Review

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Posted Date: 4 February 2025

doi: 10.20944/preprints202502.0191.v1

Keywords: PEG; nanoparticles; protein PEGylation; cancer; PEG density doi:10.20944/preprints202502.0191.v1

Review

The Art of PEGylation: "From Simple Polymer to Sophisticated Drug Delivery System"

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Abstract: The development of effective drug delivery systems is a major challenge in cancer therapy, gene therapy, and infectious disease treatment because of its low bioavailability, rapid clearance, and toxicity towards non-targeted healthy tissues. This review discusses how PEGylation, the covalent attachment of poly(ethylene glycol) (PEG), enhances the pharmacokinetic profiles of the drug-containing nanosystems through the "stealth effect" that avoids immune system detection and improves circulation times in different nano-delivery systems. The review provides an overview of the synthetic methods of PEG derivatives, their conjugation with nanoparticles, proteins, and drugs, and their characterization using modern analytical tools. The paper explores various PEGylation strategies, including covalent conjugation and self-assembly, and discusses the influence of PEG chain length, density, and conformation on drug delivery efficiency. Despite its advantages, there are several challenges associated with PEGylation such as the immunogenicity of anti-PEG responses, the potential for accelerated clearance of PEGylated drugs, reduced therapeutic efficacy, and the possibility of allergic reactions. Consequently, the balance between the benefits of PEGylation and its immunogenic risks remains a critical area of investigation.

Keywords: PEG; nanoparticles; protein PEGylation; cancer; PEG density

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1. Introduction

Today, cancer remains one of the biggest challenges in medicine, the cure of which is associated with great difficulties, despite the current development in chemotherapy, radiation and targeted therapy. The major obstacle to cancer treatment is the effective delivery of therapeutic drugs so that healthy cells are not damaged. Many anticancer drugs are characterised by poor bioavailability, and rapid clearance, which limits their therapeutic effect. Consequently, nano-based drug delivery systems have gained attention which offers efficient drug delivery and are characterised by non-toxicity. Nanoparticles have attracted special attention due to their biocompatibility, biodegradability and ability to encapsulate hydrophobic drugs which overcomes drug limitations. However, when nanosystems are in a physiological environment, they interact with the surrounding biological components, such as proteins, lipids and other biomolecules, which significantly influence their overall behaviour [1]. PEGylation improves the pharmacokinetic properties of NPs by enhancing their stability [2], circulation time, and avoids immune system detection by a phenomenon known as the "stealth effect". Many anticancer reagents, especially those of a hydrophobic nature, such as docetaxel, paclitaxel, and doxorubicin (DOX) are characterised by low bioavailability, rapid metabolism and high systemic toxicity, which prohibits their therapeutic activity. NP PEGylation [3] or direct drug PEGylation [4] has been shown to enhance the effectiveness and safety of those drugs. Recent reviews have discussed important elements of PEGylation. For instance, Gao et al. report the clinical significance of PEGylation technology and highlight the benefits and uses of PEGylated treatments in medicine [5]. Belen et al. provide an analysis of protein PEGylation techniques, emphasising site-specific PEGylation for proteins [6]. Similarly, Li et al. discuss recent progress in the development of PEGylated therapeutic proteins and peptides [7]. Moreover, recent works have focused on the challenges of PEGylation, such as the immunogenicity in PEGylated products [8,9]. PEGylation offers numerous benefits, however, in some cases, it also has notable drawbacks, one significant issue is the immunogenic response to PEG, referred to as 'anti-PEG antibodies' (APA) which can limit NPs efficacy and safety. The review provides a comprehensive analysis of PEGylation for drug delivery systems starting from synthetic strategies of various PEG derivatives, their applications in nanoparticle and protein PEGylation, and their pharmacokinetic implications. In general, a combination of nanoparticle-based delivery systems with PEGylation results in better drug delivery profiles and the goal of this review is to demonstrate the benefits which is associated with PEGylation generally for cancer treatment.

2. Functionalization on PEG

PEG is a hydrophilic, biocompatible polymer widely used in biomedical and pharmaceutical applications because of its unique physicochemical properties, such as high solubility in water and organic solvents, non or low toxicity, and low immunogenicity, cryoprotective [10], etc. The terminal hydroxyl group of PEG OH-$(CH_2CH_2O)_n$-H, can be modified with various chemical moieties (Table 1). Unmodified PEG is biologically inert and lacks functional groups that enable it to interact specifically with biological targets, while functionalized PEG can be covalently attached to drugs [11], proteins [12], and other biomolecules that are widely used for constructing hydrogels, nanoparticles, and other materials that benefit from PEG's antifouling and stabilizing properties. Functional PEGs can be divided into homobifunctional and heterobifunctional types. Homofunctional PEG can refer to both monofunctional (one functional group at one end) or bifunctional PEG derivatives. The key characteristic of homobifunctional PEGs is identical reactive groups at both ends of the polymer chain, which enables crosslinking, essential in hydrogel preparation [13,14]. Heterobifunctional PEGs, on the other hand, have different reactive groups present on each side that allow selective coupling to different substrates or biomolecules, which is particularly useful for site-specific conjugation.

Table 1. PEG Derivatives and their application.

PEG Derivative	Primary Application	Reaction	Benefits
PEG-Amine	Protein, peptide conjugation	N-terminal, Lysine PEGylation	Increased stability and half-life [55–57]
PEG-Silane	Surface functionalization, drug delivery	Highly reactive with hydroxyl groups on surfaces	Stable surface functionalization, increased circulation time [58]
PEG-Aldehyde	Protein conjugation	Nucleophilic addition with hydroxyl or amine groups	Improved half-life [59,60]
PEG-Azide	Click chemistry	Click reaction with alkyne-functionalized molecules	High specificity, bioorthogonality
PEG-Acrylate	Tissue engineering and hydrogel preparation	Michael addition, radical Polymerization	Hydrogel scaffolds for 3D cell culture[61,62], wound dressing [63,64],tissue engineering [65]
PEG-Maleimide	Protein, drug, NP conjugation	Reacts with thiols (cysteine) in proteins, gold NP surface	Increased stability, half-life [66–69]
PEG-Nitrophenyl	Protein conjugation, crosslinking	Nucleophilic substitution reactions	Rapid and simple modification of nanocarriers for protein conjugation

