati, A.S. Pitek, M.P. Monopoli, K. Prapainop, F.B. Bombelli, D.R. Hristov, P.M. Kelly, C. Aberg, E. Mahon, K.A. Dawson, Transferrin-functionalized nanoparticles lose their targeting capabilities when a biomolecule corona adsorbs on the surface, Nat Nanotechnol, 8 (2013) 137-143.
[7] N. Bertrand, P. Grenier, M. Mahmoudi, E.M. Lima, E.A. Appel, F. Dormont, J.-M. Lim, R. Karnik, R. Langer, O. Farokhzad, Mechanistic understanding of in vivo protein corona formation on polymeric nanoparticles and impact on pharmacokinetics, Nat Commun, 8 (2017) 777.
[8] V.C.F. Mosqueira, P. Legrand, J.-L. Morgat, M. Vert, E. Mysiakine, R. Gref, J.-P. Devissaguet, G. Barrat, Biodistribution of long-circulating PEG-grafted nanocapsules in mice: effect of PEG chain length and density, Pharm Res, 18 (2001) 1411-1419.
[9] R. Gref, Y. Minamitake, M.T. Peracchia, V. Trubetskoy, V.P. Torchilin, R. Langer, Biodegradable long-circulating polymeric nanospheres, Science (Washington, D. C., 1883-), 263 (1994) 1600-1602.
[10] S.D. Perrault, C. Walkey, T. Jennings, H.C. Fischer, W.C. Chan, Mediating tumor targeting efficiency of nanoparticles through design, Nano Lett, 9 (2009) 1909-1915.
[11] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo, Biochim Biophys Acta, 1066 (1991) 29-36.
[12] M.J. Harris, R.B. Chess, Effect of Pegylation of Pharmaceuticals, Nat Rev Drug Discov, 2 (2003) 214-221.
[13] M.A. Gauthier, H.-A. Klok, Polymer-protein conjugates: an enzymatic activity perspective, Polymer Chem, 1 (2010) 1352-1373.
[14] M. Hamidi, A. Azadi, P. Rafiei, Pharmacokinetic consequences of pegylation, Drug Delivery, 13 (2006) 399-409.
[15] M.T. Peracchia, C. Vauthier, C. Passirani, P. Couvreur, D. Labarre, Complement consumption by poly(ethylene glycol) in different conformations chemically coupled to poly(isobutyl 2-cyanoacrylate) nanoparticles, Life Sci, 61 (1997) 749-761.
[16] Q. He, J. Zhang, J. Shi, Z. Zhu, L. Zhang, W. Bu, L. Guo, Y. Chen, The effect of PEGylation of mesoporous silica nanoparticles on nonspecific binding of serum proteins and cellular responses, Biomaterials, 31 (2010) 1085-1092.
[17] Z. Liu, C. Davis, W. Cai, L. He, X. Chen, H. Dai, Circulation and long-term fate of functionalized, biocompatible single-walled carbon nanotubes in mice probed by Raman spectroscopy, Proc Natl Acad Sci USA, 105 (2008) 1410-1415.

[18] A. Abuchowski, J.R. McCoy, N.C. Palczuk, T. Van Es, F.F. Davis, Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase, J Biol Chem, 252 (1977) 3582-3586.
[19] R.P. Garay, R. El-Gewely, J.K. Armstrong, G. Garratty, P. Richette, Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents, Expert Opin Drug Deliv, 9 (2012) 1319-1323.
[20] Q. Yang, T.M. Jacobs, J.D. McCallen, D.T. Moore, J.T. Huckaby, J.N. Edelstein, S.K. Lai, Analysis of Pre-existing IgG and IgM Antibodies against Polyethylene Glycol (PEG) in the General Population, Anal Chem, 88 (2016) 11804-11812.
[21] C. Lubich, P. Allacher, M. de la Rosa, A. Bauer, T. Prenninger, F.M. Horling, J. Siekmann, J. Oldenburg, F. Scheiflinger, B.M. Reipert, The Mystery of Antibodies Against Polyethylene Glycol (PEG) - What do we Know?, Pharm Res, 33 (2016) 2239-2249.
[22] B.-M. Chen, Y.-C. Su, C.-J. Chang, P.-A. Burnouf, K.-H. Chuang, C.-H. Chen, T.-L. Cheng, Y.-T. Chen, J.-Y. Wu, S.R. Roffler, Measurement of pre-existing IgG and IgM antibodies against polyethylene glycol in healthy individuals, Anal Chem, 88 (2016) 10661-10666.
