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Watching the Gorilla and Questioning Delivery Dogma

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1. Introduction

Numerous high-profile editorials in the *Journal of Controlled Release* and other journals have emphasized the need for drug delivery researchers to adopt new ways of thinking that consider the biological mechanisms of drug delivery [1–4]. These articles have clearly opined that the current thinking in the field of drug delivery tends to support pre-conceived notions (i.e., the invisible gorilla syndrome), and that progress requires critical examination of the status quo. In addition, it is pointed out that many researchers utilize over-engineered nanoparticles that are not commercially feasible, and ignore problems associated with immunogenicity and toxicity; an opinion with which we wholeheartedly and respectfully agree. In addition to these points, we feel the need to stress that there is an inherent conflict between the full complexity of biological systems and simple solutions to drug delivery. More specifically, we wish to “raise a flag against the majority opinion” and bring attention to the invisible gorilla associated with the technologies surrounding PEGylation and protein delivery [2, 4, 5]. Unfortunately, many in the drug delivery field believe that these technologies can be universally employed to overcome complicated and inconsistent biological barriers. While there is no denying that these technologies have been employed in products that are currently used in the clinic, a more critical analysis below suggests that these solutions are not so simple or robust.

2. PEGylation

2.1. Immunogenicity of PEGylated products

The conjugation of PEG to proteins by Frank Davis in the 1960s was shown to mitigate adverse immunological responses *to the foreign protein*, and many PEGylated proteins have been developed into clinical products. However, immune responses against the PEG portion of a PEGylated protein were reported as early as 1983 in mice, and 1984 in humans [6, 7]. The fact that most immunogenicity studies have only assessed immune responses to the active protein likely contributes to the lack of attention paid to PEG immunogenicity, but anti-PEG antibodies have been associated with several PEGylated protein products [8, 9]. Surprisingly, several clinical studies have reported that a significant fraction (25–44%) of patients test positive for anti-PEG antibodies *before treatment with PEGylated proteins* [9,

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10]. This has been attributed to the pervasive use of PEG in modern cosmetics and food products, but clearly represents a significant concern in the development of PEGylated proteins. Furthermore, 32–46% of patients developed anti-PEG antibodies in response to PEGylated asparaginase (Oncaspar®) administration [9], and antibodies to PEGylated uricase (Krystexxa®) cross-reacted with other PEGylated proteins, supporting the conclusion that antibodies were directed against the PEG portion of the conjugate. In addition, the high sensitivity reaction to PEGinesatide (Amontys®) that caused death of some patients upon the initial infusion has been linked to PEGylation, and led to the withdrawal of that product from the market in February 2013 [11]. Similarly, a recent clinical trial failure of a PEGylated aptamer has been linked to PEGylation and the presence of pre-existing anti-PEG antibodies [12, 13]. Accordingly, the U.S. Food and Drug Administration (FDA) recently updated their guidelines to screen for anti-PEG antibodies [8].

PEGylated liposomal doxorubicin (Doxil®, LipoDox® in the USA; Caelyx® in Europe) is one of the small number of clinically approved liposome formulations. These products induce complement-dependent and complement-independent hypersensitivity upon infusion in as many as 10% of patients, demonstrating that PEGylation does not effectively mitigate complement activation and hypersensitivity towards nanomedicines [14, 15]. Several studies showed potent complement activation by PEGylated liposomes, mainly through the alternative (antibody-independent) and classical (antibody-dependent) pathways [16]. Some studies demonstrated more efficient binding of complement factors and complement activation by PEGylated, as compared to non-PEGylated nanoparticles [17]. Taken together, the clinical results with PEGylated proteins, aptamers, and liposomes suggest that this simple strategy has limitations and can potentially augment immunogenicity and toxicity. Recent studies have shown that the complement response to PEGylated liposomes can *promote tumor vascularization and growth* [18, 19].

2.2. Functionality and toxicity of PEGylated formulations

PEGylation (also called “Stealth” technology) has been used to extend the circulation times of liposomal products and other nanoparticles [1, 2, 4, 20]. Although “Stealth” technology is thought to prolong circulation times by reducing interactions with serum proteins, studies on PEGylated liposomes recovered from the blood of mice suggest minimal effects on protein binding [21]. Actually, PEGylation has been shown to *increase* serum protein binding to some lipid/DNA complexes *in vitro*, and reduce gene delivery *in vivo* [22, 23]. Another important shortcoming of PEG is the apparent decrease of affinity of nanoparticle surface-tethered ligands due to the interference of the surrounding brush layer, unless significantly distanced apart from the main nanoparticle coat [24, 25].