2.1. Synthesis and Functionalization of Amine and Thiol-Terminated PEGs

Amine PEGs contain NH_2 groups at one or both ends, the polymer is suitable for conjugation with drugs, proteins, or other molecules. The first step of NH_2-PEG synthesis is the activation of terminal hydroxyl groups which is performed using halogenation [11,15] or sulfonylation [15–18] by tosylation or mesylation through reacting with tosyl (Ts) and mesyl (Ms) chlorides, producing activated PEGs which are a precursor for functionalized PEG synthesis, those contain effective leaving groups for nucleophilic substitution with amines and thiols (Figure 1). The sulfonylation reaction requires bases such as pyridine [15] and triethylamine (TEA) [15,19] as a hydrochloric acid acceptor. Activated PEGs are treated with ammonia [20–23] forming NH_2-PEGs. However, by-products such as secondary/tertiary amines can be produced as side reactions, which reduce the yield of PEG-amine and complicate the purification of the final product. On the other hand, sulfonylated PEGs can then be further modified into PEG azide by reacting with NaN_3, which is an intermediate in the synthesis of NH_2-PEGs using the Staudinger reaction [20,24,25] generally leading to a product with higher purity and yield. Zhang et al. reported the synthesis of homofunctional and bifunctional amino-PEGs using 3 step methodologies: 1) mesylation 2) the substitution reaction of activated mPEG-OMs with $(Boc)_2NH$ to obtain mPEG-$N(Boc)_2$ followed by deprotection of the Boc groups with trifluoroacetic acid to obtain mPEG-NH_2 finally, the reaction yielded in 95–99% purity [26]. Thiol-functionalized PEGs are useful for creating self-assembling structures on gold surfaces and other metal surfaces and for protein PEGylation through Michael-addition reactions. The simplest method to introduce a thiol group into PEG is through nucleophilic substitution between PEG-OTs and a thiol nucleophile namely sodium hydrosulfide [27] or through an esterification reaction between mercapto acids, such as mercaptoacetic acid [28], mercaptohexadecanoic acid [29], 3-mercaptopropionic acid and a PEG-OH in the presence of sulfuric acid [30–32]. Mulay et al. described an environmentally friendly approach for PEG-thiol synthesis using an enzymatic process by transesterification reaction with methyl 3-mercapto propionate in the presence of *Candida antarctica* lipase B enzyme, the reaction yields were approximately 100% for both PEG_{1000} and PEG_{2050} monothiols [33].

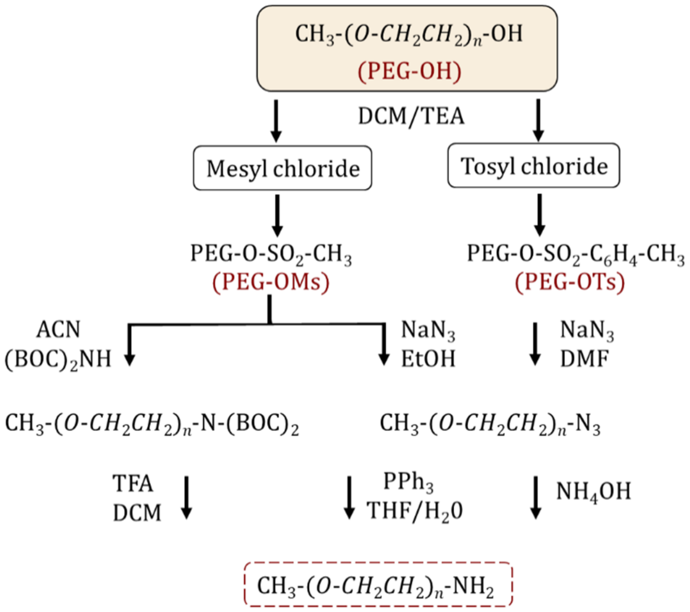

Figure 1. Synthetic pathways for NH_2-PEG preparation.

2.2. Alkyne Functionalized PEGs

Alkyne PEGs (Figure 2) are widely used in PEGylation reactions, several alkyne functional groups can be attached to PEG molecules like propargyl or strained alkynes, such as dibenzocyclooctyne or bicyclononyne. However, Propargyl PEGs are commonly used in copper-catalyzed azide-alkyne cycloaddition, while strained alkynes are employed for copper-free azide-alkyne cycloaddition also known as SPAAC, which is excellent for in vivo applications as it reduces the potential cytotoxicity associated with copper [34]. SPAAC is a bio-orthogonal reaction and minimizes interference with biological processes, the reaction occurs rapidly under physiological conditions useful for imaging [35–37] hydrogel [38,39] and drug delivery systems preparation. During the synthesis of propargyl derivatives, the hydroxyl group of PEG is first activated by a strong base (sodium hydride, potassium hydroxide) to deprotonate the alcohol group followed by a nucleophilic substitution reaction with propargyl bromide [40,41]. Herzberger et al. developed a novel procedure where glycidyl propargyl ether (GPgE) was used as a monomer in anionic copolymerization with ethylene oxide [42], which incorporated propargyl groups into the polymer chain without the need for protection of the alkyne group. Alternatively, Lu et al. used α-hydroxy-ω-carboxyl PEGs for alkyne-PEG synthesis, initially, the carboxyl group was converted to a propargyl group using propargyl bromide, whereas hydroxy ends were modified with amine, mercapto, and hydrazide groups at the other end to synthesize heterobifunctional PEGs. Those heterobifunctional PEG derivatives are useful for bioconjugation applications [43], particularly with azides in copper-catalyzed reactions.

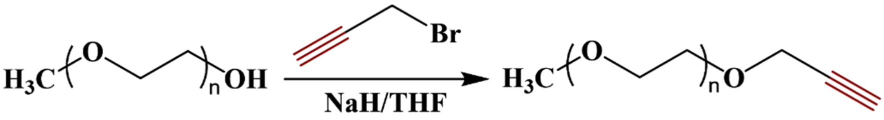

Figure 2. Synthesis of alkyne-functionalized PEG.

Aldehyde-modified PEGs are often used in conjugating with biologically active molecules such as proteins [44], and antibodies, during the conjugation Schiff bases are formed that can revert to the original aldehyde and amine in the presence of water or under acidic conditions. A common strategy to overcome this problem is to reduce the Schiff base to an amine by reducing the reagent which forms more stable C-N bonds. Only a few literature sources are available for the synthesis of aldehyde-functionalized PEG because of the challenges associated with the low reactivity of the hydroxyl groups. The most commonly used method is the oxidation of hydroxyl groups [45,46]. In 2015, Mauri et al. reported the two-step synthesis of PEG-aldehydes, oxidation methods often struggle with the low reactivity of PEG's hydroxyl groups and result in low yields with unwanted by-products. In contrast, in the new method (Figure 3), PEG was functionalized with terminal alkenes, followed by ozonolysis and subsequent reduction with dimethyl sulfide which forms chemically stable, reactive PEG-aldehyde derivatives without changing the structural integrity of the polymer [47].

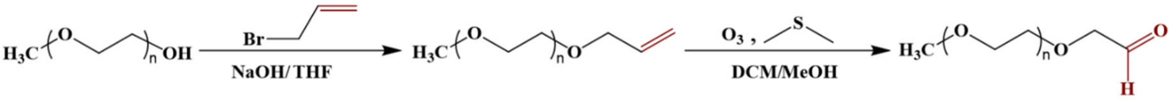

Figure 3. Synthesis of PEG-aldehyde by ozonolysis.

