Review Article

LIMITATIONS OF PEGYLATED NANOCARRIERS: *UNFAVOURABLE PHYSICOCHEMICAL PROPERTIES, BIODISTRIBUTION PATTERNS AND CELLULAR AND SUBCELLULAR FATES*

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ABSTRACT

Assuming that polyethylene glycol (PEG)-conjugated or PEGylated nanocarriers always offer outstanding physicochemical properties and pharmacokinetics profiles when compared to non-PEGylated ones, is not always accurate. For example, drug-loaded PEGylated nanocarriers for the treatment of cancer will not magically escape the reticuloendothelial system (RES) sequestration and clearance, benefit from the enhanced permeability and retention (EPR) effect of the tumor leaky vasculature and preferentially accumulate in the target tissue or cells. This is too good to be true. In this review, several drawbacks of PEGylation will be discussed; for example, how PEGylation can give rise to unfavourable physicochemical characteristics (e. g. particle size and release patterns) and post *in vivo* administration limitations of the formulated nanocarriers (e. g. limited evasion of RES uptake, development of hypersensitivity reactions, reduced intracellular accumulation and interference with the subcellular processing of nanocarriers necessary to produce the intended pharmacological effect).

This review aims at providing better understanding of the pros and cons of PEGylation, encouraging the use of PEGylation with caution, avoiding the assumption that PEGylation will provide all advantages needed to deliver nanocarriers to the target tissue and looking for alternatives to optimize nanocarriers' utilization especially in the delivery of chemotherapeutic agents for the treatment of different types of cancer. This review comprises a summary of some of the reported literature between 2013 and 2018 using different search engines; PubMed, Science Direct and Google Scholar, and the keywords listed below.

Keywords: PEG, PEGylation, Nanoparticles, Nanovesicles, Drawbacks, Limitations, Alternatives

INTRODUCTION

PEGylation, or the use of PEG, is one of the most common approaches utilized for improving nanocarriers' e. g. nanoparticles (NPs') or nanovesicles' (NVs') properties for *in vivo* administration. PEG, as a hydrophilic biocompatible stealth polymer, can decrease NPs sequestration by the RES organs (liver, spleen, kidneys). It prevents adsorption of proteins (opsonins) that tag the NPs or NVs to be engulfed by phagocytic cells in a process named opsonisation by providing steric hindrance preventing their interaction with macrophages necessary for the process of phagocytosis [1–5].

A closer insight to the mechanism by which PEG extends circulation half-life revealed that PEG could preferentially bind to other proteins that function as dysopsonins, which are proteins capable of preventing opsonisation or other protein fragments in place of opsonins. In one study, PEGylated lipoplexes have shown the higher extent of serum protein (but not opsonins) binding compared to non-PEGylated lipoplexes [6].

Also, PEG is a non-ionic (neutral) polymer that can mask surface charge and develop a near-zero zeta potential which eventually reduces non-specific interaction with macrophages and favour long circulation time in blood [7–10]. While highly charged, positive or negative, or hydrophobic NPs were found to attract complement proteins and be highly sequestered by RES organs. Positively charged NPs have even showed the highest extent of sequestration by RES organs; where they preferentially interact with negatively charged plasma proteins leading to higher RES uptake [9, 11].

Therefore, PEG can increase blood circulation time and reduce sequestration in the organs of the RES making NPs more available for uptake by their target organs or tissues e. g. tumor tissues [1–4, 12]. In other words, long-circulating nanocarriers have higher chance to benefit from the EPR promoted by the tumor leaky vasculature [4, 13–15].

Another interesting advantage for the PEGylated nanocarriers is their high ability to cross the blood-brain barrier (BBB) and accumulate in brain tissue more effectively than non-PEGylated ones possibly by transcytosis or receptor-mediated endocytosis mechanisms which need further to be elaborated. Diffusion through the brain parenchyma has also turned out to be a function of PEG coating density; i.e. the higher the PEG coating density, the higher the brain parenchymal diffusion [4, 16–19]. In this regard, PEGylated nanocarriers have shown enhanced accumulation in pathological conditions characterized by BBB hyperpermeability e. g. gliosarcoma and multiple sclerosis (MS)-induced inflammation and brain tumors-induced EPR effect [16].