In the clinic, PEGylated liposomal doxorubicin has been shown to possess equal efficacy but with reduced cardiotoxicity as compared to free doxorubicin [26]. Furthermore, PEGylated liposomal doxorubicin appeared to accumulate in tumors to a greater degree than free drug, but the absence of improved efficacy is attributed to the retention of doxorubicin within the PEGylated liposome [27]. At the same time, the prolonged circulation of the PEGylated liposomes (Doxil®) has been linked to dose-limiting skin toxicity due to liposomal accumulation in the skin [27–29], and a recent study showed a much higher skin

accumulation of PEGylated versus non-PEGylated liposomes [30]. In addition to PEGylated liposomes, other PEGylated nanoparticles showed significant skin accumulation [31]. It appears that PEGylation does not prevent non-specific interactions with the skin, suggesting limitations of the stealth properties. It is worth noting that liposomal doxorubicin is also marketed as a non-PEGylated formulation (Myocet®) that also exhibits altered pharmacokinetics and reduced cardiotoxicity, but lacks the skin toxicity associated with PEGylated liposomal doxorubicin.

The dose-limiting cardiotoxicity associated with free doxorubicin is greatly reduced with both liposomal formulations regardless of PEGylation, and the maximum tolerated dose for the PEGylated liposomal formulation is comparable to that for both the non-PEGylated liposomal formulation and the free drug [27]. Recently, the FDA approved a non-PEGylated anionic liposome for leukemia therapy [32]. This liposome shows high accumulation in the bone marrow but apparently lacks skin toxicity. Therefore, it appears that PEGylation technology is not the universal remedy to improve pharmacokinetics of nano-drugs, and that long-circulating properties come at the expense of reduced functionality and other forms of toxicity.

2.3. Is there a need for long circulation to achieve therapeutic goals of nanomedicines?

PEGylation is often justified by the need for extended circulation in plasma. Indeed, the idea that extended circulation times provide more opportunity for uptake by target tissues (tumors, and presumably other organs) due to increased area under the curve (AUC) is remarkably compelling. Only few tumor types, however, have demonstrated a time-dependent increase in particle accumulation in patients. Actually, some of the nanoparticle-mediated siRNA formulations showing promise in clinical trials are not long-circulating [33, 34]. Admittedly, those formulations are not designed for tumor delivery, where prolonged circulation is thought to enhance delivery to tumors via the Enhanced Permeation and Retention (EPR) effect [35]. However, the EPR effect has been called into question by numerous researchers, and its applicability to the treatment of human patients has been challenged [36]. In this respect, it is important to recognize that the two liposomal doxorubicin preparations marketed for the treatment of cancer (Doxil®, Myocet®) differ markedly in their circulation half-life, but exhibit comparable efficacy [36]. Another clinically approved liposomal product, Onivyde® (liposomal irinotecan) is non-PEGylated and is not designed to exploit the EPR effect [37]. As previously noted, prolonged circulation can also induce other toxicities that are not seen in formulations with short circulation half-lives [18, 19, 27, 38].

Despite extended circulation times, the limited quantity of receptors on the target cells may limit the ability of delivered liposomes and other nanoparticles to exhibit progressive accumulation in tumors [39]. At the same time, delivery of formulations to the target cells is restricted due to the limited ability of particles to diffuse into tumors against the interstitial fluid pressure gradient [40, 41]. Considering that prolonged circulation and EPR are the main reasons cited for employing PEGylation, the evidence challenging these effects should trigger more questions about the rationale for this ubiquitous strategy [5].

3. Protein Delivery Systems

The sustained, constant release of therapeutic peptides and proteins offers tremendous advantages in terms of efficacy and patient compliance [42]. Despite the consistent, decades-long effort to develop sustained release protein formulations, the syringe and the pump remain the only delivery systems currently marketed for protein delivery. This fact is not surprising to researchers that have struggled to maintain the native conformation of protein therapeutics during production, processing and storage. Many scientists in drug delivery, however, often approach encapsulation and release with no appreciation for the significant challenges involved in protein formulation. In addition to the loss of protein function associated with instability, the potential immunogenic consequences of protein unfolding/aggregation is underscored by patients required to receive life-long treatment for red cell aplasia after receiving recombinant erythropoietin [43–47].

3.1. Biodegradable polymer matrices

The development of hydrophobic polymers for sustained protein release in the 1970s was considered a major breakthrough that would allow for depot formulations of proteins [48]. When one reads reviews about this technology, the idea does indeed seem quite simple: proteins are encased within a polymer matrix, and are gradually released as the matrix degrades. However, this portrayal greatly oversimplifies the difficult task of maintaining native protein structures, complex mechanism of release, and ignores the significant effects of glass transition temperature, crystallization, collapse and polymer swelling/hydration that can profoundly influence release kinetics [49, 50]. For protein depot formulations, biodegradable polymers, e.g., poly(lactide-co-glycolide) (PLGA), are used because of their demonstrated biocompatibility and prior clinical use. It is now well established that the mechanism of polymer degradation can cause the matrix to acidify to pH as low as 2 [51]. This extreme acidification is similar to what is achieved in the stomach to promote digestion, and thus it should not be surprising that many organic molecules (e.g., small molecules, proteins, nucleic acids) cannot withstand prolonged exposure to such conditions [50–52]. Furthermore, it must be stressed that the interior of these polymer matrices are strongly hydrophobic, and thereby promotes protein unfolding and aggregation, especially under hydrated conditions and high protein concentration (discussed in more detail below).