In addition to aldehyde-functionalized PEGs (Figure 4), nitrophenyl-functionalized PEGs are commonly employed in the development of PEGylated systems, which are synthesized by a reaction between nitrophenyl chloroformate (NPC) and PEG hydroxy ends in organic solvents like THF, DMSO, and DCM in the presence of base catalysts, triethylamine [48–51] or dimethylamino pyridine (DMAP) [52]. Generally, NPC is taken in a slightly excess amount relative to PEG to ensure a high conversion of hydroxyl groups. Acylation reaction between PEG and NPC is typically performed at low temperatures (0–5 °C) which reduces the side reactions, however, the presence of water in the reaction system can trigger secondary reactions resulting in the formation of 4-nitrophenol which is a major by-product of hydrolysis and the purification presents difficulties. The NPC-PEG product is often conjugated with various molecules such as dendrimers [53,54], proteins or micelles, that contain amine groups forming a stable urethane linkage.

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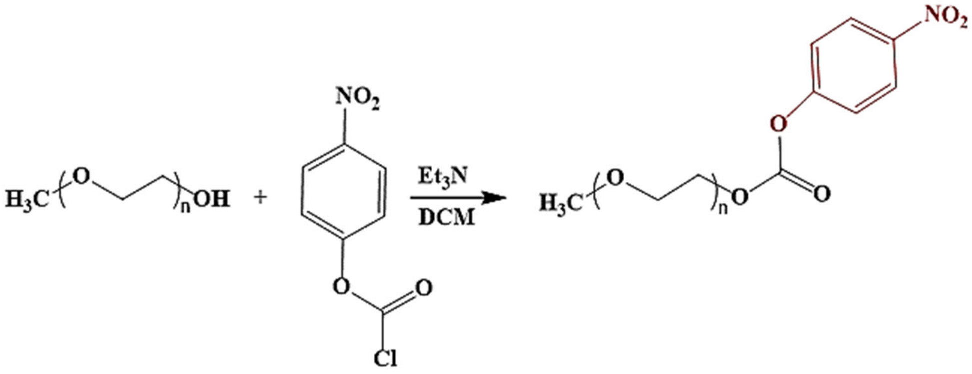

Figure 4. Synthesis of p-nitrophenyl-activated PEG via acyl substitution reaction.

3. PEGylation Strategies for Nanosystems

PEGylation of nanosystems can generally be achieved through different methods, including covalent conjugation, physical adsorption and self-assembly. Non-covalent PEGylation is performed by incubating nanoparticles in a PEG solution. However, this simple incubation creates weak interactions between PEG and NPs surfaces and PEG can detach over time, especially under physiological conditions when parameters can fluctuate. In contrast, the covalent attachment of PEG provides a more stable modification. Covalent PEGylation can be accomplished by direct conjugation of PEG molecules to the surface of the nanoparticles, for example, PEG molecules with thiol groups can react with the metal nanoparticle surface like gold or silver with high affinity, forming stable Au-S [70,71], Ag-S [72] bonds. Often PEGylation is performed through a non-direct approach, when PEG is firstly modified into amphiphilic forms as part of di-block copolymers like PEG-PLA, PEG-PCL or even tri-block copolymers [73] also known as pluronics, which forms self-assembled structures on NPs. In our previous work, we reported the synthesis of PEG-grafted poly(ester amide) (PEG-PEA) using a thiol-Michael addition reaction, the NPs were prepared by a solvent-displacement method where the PEG-PEA was used as a surfactant, forming self-assembling structures (Figure 5) which were confirmed by transmission electron microscopy [74].

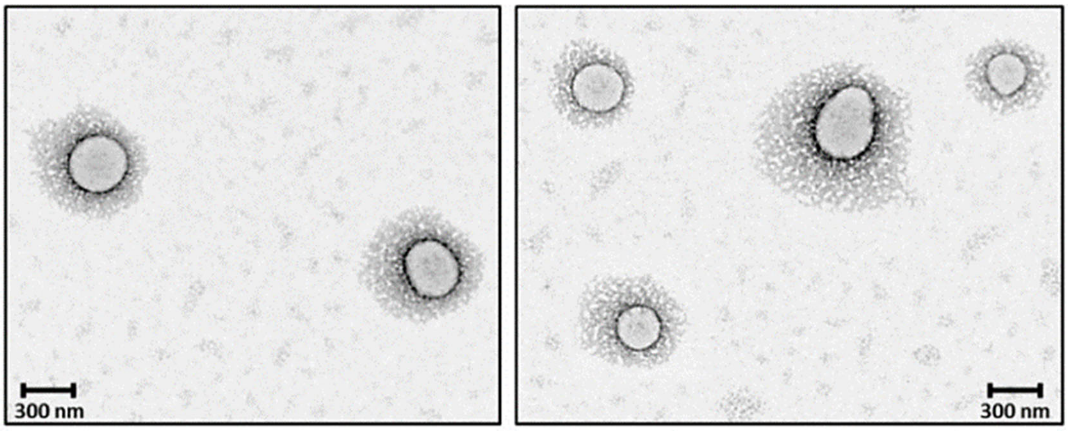

Figure 5. TEM micrographs core-shell PEGylated NPs. Reprinted with permission from Ref. [74].

As a surfactant, PEG can stabilize emulsions, suspensions, and other systems. However, only PEG can not assemble on NP's surface because of its hydrophilic nature, except when in combination with hydrophobic segments (Figure 6). PEG-containing copolymers can self-assemble into well-organized structures due to molecular interactions. In aqueous environments, hydrophobic segments of amphiphilic PEGs tend to avoid water and interact with the core, through Van der Waals forces or electrostatic interactions for the charged polymers [75]. The ratio of hydrophilic and hydrophobic blocks and their molecular weight can be adjusted to control the stability and biological properties of

NPs. Ueya et al. showed that increasing the PEG ratio in a PEG-PLGA[78–80 block copolymer decreased the stability of micellar NPs due to the increased hydrophilic nature, by increasing the molecular weight of PLGA, water diffusion into the core was hindered, resulting in enhanced stability [76]. The ratio of hydrophilic/hydrophobic (H/H) blocks of PEG-PLA was shown to affect the cellular internalization of self-assembled NPs in HepG2 tumour cells. The results for the HepG2 line show that clathrin-mediated endocytosis is the predominant pathway for micelles with a 5:5 H/H ratio whereas micelles with H/H ratios closer to 2:8 showed the lowest cellular uptake, which was attributed to the formation of less dense protein corona, which in turn facilitates better cellular uptake [77]. PEG content can significantly influence the drug release profile from nanoparticles, high PEG generally leads to faster and more extensive drug release, due to the increased hydrophilicity of the nanoparticle surface, which can lead to enhanced water uptake and facilitate faster drug release, especially for hydrophilic drugs [78–80]. Certain structures, like micelles, form self-assembly structures above a certain concentration known as the critical micelle concentration (CMC), which is the level of surfactant molecules in a solution above which they spontaneously form organized structures, high-CMC surfactants tend to form micelles that are less stable and more susceptible to dissociation when diluted [81]. The PEG-block copolymer surfactants tend to have lower CMC values compared to conventional surfactants. Long hydrophobic blocks lower the CMC [82] because they enhance the tendency for self-assembly by reducing solubility in aqueous environments while in turn a long PEG block increases the CMC, however, a balanced ratio is important to decrease CMC and maintains other physical properties which are necessary for drug delivery purposes.