For these advantages, PEG has been regarded as a gold standard polymer in various applications and could not be neglected; however, on the other hand, many drawbacks have been reported over the years that could limit its applications (fig. 1);

Major hurdles of PEGylation

PEGylation that has been widely used to improve the physical and chemical properties of nanocarriers showed unfavourable profiles of an abrupt release of the loaded cargos before or right after *in vivo* administration, even before NPs sufficiently accumulate in target tissues.

Also, particle size enlargement has been observed on comparison of PEGylated and non-PEGylated nanocarriers which produced a reversed effect on the intended RES evasion and accumulation in tumor tissue by the EPR effect [6].

Deeper analysis of the effect of PEGylation on the cellular and subcellular fate showed reduced uptake and interference with NPs processing which could hinder the optimized pharmacological effect of the loaded drug [13]. Also, there has been evidence of the occurrence of PEG-containing vacuoles in cells. Reports so far also show the potential risk of PEG itself being a non-biodegradable polymer that can produce an immune response or cause hypersensitivity where PEG could generate antibodies that can neutralize their efficacy upon repeated administration [6].

Also, PEG synthesis and purification are difficult processes that give rise to an inherent polydispersity of the polymer which eventually causes batch-to-batch variability [20].
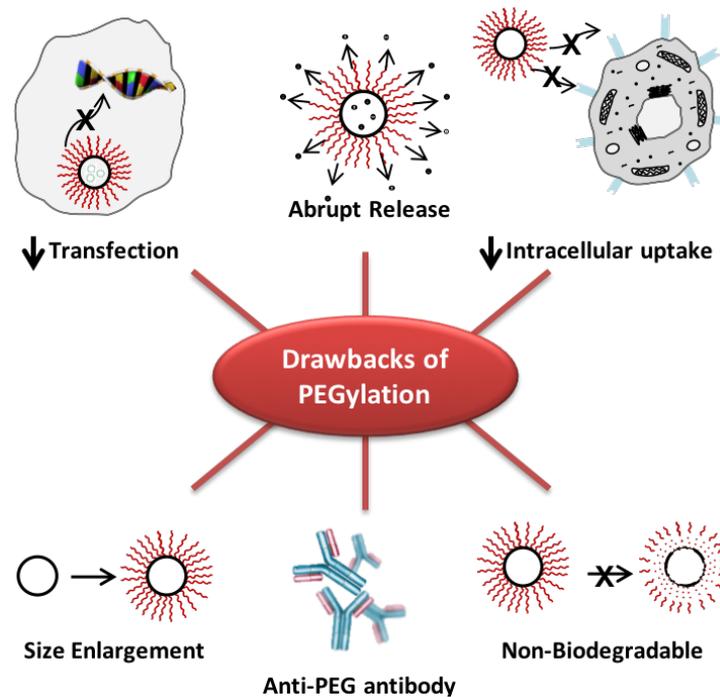
Fig. 1: Main drawbacks of PEGylation of nanocarriers