[23] C.-J. Chang, C.-H. Chen, B.-M. Chen, Y.-C. Su, Y.-T. Chen, M.S. Hershfield, M.-T.M. Lee, T.-L. Cheng, Y.-T. Chen, S.R. Roffler, J.-Y. Wu, A genome-wide association study identifies a novel susceptibility locus for the immunogenicity of polyethylene glycol, Nat Commun, 8 (2017) 522.
[24] T. Ishida, R. Maeda, M. Ichihara, K. Irimura, H. Kiwada, Accelerated blood clearance of PEGylated liposomes in rats after repeated injections, J Control Release, 88 (2003) 35-42.
[25] E.T.M. Dams, P. Laverman, W.J.G. Oyen, G. Storm, G.L. Scherphof, J.W.M. Van der Meer, F.H.M. Corstens, O.C. Boerman, Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes, J Pharm Exp Ther, 292 (2000) 1071-1079.
[26] H. Koide, T. Asai, K. Hatanaka, S. Akai, T. Ishii, E. Kenjo, T. Ishida, H. Kiwada, H. Tsukada, N. Oku, T cell-independent B cell response is responsible for ABC phenomenon induced by repeated injection of PEGylated liposomes, Int J Pharm, 392 (2010) 218-233.
[27] T. Ishida, M. Ichihara, X.Y. Wang, H. Kiwada, Spleen plays an important role in the induction of accelerated blood clearance of PEGylated liposomes, J Control Release, 115 (2006) 243-250.
[28] T. Ishida, X.Y. Wang, T. Shimizu, K. Nawata, H. Kiwada, PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner, J Control Release, 122 (2007) 349-355.
[29] R. Saadati, S. Dadashzadeh, Z. Abbasian, H. Soleimanjahi, Accelerated Blood Clearance of PEGylated PLGA Nanoparticles Following Repeated Injections: Effects of Polymer Dose, PEG Coating, and Encapsulated Anticancer Drug, Pharm Res, 30 (2013) 985-995.
[30] T. Tagami, K. Nakamura, T. Shimizu, N. Yamazaki, T. Ishida, H. Kiwada, CpG motifs in pDNA-sequence increase anti-PEG IgM production iduced by PEG-coated pDNA-lipoplexes, J Control Release, 142 (2010) 160-166.
[31] M.D. McSweeney, Z.C. Versfeld, D.M. Carpenter, S.K. Lai, Physician Awareness of Immune Responses to Polyethylene Glycol-Drug Conjugates, Clinical and Translational Science, 11 (2018) 162-165.
[32] J.K. Armstrong, G. Hempel, S. Koling, L.S. Chan, T. Fisher, H.J. Meiselman, G. Garratty, Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients, Cancer, 110 (2007) 103-111.
[33] N.J. Ganson, S.J. Kelly, E. Scarlett, J.S. Sundy, M.S. Hershfield, Control of hyperuricemia in subjects with refractory gout, and induction of antibody against poly(ethylene glycol) (PEG), in a phase I trial of subcutaneous PEGylated urate oxidase, Arthritis Research & Therapy, 8 (2005) R12.
[34] N.J. Ganson, T.J. Povsic, B.A. Sullenger, J.H. Alexander, S.L. Zelenkofske, J.M. Sailstad, C.P. Rusconi, M.S. Hershfield, Pre-existing anti–polyethylene glycol antibody linked to first-exposure allergic reactions to pegnivacogin, a PEGylated RNA aptamer, J. Allergy Clin Immunol, 137 (2016) 1610-1613.e1617.

[35] Q. Yang, S.K. Lai, Anti-PEG immunity: emergence, characteristics, and unaddressed questions, WIRES Nanomed Nanobiotechnol, 7 (2015) 655-677.
[36] A. Gabizon, R. Isacson, O. Rosengarten, D. Tzemach, H. Shmeeda, R. Sapir, An open-label study to evaluate dose and cycle dependence of the pharmacokinetics of pegylated liposomal doxorubicin, Cancer Chemother Pharmacol, 61 (2008) 695-702.
[37] T. Ishida, K. Atobe, X.Y. Wang, H. Kiwada, Accelerated blood clearance of PEGylated liposomes upon repeated injections: Effect of doxorubicin-encapsulation and high dose first injection, J Control Release, 115 (2006) 251-258.
[38] T.C. Chang, H.S. Shiah, C.H. Yang, K.H. Yeh, A.L. Cheng, B.N. Shen, Y.W. Wang, C.G. Yeh, N.J. Chiang, J.Y. Chang, L.T. Chen, Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients, Cancer Chemother Pharmacol, 75 (2015) 579-586.