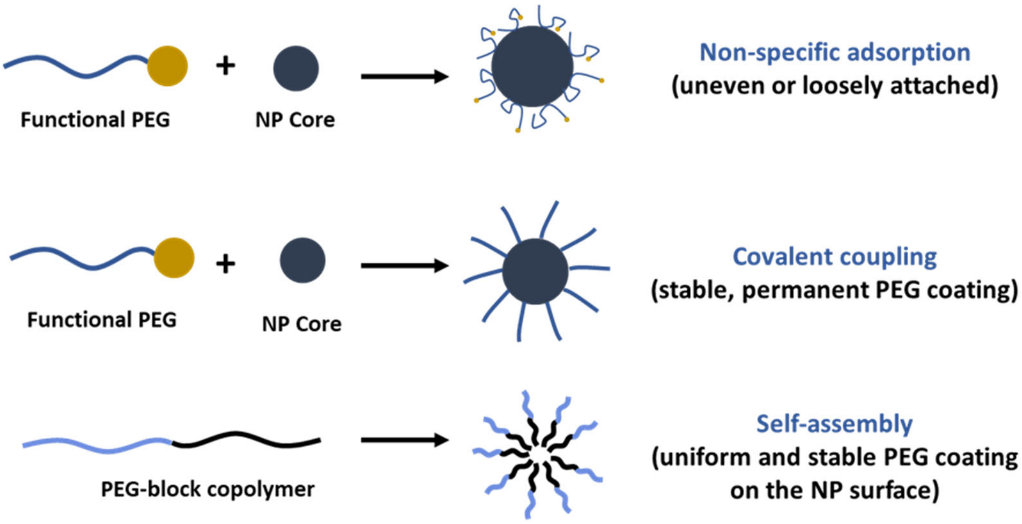

Figure 6. Comparison of PEGylation strategies for nanoparticle surface modification.

4. Flory Radius, PEG Chain Length, and Density: Influence on PEG Conformation and Biological Interactions

PEGylation can lead to mushroom and brush structures on the NP surfaces depending on factors such as solvent, molecular weight and density of PEG chains (Figure 7). The conformational regime of PEG can be predicted from the Flory radius using the following relation: $R_f=\alpha N^{3/5}$ where N is the number of monomeric units in the PEG polymer chain and α is the length of monomeric unit (0.35 nm), and the exponent value (3/5) serves only for a good solvent, that can change based on factors like molecular weight and solvent type. The grafting density or the average distance between adjacent PEG chains is calculated by the following formula $D=(A/\pi)^{1/2}$, where A is an area occupied in nanoparticle occupied per PEG chain, using these two formulas one can predict PEG conformation, whether the PEG adopts a brush-like ($D<2R_f$) or mushroom-like ($D>2R_f$) structures. At a higher PEG density, in a brush configuration, the PEG chains are extended and occupy more space due to higher R_f relative to D and vice versa in a mushroom configuration, the chains are more compact and the effective size is smaller. High PEG surface density is generally preferred for biological applications which show biocompatibility, and low protein adsorption [83] inhibiting phagocytic uptake [84,85]. However, as brush conformation avoids phagocytic uptake it does not necessarily guarantee full protection from macrophage uptake, it was shown that a minimal brush PEG coating could not evade THP-1 macrophage endocytosis and sometimes dense-brush conformation is required for an effective reduction in protein adsorption [86] and macrophage uptake [87]. Wang et al. showed the importance of PEG chain size in the comparative study while the terminal PEG density was constantly regulated by changing the PEG-PCL block copolymer molecular weight in PEG_n-PCL_n/PCL_{3500} core-shell NPs, increasing PEG length from 10.7 nm (3.4 kDa) to 13.8 nm (5 kDa) improved tumour growth inhibition from 73.4% to 88%, however, further increase in PEG length to 24.5 nm reduced efficacy to 54%. PEG 5 kDa, particularly between PEGs 3.4 kDa and 8 kDa, showed enhanced stealth properties, longer circulation time and reduced macrophage interactions, which indicates that optimizing PEG length is crucial for designing effective drug delivery systems [88]. Gref et al. studied the impact of PEG Mw in PEG-PLA 45 kDa/PLA 40 kDa core-shell NPs on protein adsorption. A steep decrease was observed when increasing PEG Mw from 2000 to 5000 g/mole. PEG 5 kDa was found to be the most optimal for protein resistance and no significant benefits were observed when increasing PEG Mw above 5000 compared to bare PLA 40 kDa NPs. The reduced protein adsorption for PEG content in PEG 5 kDa-PLA 20 kDa/PLA 40 kDa NPs was between 2-5 weight %, however, no complete protection against plasma protein adsorption was achieved [89]. While long PEG chains improve stability and circulation, they can also inhibit cellular uptake of the nanoparticles [90–92].

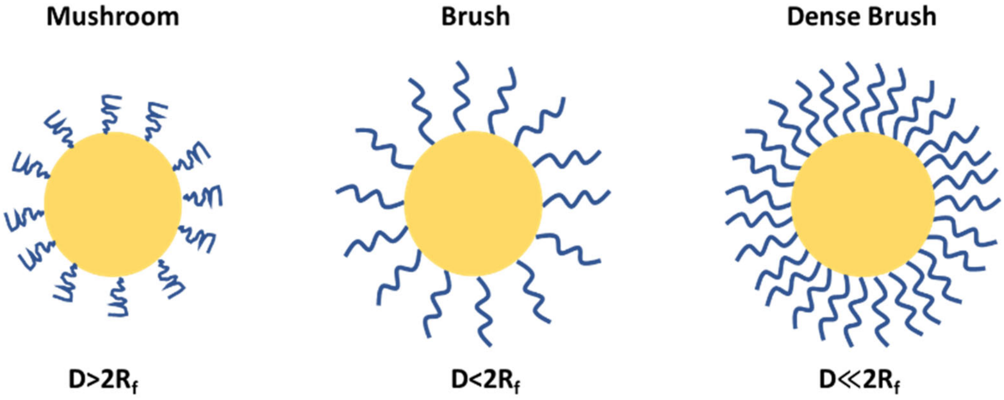

Figure 7. PEG conformational regime on NP surface.

5. Quantification of PEG Surface Density on Nanoparticles

There are several techniques available to study and evaluate PEG density around the NP surface. Quantification of PEG surface density is important to determine the PEG conformational regime, especially in the area of drug delivery, which, as shown before, directly influences NP properties such as stability, solubility, and interaction with the immune system. One of the methods to study the PEG surface density is a fluorescence-based assay, PEG molecules are labelled with a fluorescent dye, and the PEG surface density is measured by the fluorescence intensity of the dye [82,93]. NPs are typically incubated with fluorescent dyes, followed by a washing step to remove unbound labels. The signal is measured, and PEG surface density is calculated from the standard calibration curve.