Unfavorable physicochemical properties of PEGylated nanocarriers

PEGylation was reported to affect the physicochemical properties of NPs adversely causing enlargement of particle size-that could enhance RES uptake especially with large molecular weight PEG-and abrupt release of the drug loads from NPs over a short period of time. For example, PEGylated liposomes loaded with doxorubicin have shown a faster release of drug cargos than the non-PEGylated counterparts, where 90% of the loaded doxorubicin was released in 3 h post intravenous (IV) administration while the emptied liposomes were still circulating in blood before reaching the target site. This made the assumption that the loaded drug cargo is released after being up-taken by target cells not accurate. Similarly, the release of morphine from poly (L-lactide) or PLA-PEG block copolymer particles have shown to be much faster than release from PLA polymer ones. Also, the release rate has been shown to be a function of PEG percentage (%) in the copolymer i.e. the initial burst release is more evident in particles with PEG content of 5% compared to 3%. While particles containing 0-1% of PEG showed no burst release, but rather a continuous sustained release over time. Upon estimating the $T_{80\%}$, which represents the time when the cumulative release percentage of morphine reached about 80%, PEG-free particles showed around 15 and 30 times longer $T_{80\%}$ than particles containing 3 and 5% of PEG respectively. This reflects that loaded cargos could be released from PEG-containing particles before degradation of the polymer. In other words, particles could become empty of the loaded cargos while they are still in circulation before their degradation. Moreover, a similar trend was reported for PEGylated and non-PEGylated poly (lactic-co-glycolic acid) (PLGA) and PLA NPs. Faster release profiles of hydrophilic drugs loaded in PEGylated NPs could be attributed to the hydrophilicity of PEG chains at the surface of the nanocarriers which act to attract water resulting in pronounced wetting/hydration of PEGylated NPs [9, 10, 21, 22].

RES evasion capacity not as expected or even worsened and reversed

As reported earlier in the advantages of PEGylation, the hydrophilic polymer provides steric hindrance and masking of surface charge that prevents adsorption of tagging proteins (opsonisation) which is a prerequisite of uptake by RES macrophages; however, PEGylation capacity of prevention of opsonisation has shown to be limited, and

eventual opsonisation and RES clearance has been repeatedly reported. In one study, for example, temoporfin (photosensitizer used in photodynamic cancer therapy) was loaded in both PEGylated and non-PEGylated PLGA NPs and followed for *in vivo* behaviour. It has been reported that similar amounts of both NPs accumulated in the spleen and that the RES tissue distribution differed only slightly between PEGylated and non-PEGylated NPs showing inefficiency of PEGylation in RES evasion [23].

In another study, PEGylation of thiolated gelatin was observed to reduce mean residence time (MRT) and half-life of NPs in blood. In other words, the majority of NPs were cleared from the blood by circulating phagocytes and tissue macrophages (hepatic kupffer cells and macrophages in the spleen) and the NPs eventually accumulated in the liver and spleen [10]. A similar trend was reported for PEGylated and albumin-conjugated PEGylated liposomes, where results showed that albumin conjugation produced the longest circulation time [24]. Many factors control the effect of PEGylation on NPs' fate; these include the molecular weight, PEG polymer identity, composition and hydrophobicity, density and stability of the PEG in the coating layer, NP core properties, and repeated administration. These criteria if not properly controlled lead to unfavourable effects upon PEG coating [6, 13, 25–27].

The limited RES evasion capacity by PEGylation has been attributed to many phenomena; most importantly, size enlargement of nanocarriers, desorption of PEG from nanocarriers' surfaces and production of anti-PEG antibodies and development of an immune response that causes accelerated blood clearance (ABC) of PEGylated nanocarriers. These are explained in detail as follows;

PEGylation causes enlargement of particle size

Formulations possessing average particle size ≤ 150-200 nm were reported to be suitable for IV application [7, 28, 29]; being large enough to avoid filtration in the kidney, and small enough to avoid sequestration in the RES organs (liver, spleen, kidneys), and suitable for selective accumulation and retention in tumor by EPR effect [30–34].

In one study, PEGylation of PLGA NPs led to a larger particle size that had an effect on the pharmacokinetics of NPs and accounted for the abnormal decrease in the MRT of NPs in blood [10]. This means evasion of RES by PEG is not absolute. Larger size conferred by PEG

layer could make them more accessible to RES organs than the smaller non-PEGylated PLGA NPs [9].

In another study, increasing the % of PEG in PLGA NPs from 0% to 9% to 15% increased particle size from ~740 nm to 1170 nm to 1995 nm respectively [35]. A similar observation was recorded in chitosan NPs; where PEGylated chitosan NPs possessed larger particle size (~290 nm) than non-PEGylated ones (~200 nm) [36]. Also, PEG coating of nanostructured lipid carriers (NLCs) led to size enlargement from (~72 nm) to (~92 nm) [37].