[39] E.J. Feldman, J.E. Kolitz, J.M. Trang, B.D. Liboiron, C.E. Swenson, M.T. Chiarella, L.D. Mayer, A.C. Louie, J.E. Lancet, Pharmacokinetics of CPX-351; a nano-scale liposomal fixed molar ratio formulation of cytarabine:daunorubicin, in patients with advanced leukemia, Leukemia Research, 36 (2012) 1283-1289.
[40] S. Ashton, Y.H. Song, J. Nolan, E. Cadogan, J. Murray, R. Odedra, J. Foster, P.A. Hall, S. Low, P. Taylor, R. Ellston, U.M. Polanska, J. Wilson, C. Howes, A. Smith, R.J.A. Goodwin, J.G. Swales, N. Strittmatter, Z. Takáts, A. Nilsson, P. Andren, D. Trueman, M. Walker, C.L. Reimer, G. Troiano, D. Parsons, D. De Witt, M. Ashford, J. Hrkach, S. Zale, P.J. Jewsbury, S.T. Barry, Aurora kinase inhibitor nanoparticles target tumors with favorable therapeutic index in vivo, Sci Transl Med, 8 (2016) 325ra317-325ra317.
[41] X.Y. Wang, T. Ishida, H. Kiwada, Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes, J Control Release, 119 (2007) 236-244.
[42] T.M. Allen, C. Hansen, Pharmacokinetics of stealth versus conventional liposomes: effect of dose, Biochim Biophys Acta, 1068 (1991) 133-141.
[43] T. Yamaoka, Y. Tabata, Y. Ikada, Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice, J Pharm Sci, 83 (1994) 601-606.
[44] M. Mahmoudi, N. Bertrand, H. Zope, O.C. Farokhzad, Emerging understanding of the protein corona at the nano-bio interfaces, Nano Today, 11 (2016) 817-832.
[45] M.P. Monopoli, D. Walczyk, A. Campbell, G. Elia, I. Lynch, F. Baldelli Bombelli, K.A. Dawson, Physical−Chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles, J Am Chem Soc, 133 (2011) 2525-2534.
[46] M.R. Ehrenstein, C.A. Notley, The importance of natural IgM: scavenger, protector and regulator, Nat Rev Immunol, 10 (2010) 778-786.
[47] M.J. Walport, Advances in immunology: Complement: First of two parts, New Engl J Med, 344 (2001) 1058-1066.
[48] Y. Hashimoto, T. Shimizu, A.S. Abu Lila, T. Ishida, H. Kiwada, Relationship between the Concentration of Anti-polyethylene Glycol (PEG) Immunoglobulin M (IgM) and the Intensity of the Accelerated Blood Clearance (ABC) Phenomenon against PEGylated Liposomes in Mice, Biological and Pharmaceutical Bulletin, 38 (2015) 417-424.
[49] T. Shimizu, Y. Mima, Y. Hashimoto, M. Ukawa, H. Ando, H. Kiwada, T. Ishida, Anti-PEG IgM and complement system are required for the association of second doses of PEGylated liposomes with splenic marginal zone B cells, Immunobiology, 220 (2015) 1151-1160.
[50] M.A. Kock, B.E. Hew, H. Bammert, D.C. Fritzinger, C.-W. Vogel, Structure and Function of Recombinant Cobra Venom Factor, Journal of Biological Chemistry, 279 (2004) 30836-30843.
[51] F. Brandl, N. Bertrand, E.M. Lima, R. Langer, Nanoparticles with photoinduced precipitation for the extraction of pollutants from water and soil, Nat Commun, 6 (2015) 7765.

[52] H.T. Qian, A.R. Wohl, J.T. Crow, C.W. Macosko, T.R. Hoye, A Strategy for Control of "Random" Copolymerization of Lactide and Glycolide: Application to Synthesis of PEG-b-PLGA Block Polymers Having Narrow Dispersity, Macromolecules, 44 (2011) 7132-7140.
[53] J. Cheng, B.A. Teply, I. Sherifi, J. Sung, G. Luther, F.X. Gu, E. Levy-Nissenbaum, A.F. Radovic-Moreno, R. Langer, O.C. Farokhzad, Formulation of functionalized PLGA–PEG nanoparticles for in vivo targeted drug delivery, Biomaterials, 28 (2007) 869-876.
[54] N. Bertrand, P. Simard, J.-C. Leroux, Serum-stable, long-circulating, pH-sensitive PEGylated liposomes, in: G.G. D'souza (Ed.) Liposomes: methods and protocols, Humana Press, Clifton, NJ, 2017, pp. 193-207.