X-ray photoelectron spectroscopy (XPS) examines the chemical and elemental states of a material surface, and it is commonly used to confirm the successful PEGylation of the NP surface. The existence and relative abundance of oxygen and carbon atoms indicate the presence of PEG molecules on the NP surface to analyze the PEG cloud around the NPs [25,28,29,32,94,95]. XPS is particularly useful for verifying the modification of NPs with PEG and its efficacy in reducing protein adsorption [83][96]. The surface density of PEG can be calculated by comparing the intensity of the PEG-related peaks to the total surface area of the NPs [97]. However, XPS is a surface-responsive technique, that usually covers up to 10 nanometers of the sample, therefore experimental results correspond to the outer region of the material, and PEG coverage below this surface region can not be detected.

Thermogravimetric analysis is a useful technique for evaluating the PEG density on a nanoparticle surface. The PEG number is quantified by analyzing the weight loss as a function of temperature, [94,95,98,99] based on the calibration of the pure PEG molecule which has been used for NP preparation. However, TGA provides information about the total PEG content but it does not directly reveal how PEG molecules are distributed on the surface of the nanoparticles [100].

NMR spectroscopy provides quantitative information about the surface density of PEG on NPs. The average number of PEG molecules for each NP and chains per surface area can be calculated from ^{1}H-NMR spectra by integration of the CH_2 groups of PEG peak typically observed at ~3.5 ppm which can be compared to internal [94] or the external standard signal [101], the conformation of PEG chains can vary from mushroom to brush conformations that impact the mobility and dynamics of the polymer chains, moreover, the measurement of the spin relaxation time promotes a better understanding of PEG chain conformation around the NPs, with the increased mobility of the polymer chains the relaxation time decreases [101].

6. Protein PEGylation

6.1. N-terminus and Lysine PEGylation

PEG molecules can be conjugated to functional groups in proteins and peptides which are presented in the amino acid sequence (Figure 8). The PEGylation of amino groups is the most commonly used technique for protein PEGylation that can be divided into two categories: site-specific N-terminal PEGylation, targeting the amino terminus (N-terminus) and Lysine PEGylation. N-terminus PEGylation is often employed by maleimide or NHS ester-activated PEGs. Peng et al. showed covalent attachment of PEG 5 kDa molecules to immunostimulatory peptide Thymosin alpha 1 (Tα1), peptides with PEG attached to the N-terminus exhibited a longer half-life and improved immune activity compared to those modified with a cysteine thiol (Cys-Tα1) group. The authors suggest that the degradation of Cys-Tα1 over time could be attributed to interactions between the cysteine-modified Tα1 peptide and proteins, resulting in the breakdown of the structure [66]. Ming Yu et al. reported N-terminal PEGylation of recombinant human interleukin (rh-PEG-IL11) using PEG aldehyde, which significantly increased the half-life of the drug during subcutaneous injection, approximately from 3 hours for non-PEGylated rh-IL11 to 67 hours for PEGylated-IL11 in monkeys [59]. On the other hand, PEGylation of lysine residues occurs through a nucleophilic substitution reaction, where the amine group of the lysine side chain reacts with activated PEG derivatives, however, the reaction is non-specific and can lead to the modification of other moieties of proteins. PEG conjugation to lysine has proven to be effective in reducing proteolytic degradation of the fibronectin (FN) protein which plays a major role in cell adhesion, growth, migration, and differentiation, the authors studied the influence of PEG molecular weight on the stability and biological activity of FN. Cell properties (adhesion, cell spreading) were shown to deteriorate by increasing PEG molecular weight [102]. PEG attachment to lysine side chains is a popular technique for uricase PEGylation, which diminishes blood uric acid levels by converting them into allantoin. It provides multiple benefits, including increased solubility, reduced immunogenicity, and improved enzymatic stability [103]. Lysine group PEGylation is often employed for modification interferon alfa-2a with PEG which is used in combination with the anti-viral medicine ribavirin, for a treatment for chronic hepatitis C [104]. Lysine PEGylation is commonly used to modify nitrophenyl-PEGylated liposomes and micelles for targeted drug delivery. Amine groups of proteins readily react with PEG, facilitated by the excellent leaving properties of the nitrophenol group, which forms stable urethane linkages, the reaction is conducted at a slightly basic pH (7.5-8.5) [105–107] and the residual nitrophenol and unbound proteins are removed by dialysis.

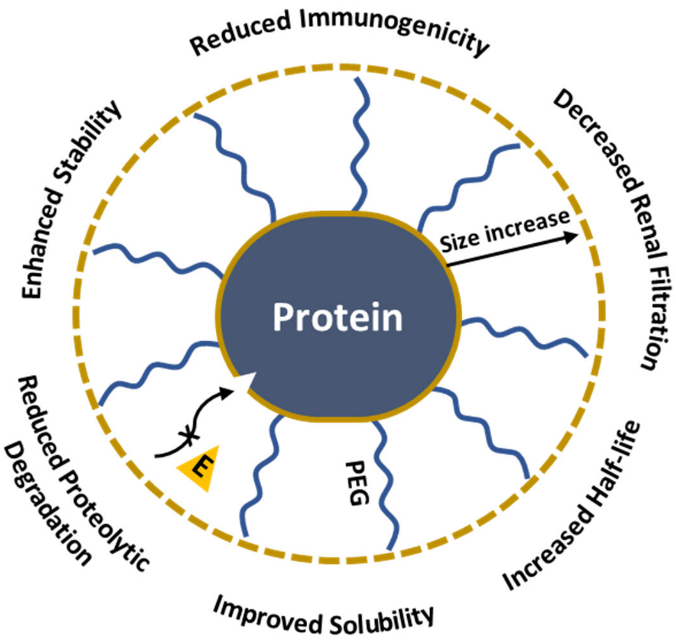

Figure 8. PEGylation improves the pharmacokinetics of proteins.

6.2. Cysteine (Thiol) PEGylation

Cysteine PEGylation has been effectively used in modifying proteins such as enzymes and monoclonal antibodies [108,109] for improved tumour targeting which is based on maleimide chemistry. In neutral or slightly basic buffers, maleimide reacts with the thiol groups of cysteine creating strong thioether bonds. Thiol PEGylation was successfully used for site-specific modification of granulocyte colony-stimulating factor (G-CSF) which demonstrated prolonged biological activity during neutropenia in a mice model, whereas increasing PEG molecular weight improved leukocyte proliferation [68]. In another study, authors demonstrated that PEGylated G-CSF showed significantly longer circulating half-lives compared to unmodified G-CSF, as observed in the previous work [63], the half-life of cysteine PEGylated proteins was enhanced by increasing the molecular mass of the PEG [69]. Furthermore, thiol PEGylation was used to crosslink L-asparaginase, an enzyme used in leukemia treatment. Researchers introduced recombinant cysteine residues on the enzyme surface and conjugated them with different molecular weights PEGs. The study revealed that intramolecular crosslinking of L-asparaginase with PEG stabilized the enzyme active site resulting in improved catalytic activity. Additionally, PEGylation increased the enzyme hydrodynamic radius, reducing the probability of glomerular filtration and thereby extending the therapeutic half-life [110].