Insufficiency or desorption of PEG layer

As reported earlier in the advantages of PEGylation, the hydrophilic polymer provides steric hindrance that prevents adsorption of tagging proteins (opsonisation) which is a prerequisite of uptake by RES macrophages; however, it has also been observed that PEG polymers could also desorb, leaving holes in surface coverage where opsonins can bind [38] or not provide full prevention of opsonisation at low density of PEG or using low molecular weight polymer. For example, with 5 nm gold NPs, the protective effect of PEG diminishes within hours [39].

Immunogenicity of PEG

PEG, the long-believed polymer to be non-antigenic could develop an immune response resulting in ABC of PEG-modified materials e. g. PEGylated proteins, liposomes, micelles, and biodegradable and non-biodegradable nanocarriers. Where an anti-PEG antibody could be formed that results in the faster elimination of the second or further IV injected dose in animal studies and clinical settings [39]. This has been clarified by the formation of immune complexes which can be rapidly bound and cleared by macrophages via Fc receptor-mediated phagocytosis [40].

The ABC phenomenon is believed to result from anti-PEG IgM antibody produced by the spleen in response to a previously-administered dose. This type of antibody is, however, unable to solely promote sequestration by RES macrophages. However, these antibodies were hypothesized to bind to the second injected dose of PEG conjugates and form a complex with other serum components eventually promoting sequestration by RES [6, 13]. For instance, the second injection of PEGylated liposomes witnessed reduced circulation time and increased hepatic and splenic accumulation in comparison to the first injected dose of the same formulation in the same animal [6]. Moreover, anti-PEG antibodies of both IgG and IgM isotypes were found in 25% of 250 healthy blood donors. This extraordinary evidence of anti-PEG antibodies was explained by the increasing exposure of the general population to PEG in different cosmeceutical and pharmaceutical products for many years [41]. Other reports show the incidence of anti-PEG antibodies in the range of 0.4% to 36% in naïve individuals [40].

In another study, a more severe adverse effect to the immunogenicity of PEG, hypersensitivity, has been observed clinically where three cases (0.002% of the treated patients with advanced coronary artery disease) administered a commercial PEG-containing contrast agent, SonoVue, developed life-threatening anaphylactic shock [6, 15, 42].

The loss of activity of PEGylated pharmaceutical products upon repeated injection has also extended to anticancer therapeutics. PEGylated asparaginase is an integral part of therapy for acute lymphoblastic leukaemia (ALL) that is administered either intravenously (IV) or intramuscularly (IM). In a study, 46% of patients were reported to develop anti-PEG antibodies in response to IV administration that resulted in accelerated clearance of subsequent doses eventually causing silent inactivation of the treatment. In another study, 25% of patients were identified with pre-existing anti-PEG antibodies prior to treatment that could be attributed to their pre-exposure to PEG from other pharmaceutical or cosmeceutical products that are widely available. Another study showed the development of anti-PEG antibodies after IM administration of the PEGylated asparaginase [13, 43, 44].

Therefore, it could be concluded that PEG effect does not last for a long time, and eventually, opsonisation and clearance by RES organs still occur [45].

The prevalence of anti-PEG antibodies has shown to be higher in females than males; this could be explained by their higher exposure to PEG-containing products e. g. skin lotions, deodorant sticks, hair care products and face makeup products or their higher tendencies to have autoimmune responses [40].

Unfavourable cellular and subcellular fate

Another major drawback of PEGylation is the reduced intracellular uptake of the PEGylated NPs in comparison to non-PEGylated ones due to the steric hindrance caused by the projecting chains of PEG polymers and the hydrophilicity of NPs' surfaces as well. In other words, neutral and hydrophilic NPs e. g. PEG-modified NPs show slower and lower internalization in target cells than both of positively and negatively charged ones. For example, intracellular uptake of PEGylated NPs was noted to drop to one-third of the uptake of non-PEGylated ones [46, 47]. Also, PEGylation of folic acid (FA)-targeted dendrimers has been also reported decreasing the tumour-specific targeting by FA [20].