The only product that utilized PLGA for the sustained release of a protein (recombinant human growth hormone) was marketed in 1999 (Nutropin Depot®), but withdrawn in 2004 [53, 54]. Pre-clinical studies in mice and primates indicated that the immunogenicity of the depot formulation was equivalent to non-encapsulated protein, but subsequent human studies revealed a much higher level of immunogenicity associated with protein *instability within the PLGA matrix* [55, 56]. This case study demonstrates how difficult it is to develop sustained-release protein delivery systems, yet numerous researchers and start-up companies continue to claim that this approach can be readily employed to deliver protein therapeutics. *There are no other marketed sustained release protein products that utilize the PLGA microparticle technology.* Similar to our conclusions regarding PEGylation technology, researchers need to understand the real-world limitations of biodegradable polymer

technology, especially as it pertains to the sustained release of biological macromolecules, before pursuing this approach to overcome delivery barriers.

3.2. Maintaining protein stability

Protein stabilization can be achieved by trapping proteins in their native conformation and by reducing molecular mobility. This can be accomplished by freezing and storage at cold temperatures, but successful cryopreservation of proteins requires optimization of many factors including freezing rate, thawing rate, and stabilizing excipients [57–59]. Alternatively, proteins can be immobilized to maintain their native structure at higher temperatures by dehydration/lyophilization [60–62]. Not surprisingly, the lyophilization of proteins is a science in and of itself, and the removal of water presents different stresses than that encountered by freezing [63–65]. This strategy was used by scientists at Alkermes where proteins were dried with stabilizing excipients prior to suspending powders in organic solvents at low temperature [66]. This very clever approach allowed dried proteins to remain immobilized and retain their conformation during processing. A similar approach was used to avoid shearing and preserve DNA structure during encapsulation [67]. We feel that the ingenuity of this breakthrough approach is greatly under-appreciated, and most researchers attempting to encapsulate proteins into sustained release formulations commonly utilize emulsification technology that exposes the protein to high surface area, hydrophobic solvents, and significant shear. It is critical to recognize that even if only native, active protein is incorporated in a dry matrix during preparation, exposure to water during reconstitution and/or within the body (after administration) causes swelling of the polymer matrix and protein rehydration. The resurrected mobility of the entrapped protein allows degradation (e.g., deamidation, aggregation, unfolding) to occur within the matrix prior to release.

More recent studies have clearly demonstrated the ability of protein aggregates to elicit immune responses that have been linked to the diminished effectiveness of protein therapeutics [68–74]. It is important to recognize that the UV absorbance measurements typically used to monitor release cannot distinguish between native, unfolded, and aggregated protein. Instead, direct measurements of specific activity (i.e., activity per mole of protein) and structural characterization (i.e., with Fourier Transform infrared spectroscopy, circular dichroism, analytical ultracentrifugation, and/or NMR) would be needed to demonstrate that released protein is fully native and functional - analyses that are rarely performed in drug delivery studies. More typically, in the few cases where activity of the released protein is measured, the presence of any activity is touted as evidence of successful encapsulation. However, it must be appreciated that an immune response only requires a small fraction of the protein to be aggregated/unfolded, and thus successful delivery systems will need to demonstrate that virtually all encapsulated protein is released and functional – a feat that is almost insurmountable [73, 75, 76]. While the methods developed by scientists at Alkermes that enabled stable protein encapsulation into PLGA matrices were indeed revolutionary, Nutropin Depot® failed despite the significant resources dedicated to manufacturing and commercialization [53, 77]. Indeed, the ease with which protein structure can be disrupted by surfaces (e.g., the air-water interface), heat, adsorption, mechanical shock, freezing and exposure to organic solvents must be more fully appreciated.

Furthermore, it should be understood that instability is a fundamental barrier to the incorporation of proteins (e.g., interferons and growth factors) into any material (e.g., drug delivery systems, hydrogels for tissue engineering) [43–49]. Practically speaking, the significant and fundamental differences between small molecule therapeutics and proteins in terms of chemical stability, potential for immunogenicity, aggregation, and structural perturbation impose a formidable barrier to the development of protein delivery systems [41, 42].

3. Conclusions

We completely agree with the opinions expressed in prior editorials regarding the need for drug delivery scientists to better understand biological mechanisms and focus on simple solutions that have a realistic potential for commercialization. This perspective attempts to bring attention to the limitations of approaches that are widely viewed as successful, breakthrough technologies. While we do not intend to diminish the significant accomplishments involved in the development of PEGylation, biodegradable polymer matrices, and protein delivery systems, we feel it is important to encourage drug delivery scientists to escape from the current dogma and to think “out of the box” and explore new strategies that further improve safety and efficacy.

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