6.3. Carboxyl PEGylation

Carboxyl PEGylated products have better solubility and reduced immunogenicity [111], PEGylation targets carboxyl groups typically found in aspartic and glutamic amino acids. Carboxyl group PEGylation is employed by EDC/NHS activation, firstly, the carboxyl group is activated to react with a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), which converts the carboxyl group into O-acylisourea reactive form reacting with the amine groups of PEG forming an amide bond between PEG and the protein. However, the reaction is limited to buffers that do not contain carboxyl or amine groups. In 1997 study demonstrates the first carboxyl-specific PEGylation of BDNF neuropeptide which plays a crucial role in neuron survival, growth, and differentiation. PEGylation improved the pharmacokinetic properties of the peptide in terms of decreased plasma and hepatic clearance, however, authors emphasize that PEGylation increased the renal clearance of BDNF-PEG_{2000} compared to non-PEGylated BDNF, attributed to the fact that PEGylation enhanced the cationic properties of the peptide followed by kidney clearance, but BDNF-PEG$_{5000}$ showed a decrease in removal because of the increased size of the peptide [112]. In conclusion, PEGylation is a powerful tool used in preparing therapeutic proteins because of its ability to enhance half-life, solubility, and immunogenicity, and at the same time, reduce the clearance of proteins (Figure 8). However, PEGylation might alter the bioactivity or functional properties of the protein because PEG could interfere with the active site or overall structure of the protein [113–116], therefore, selecting the appropriate modification method is important to maintain the protein functional integrity.

7. PEG Immunogenicity

PEGylation enhances drug properties by increasing circulation time, improving stability, and solubility, however, the repeated use of PEG has led to an unexpected consequence, the development of anti-PEG antibodies (APA). The recognition of PEG by immune system seems to be a complex mechanism. When the body encounters PEGylated compounds, immune cells can generate antibodies specifically targeting PEG epitopes. These antibodies may be of different isotypes, predominantly IgM [117–119] and IgG [120] followed by a quick removal and immune responses. Studies have revealed the prevalence of APA in the general population, 25% of healthy blood donors possessed it [121]. In another study, APA were detected in 44.3% of healthy donors both antibodies IgM (27.1%) and IgG (25.7%), antibodies were significantly more common in females as compared to males, which is in accordance with the separate study [122], likely due to cumulative exposure to PEG containing products in daily life. The presence of these antibodies has been associated with accelerated blood clearance (ABC) phenomenon, reduced therapeutic efficacy, and potential hypersensitivity reactions in patients receiving PEGylated medications [8,123]. Several key factors influence the development of anti-PEG immunity and ABC phenomenon such as previous exposure to PEG, administration routines, PEG characteristics, etc. Münter et al. compared the antibody generation against PEGylated mRNA-carrying NPs by different administration routes. Intramuscular injections in mice were found to generate overall low and dose-independent levels of antibodies, while intravenous and subcutaneous injections generated substantial levels of IgG and IgM antibodies [124]. In separate research by Takata et al., all tested routes for mPEG$_{2000}$-DSPE induced anti-PEG IgM production, however, the maximum production of anti-PEG IgM antibodies was observed after intravenous administration and anti-PEG IgM production was significantly reduced after splenectomy for all administration routes, indicating the spleen key role in antibody generation [125]. Opinions vary on how PEG chain density affects ABC phenomena, for example, Li et al. reported that liposomes with a higher PEG grafting density (e.g., 9%) tend to induce enhanced ABC phenomenon compared to those with a lower PEG density (e.g., 3%).

Both high and low PEG formulations induced similar levels of anti-PEG IgM following the first dose. However, the higher PEG density results in stronger recognition and neutralization during subsequent doses, leading to faster clearance [126]. On the contrary, Ishida et al. showed that a PEG surface density of 5 mol% on mPEG$_{2000}$-DSPE liposomes was found to induce ABC phenomena at the first dose, while the PEG density way below or higher this threshold reduced the ABC phenomenon, authors suggest that a high density of PEG reduced the activity of splenic B cells, resulting in less clearance of subsequent dose and increasing PEG Mw from 2000 up to 5000 g/mol, did not affect ABC phenomenon [127]. In addition to PEG Mw, the immunogenicity of PEG is influenced by its terminal group [128,129]. The molecular basis of anti-PEG antibody formation involves complex immunological mechanisms. Research by Ishida et al. has shown that empty PEGylated liposomes induce an anti-PEG IgM response in rats and mice, even in T-cell-deficient mice, indicating that the response is T-cell-independent [130].

APA detection methodologies have evolved significantly to name some of them immunosorbent assay ELISA, flow cytometry, and surface plasmon resonance. Ehlingeret et al. demonstrated high sensitivity in detecting clinically relevant anti-PEG antibody levels in a large analysis involving 200 healthy patients the ELISA method detected 97.5% pre-existing antibodies in human serum [131]. The existence of anti-PEG antibodies remains challenging in the vaccination process, cases of immediate hypersensitivity reactions including anaphylaxis were reported following the administration of PEG-containing COVID-19 vaccines, particularly those employing PEGylated lipid nanoparticles (LNPs), in the study 130 adults received either the BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna) mRNA vaccines against SARS-CoV-2, ELISA analysis showed anti-PEG IgG was detectable in 71% of subjects prior to vaccination. However, APA were boosted after 2 doses vaccination: Pfizer-BioNTech showed tiny 1.78-fold change for IgG and 2.64 for IgM, while Moderna vaccine showed 13.1 for IgG and 68.5 for IgM and no increase was observed in unvaccinated controls [122]. In 2023, Kozma et al. studied the potential role of APA in allergic reactions to PEG COVID-19 vaccines, results showed elevated levels of IgG and IgM antibodies indicating that individuals with higher levels are more likely to experience allergic reactions upon receiving PEG-containing vaccines [132].

8. PEGylated Nanocarriers in Cancer Therapy

8.1. PEGylated liposomes

PEGylated liposomes are lipid bilayers that are coated by PEG on the surface, which reduces aggregation and enhances the colloidal stability of this formulation. Due to the enhanced permeability and retention effect, PEGylated liposomes accumulate in tumour tissues more effectively than conventional drug formulations (Table 2). One of the first stealth liposomal formulations coated with PEG was reported by Klibanov et al. who demonstrated the positive effect of liposome PEGylation on prolonged circulation time in the blood [133], which were capable of evading the RES, since then new strategies have been developed to increase the efficacy of that system, such as pH-sensitive design, which contains acid labile bonds these liposomes remain stable under physiological pH characteristic of healthy tissues and the bloodstream, but they degrade in acidic conditions which are found in tumour microenvironment where encapsulated drugs are released [134–137].