Therefore, even that PEGylation could sometimes evade sequestration of drug-loaded nanocarriers by RES cells and increase the probability of extravasation of NPs through the tumor leaky vasculature, it reduces interaction with target tissues e. g. tumor cells, whereby PEG shields internalization by non-immune cells by both steric hindrance and masking of NPs surface charge required for optimum cellular uptake [13].

In a similar manner, the interesting properties of PEG to enhance BBB crossing encouraged the application of PEGylated nanocarriers for brain delivery; however, it should not be neglected that there is another barrier that could hinder the successful delivery of therapeutic agents, which is the microglial cells that constitute the immune component of the central nervous system (CNS) itself. These cells are characterised by high phagocytic activity that resembles RES macrophages that could compete with other non-immune neural cell types for NPs' uptake. Jenkis et al., 2016 have been concerned with comparison of NPs' uptake in their PEGylated and non-PEGylated forms in different neural cell types; their findings confirmed that PEGylation reduces NPs' uptake in all cell types; immune and non-immune neural cells suggesting that PEGylation evades non-specific cell uptake that could outweigh its advantage of BBB and parenchymal diffusion when applied for brain drug delivery [16].

The effect of PEGylation on the target cells is not limited to reduced uptake but extends to inhibition of endosomal escape, which results in significant loss of activity of the delivery systems e. g. gene and nucleic acid delivery systems for cancer treatment where the NPs' cargos are degraded in the digestive acidic medium of the endolysosomal system [41].

The detrimental effect of PEGylation extends also to the interference with the intracellular processing of NPs required for optimum release of loaded cargos to achieve the intended goals. For example, PEGylated nanocarriers intended for gene and siRNA delivery have shown reduced transfection and silencing rates that could not be explained solely by the previously discussed reduced uptake caused by PEGylation. Therefore, it was concluded that PEGylation affects the intracellular fate of nanocarriers after being internalized by target cells. In different studies, PEGylation levels as low as 0.4-0.5% showed a drastic decrease in transfection rates indicating that the therapeutic gene expression in the target cells may be significantly compromised by PEGylation [13].

Non-biodegradability of PEG polymers

Despite that PEG has been the gold standard in pharmaceuticals' application for many years possessing the longest clinical track record amongst many other polymers and that it has been widely used as a biocompatible polymer, it should be noted that it is non-biodegradable and that its ultimate fate and potential side effects that could arise from its accumulation need further to be elaborated. Also, there are concerns about its bioaccumulation in lysosomes of healthy tissue. Therefore, limiting the molecular weight should be taken into account for *in vivo* application [13, 48–50].

Strategies to overcome PEGylation drawbacks

Proper selection of PEG polymer and conjugation chemistry

There are many available approaches to overcome the PEG dilemma (fig. 2), for example, choosing appropriate polymers with less or no antigenicity and controlling the different criteria that can affect PEGylation outcomes e. g. PEG molecular weight, density and stability of the PEG coating [6, 25, 27]. In one study, branched PEG was shown to be the least immunogenic and antigenic when compared to methoxy-PEG (mPEG) and other un-branched PEG forms [6]. Also, a more recent study by Shimizu *et al.* showed that

hydroxyl-PEG could also serve as a potential alternative to mPEG. It could attenuate the elicitation of the ABC phenomenon when conjugated to PEGylated liposomes by the production of less anti-PEG IgM antibodies [51]. In another report, the immunogenicity of different PEG derivatives showed the following order; t-butoxy-PEG (tBu-PEG)>mPEG>hydroxyl-PEG (OH-PEG). Also, two types of anti-PEG antibodies have been proposed; methoxy-specific and backbone-specific. Where OH-PEG was found to produce antibodies that have a similar affinity to both mPEG and OH-PEG, while mPEG antibodies recognize mPEG more effectively than OH-PEG [26].
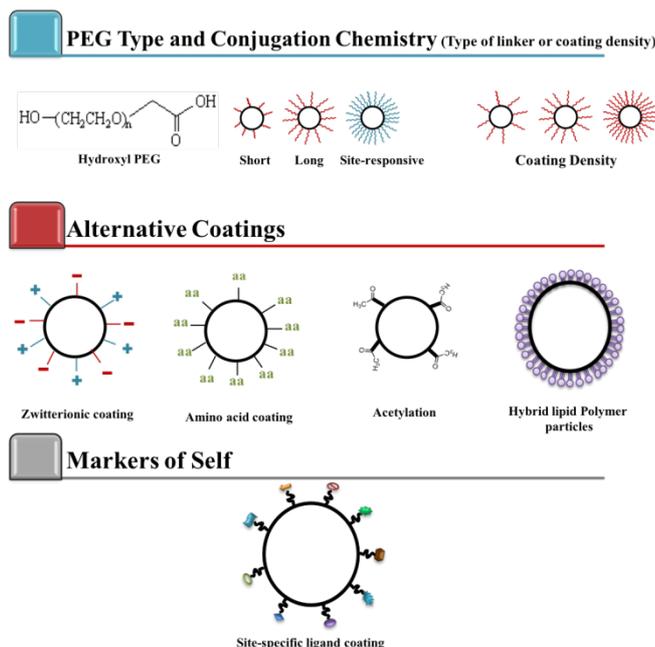

Fig. 2: Alternative strategies to overcome PEGylation drawbacks

Also, the issue of non-biodegradability of PEG could be addressed and solved; for example, by conjugating PEG to degradable PLA or poly (glycolic acid), (PGA) segments to form corresponding biodegradable copolymer [52, 53].

In the meanwhile, attempting to overcome the detrimental effect of PEGylation on gene and siRNA delivery, PEG has been conjugated to shorter lipid anchors to facilitate loaded cargos diffusion out of the lipid-based delivery systems. In another attempt, PEG was attached to site-specific cleavable linkers e. g. matrix metalloprotease (MMP)-sensitive linkers in tumors or pH sensitive linkers to be cleaved by the low pH conditions encountered after cellular uptake in the lysosomal degradation pathway [13].

Alternative coatings

In another approach to overcome PEGylation dilemma, alternative coatings of nanocarriers have been studied. For example, NPs with zwitterionic coatings were found to be able to evade brain immune components and reduce the rate of non-specific binding for proteins and lipids without steric hindrance [16].

Moreover, 5-nm gold NPs were coated with amino acids such as lysine or cysteine to constitute a mixed-charge monolayer. This coating could prevent protein adsorption in fetal bovine serum [39, 54].

Also, another study employed acetylation of dendrimers as an alternative to PEGylation. This approach offered the advantage of RES evasion without adding steric hindrance that could reduce the interaction between FA on the acetylated dendrimers and folate receptor on the surface of the target cells that could reduce the uptake of the NPs as in the case of FA-conjugated PEGylated dendrimers. The mechanism by which acetylation reduces RES clearance was hypothesized to occur by neutralizing the cationic

surface of the dendrimers and reducing the electrostatic interactions with serum proteins that are required for uptake by RES macrophages. Acetylation is also potentially applicable for modification of other types of nanocarriers and possesses an added advantage of better control over the size and polydispersity of the NP compared to PEGylation [20].

Utilization of hyperbranched polyglycerol (HPG) as an alternative to the typical PEG has shown to be promising in the brain drug delivery. HPG showed more favourable physicochemical properties e. g. higher drug loading, higher NPs' surface coverage and higher effectiveness for ligand conjugation and more prolonged circulation time [50,55]. HPG coating imparted excellent stability of magnetic Fe_3O_4 NPs in cell culture medium. These coated NPs were nontoxic to mammalian cells and showed minimal uptake by macrophages. HPG also offers a platform for staging the NPs surfaces for active targeting [49].