One of the most widely used PEGylated liposomal formulation against cancer is DOXIL which is the carrier of the chemotherapeutic drug doxorubicin (DOX), approved for the treatment of breast cancer, ovarian cancer, and multiple myeloma. Safra et al. reported that 500 mg/m^2 of liposomal DOXIL significantly reduced the cardiomyopathy risk which is generally associated with the use of free doxorubicin [138]. Several methods have been used for the preparation of liposomal formulations, such as the pre-insertion method, where PEGylated lipids are incorporated into the formulation before the liposome formation. Since the PEGylated lipids are present throughout the formulation process, the resulting liposomes have a consistent surface modification, but a lower PEG surface density [139]. Meanwhile, in the post-insertion method, liposomes are first formed without PEGylation. Afterwards, PEG-lipid conjugates are added to the pre-formed liposomes, and the PEGylated lipids spontaneously integrate into the liposomal bilayer due to hydrophobic interactions. However, repeated administration of several PEGylated liposomes was reported to lead to the generation of anti-PEG antibodies, resulting in faster clearance [140–143]. Additionally, liposomes are limited by their drug-loading ability, purification steps and high production costs.

Table 2. List of FDA-approved PEGylated liposomal formulations.

Brand Name	Active Ingredient	Cancer Type	Mechanism of Action
Onivyde	Irinotecan hydrochloride trihydrate	Pancreatic cancer, small cell lung cancer, colon cancer	Topoisomerase I inhibitor
DaunoXome	Daunorubicin	Kaposi’s sarcoma, Non-Hodgkin lymphoma	Topoisomerase II inhibitor
Doxil, Zolsketil , Caelyx, Myocet	Doxorubicin hydrochloride	Ovarian cancer, Kaposi’s sarcoma, myeloid melanoma, Breast cancer	Topoisomerase II inhibitor
Mepact	Mifamurtide	Osteosarcoma	Activating immune cells
Marqibo	Vincristine sulfate	Acute lymphoblastic leukemia (ALL), Hodgkin's disease, neuroblastoma	Microtubule synthesis Inhibition

Vyxeos	Daunorubicin, cytarabine (in combination)	Leukemia	Topoisomerase II, Nucleoside Inhibitor
DepoCyt	Cytarabine	Lymphomatous meningitis	Nucleoside Inhibitor

8.2. PEGylated Micelles

Micelles are self-assembled structures formed by amphiphilic surfactants or block copolymers. PEG block copolymers PEG-PLA and PEG-PCL form polymeric micelles by self-assembling processes, they consist of a hydrophilic outer PEG shell and a hydrophobic polymer core, that can encapsulate poorly soluble drugs (Figure 9). Polymeric micelles like liposomes are effective in solid tumours, PEGylated micelles typically range from 20 to 100 nm in size, and are ideal for passive targeting via the enhanced permeability and retention (EPR) effect. On the other hand, micelles are used for active targeting, which is achieved by functionalizing with ligands (e.g., antibodies, peptides, or small molecules) that specifically bind to overexpressed receptors on the cancer cells. PEGylated micelles are prepared by thin-film hydration: generally, the drug and polymer are dissolved in an organic solvent typically chloroform, or methanol, the organic solvents are removed by the rotary evaporation and a produced thin film is hydrated forming micelles with the drug trapped inside. The thin film method has been successfully used for the preparation of transferrin-targeted PEGylated liposomes for treating ovarian carcinoma in-vivo models [144,145].

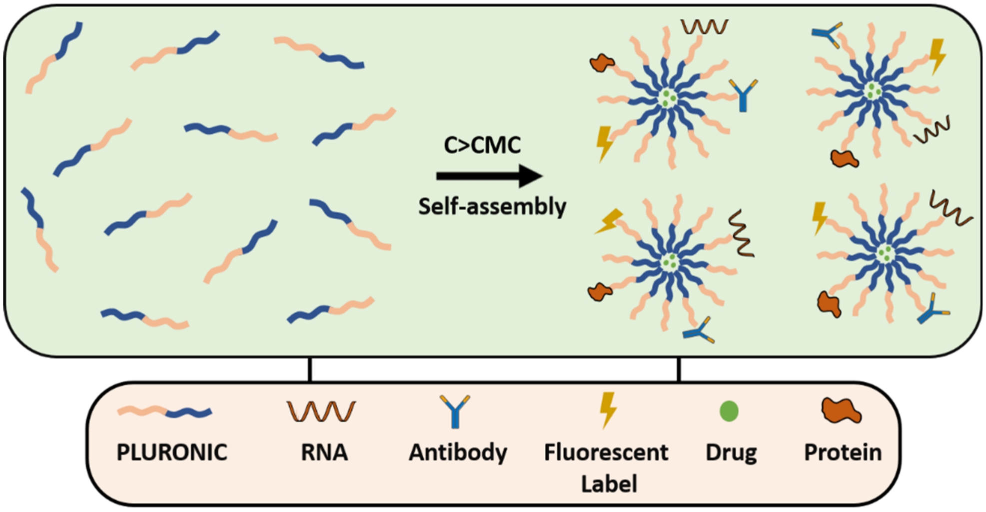

Figure 9. Polymeric micelle formation and conjugation strategies.

8.3. PEGylated Dendrimers

Dendrimers are highly branched, nano-sized three-dimensional polymeric structures with unique properties, they increase the solubility of poorly soluble drugs and their half-life [146–148]. Positively charged dendrimers are widely used in drug delivery reacting with the negatively charged cell membrane; however, the charge on the surface also causes cytotoxic effects [149] and rapid clearance. The interaction of dendrimers with biological membranes can lead to membrane disruption by creating small holes or pores in the membrane [150]. PEGylation of cationic dendrimers is an effective way to overcome cytotoxicity problems [151–153]. PEGylation of poly(amidoamine) dendrimer (PAMAM) was shown to improve the solubility and the anticancer activity of piperlongumine drug in human colon cancer with sustained drug release [154]. In another study, PEGylated DOX-loaded dendrimers showed high accumulation in C26 murine colon tumour cells (around 20 times) and reduced accumulation of DOX/dendrimers in the spleen compared to the free drug [155]. Besides reducing toxicity, PEGylation enables the further modification of dendrimers with different ligands for targeted delivery [156]. Dual-responsive PEGylated dendrimers have shown promise in glioblastoma treatment by improving the delivery of therapeutic agents across the blood-brain barrier (BBB), studies in the C6 glioma cell line have demonstrated that ligand-modified

PAMAM dendrimers conjugated with drugs like doxorubicin and tamoxifen can increase their half-life, accumulation in brain tumour and reduce non-specific toxicity [157–160].