Other stealth polymers such as chitosan, poly (carboxybetaine), polysaccharides, polypeptides, poly (vinyl alcohol) and poly (2-oxaline) have been also investigated [25, 26].

Lipids also can be used as coatings for NPs to increase their blood circulation time as an alternative to PEG, producing hybrid lipid-polymer NPs. Hybrid NPs represent a promising drug delivery platform that has shown controllable particle size, surface functionality, high drug loading yield, triggered or stimuli-responsive drug release profile, excellent in vitro and in vivo stability, biocompatibility and biodegradability. Also, co-loading of drugs with different physicochemical properties became possible. In other words, the polymeric core functions to encapsulate either water or oil-soluble drugs and to provide robust structures, whereas the external lipid coat offers the following advantages; (i) acts as a biocompatible shield, (ii) a template for surface modifications, (iii) a

barrier preventing the fast leakage of water-soluble drugs (iv) possesses favourable stability in serum (longer circulation times) and (v) good cellular accumulation and targeting ability [33, 56–61]. In another study, it was observed that the incorporation of phospholipid into polymeric NPs could significantly enhance their intracellular accumulation [62].

Utilization of markers of self; target-specific ligands

In order to address the problem of reduced intracellular uptake by target cells upon PEGylation, markers of self or target-specific ligands e. g. FA or transferrin can be attached to the surface of the NPs in order to achieve active targeting (enhance cellular uptake into target tissues and to avoid exposure of healthy cells) and also help minimize or avoid complement activation [39]. Arginine-glycine-aspartic acid (RGD) tripeptide has also shown to improve the selectivity, binding and uptake of the NPs by targeted cells because of its ability to be recognized by integrin receptors that are ovreexpressed on tumor cells. This strategy can specifically enhance the limited uptake drawback due to steric hindrance by PEG polymers [41]. In another study, targeted PEGylated liposomes produced by conjugation to the fibronectin-mimetic peptide, PR_b, to target $\alpha_5\beta_1$ in metastatic colon cancer, have shown better transfection efficiency than non-targeted ones [5]. Further optimization of PEGylated liposomes was performed by dual targeting; in a study, PR_b and AG86 peptides were used to target $\alpha_5\beta_1$ and $\alpha_6\beta_4$ respectively were conjugated with different ratios to the PEGylated liposomes and investigated for their internalization and transfection efficiencies in dual receptor expressing cancer cells. Results showed high selectivity of the employed nanocarrier system especially in ovarian cancer cells (SK-OV-3) characterized by an almost equal expression of the two receptors [63].

The same targeting strategy could be applied to optimize the brain delivery of drugs in PEGylated nanocarriers. For example, PEGylated gold NPs, when functionalized with transferrin-specific peptide, showed significantly higher accumulation in brain tumor [64]. Also, PEGylated PLGA NPs or polymersomes when conjugated with phage display peptide or lactoferrin respectively showed higher brain accumulation [64, 65]. Dual targeting has also been employed to optimize brain drug delivery; where paclitaxcel was conjugated to cyclo-[Arg-Gly-Asp-D-Phe-Lys] peptide which can interact specifically with integrin receptors, overexpressed on glioma cells. This conjugate was further loaded into PEGylated lipid-polymer hybrid NPs [66].

CONCLUSION

In conclusion, PEG has shown, for decades, the outstanding potential for enhancement of properties of drug delivery systems that made its application in pharmaceuticals and cosmeceuticals widespread. However, improper control of the PEGylation process gave rise to many drawbacks that render its application limited. Also, chronic exposure to PEG for many years made the immune system of humans and animals alert to the molecule producing antibodies; that limit the efficacy of the administered drug in most of the times and could give rise to more serious side effects e. g. anaphylactic shock. Therefore, PEGylation should be used with great caution. Also, looking for alternatives has emerged to become an ultimate necessity.

AUTHORS CONTRIBUTIONS

All the work have been carried out by me

CONFLICT OF INTERESTS

The author reports no declarations of interest

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