8.4. PEGyled Polymeric Nanoparticles

Polymeric polyester nanoparticles such as PLGA, PLA, PCL or chitosan are often used as drug delivery systems, polymer matrices can encapsulate both hydrophobic and hydrophilic drugs, protecting them from degradation and providing sustained release. In addition, the NP's PEG surface can be modified with various ligands, such as antibodies or proteins, that can specifically target receptors overexpressed on cancer cells. However, “naked” nanoparticles are easily recognised by the immune system, when foreign bodies, including NPs, enter the bloodstream, the immune system quickly detects and removes them, by the process known as opsonization. During opsonization, the surface of nanoparticles is covered with opsonin proteins, signalling macrophages to neutralize them by phagocytosis [161]. PEGylation of the NP surface overcomes this problem by forming a hydrophilic "shield" around NPs and improves the nanoparticles stability, biocompatibility, and circulation time [162]. PEGylation was shown to increase the water solubility and bioavailability of hydrophobic drugs [163]. The size of PEGylated polymeric nanoparticles is one of the most important factors that determine NP behaviour. Nanoparticles in the 10 nm to 200 nm size range are optimal for efficient cellular uptake, tissue penetration, and circulation in the bloodstream, as most nanoparticles around 10 nm are filtered by the kidneys. As it is known the stability of non-modified nanoparticles highly depends on a surface charge [164]. PEGylation of NPs seems to reduce their zeta-potential [165–167] however, this does not present a significant issue as PEG prevents nanoparticle aggregation through steric hindrance, rather than the electrostatic stabilization mechanism. PEGylation affects interactions between nanoparticles and cell membranes and their subsequent cellular internalization [168]. Generally speaking, amphiphilic nanoparticles are internalized by cells through endocytosis, which is a major pathway for cellular uptake, for particles with diameters up to 200 nm clathrin-mediated endocytosis has been a predominant pathway for cellular uptake [169].

8.4.1. Methods for Preparation of PEGylated Polymeric Nanoparticles

Nanoprecipitation

Nanoprecipitation, also known as the solvent displacement method, is the most commonly used technique for the preparation of PEGylated polymer nanoparticles (Table 3), which is very useful for encapsulating hydrophobic drugs; however, less effective for hydrophilic drugs as precipitation is performed in the aqueous phase. NPs are prepared by adding an organic to the water phase, therefore, the organic solvent must be miscible with water. The polymer and the drug are dissolved in an organic solvent and slowly added to the aqueous phase under continuous stirring, the solvent can be removed by evaporation or dialysis. The particle size of PEGylated NPs can be controlled by modifying several key parameters such as polymer concentration, antisolvent volume-flow rate, and stirring rate. The concentration of the polymer in the organic solvent impacts the size of the resulting NPs, generally speaking, the higher the polymer concentrations the larger particles are formed, and increasing the polymer concentration too much can lead to aggregation and finally precipitation. Normally, the nanoprecipitation is performed without the use of an external stabilizer as PEG itself can stabilize suspension, however, when NPs are freeze-dried, it is often associated with structural collapse and difficulty in reconstitution or changes in particles physiochemical properties, adding a small amount of stabilizer can prevent this issue [167,170–173]. The nanoprecipitation method is generally used for preparing self-assembled NPs, which consists of supersaturation, nucleation, growth, and coagulation steps [174].

Table 3. Characteristics of PEGylated NPs prepared by nanoprecipitation method.

Polymer	Encapsulated Drug	Organic Solvent	Antisolvent	EE or DL (%)	Stabilizer	Particle Size (nm)	Activity	Refs.
PEG-PLGA	Vinpocetine	Acetone	Water	60-90 •	PVA	30-290	Cerebrovascular disorder	[166]
PEG-PLGA	Docetaxel	Acetonitrile	Water	-	Without stabilizer	153.3	Antineoplastic agent	[175]
PLGA-PEG	Ciprofloxacin	DMSO	Water	1.70-3.29 *	Without stabilizer	174-205	Antibiotic	[176]
PEG-PLA	pEGFP	Acetone	Water	95.56 •	CTAB/Tween 80	128.9	Plasmid vector	[177]
HA–PEG–PCL	DOXORUBICIN	Acetone	Water	92 •	Pluronic F-68	95	Antineoplastic agent	[178]
Poly(isobutylcyanoacrylate/ PCL–PEG	Busulfan	Acetone	Water	17.0 •	Without stabilizer	152	Antineoplastic agent	[167]
PEG-PLGA	Platinum(IV)	Acetonitrile	Water	18.4 *	Without stabilizer	172	Antineoplastic agent	[179]
PEG-PLGA	Dexibuprofen	Acetone	Water	85-100 •	PVA	201-226	Anti-inflammatory drug	[180]

* Drug loading (DL), ● Encapsulation efficiency (EE).

Single (O/W) and Double Emulsion (W/O/W) Solvent Evaporation Techniques

The single-emulsion solvent evaporation method can mainly encapsulate hydrophobic drugs in PEGylated polymeric nanoparticles (Table 4). The PEG-block-copolymer and hydrophobic drug are dissolved in a volatile organic solvent, which is added to the stabilizer-containing water under high-speed stirring or sonication, creating an oil-in-water emulsion (O/W). The organic solvent can be removed by stirring at room temperature; however, the boiling point should be less than water to ensure a complete evaporation process. As the solvent evaporates, the polymer precipitates and forms solid, drug-encapsulated NPs, resulting nanoparticles can be collected by centrifugation, filtration or lyophilization. NP size like in the nanoprecipitation method depends on several factors, such as polymer concentration that affects the viscosity of the organic phase, higher polymer concentrations typically result in larger nanoparticles. The choice of organic solvent plays a significant role in determining the final size of the PEGylated NPs, particularly, the evaporation rate of the solvent affects the rate at which the polymer precipitates to form nanoparticles. Solvents like DCM or chloroform are commonly used as they evaporate relatively quickly, promoting the rapid formation of nanoparticles, slower evaporation rates, on the other hand, allow the polymer to precipitate more slowly, leading to the formation of larger particles. Matsumoto et al. demonstrated that the release of the drug from nanoparticles was affected by PEG content in PLA-PEG-PLA and its molecular weight, copolymers with higher PEG content (25.8%), showed faster drug release (95% in 24 hours). In contrast, copolymers with lower PEG content (5.2%) exhibited slower release (44% in 24 hours), additionally, lower MW of copolymers and higher PEG Mw enhanced the initial and overall drug release [181]. In a different study, Xu et al. demonstrated the difference in paclitaxel drug release profiles between micellar PLGA 45 kDa-PEG 5kDa NPs and the core-shell PLGA 45 kDa/PLGA 20 kDa-PEG 5 kDa NPs which showed sustained release, whereas it seems that PEG content and matrix structure of the nano-system is detrimental for the drug release [182]

Table 4. Characteristics of PEGylated NPs prepared by single emulsion-solvent evaporation method.

Polymer	Encapsulated Drug	Organic solvent	Emulsifier	Particle size (nm)	Encapsulation Efficiency (%)	Refs.
PLGA-PEG	Cyclosporine	DCM	PVA	212	91.90	[183]
PLA-PEG-PLA	Progesterone	DCM	PVA	193-335	65-71	[181]
PLGA-PEG	Curcumin	Ethyl Acetate /DCM	PVA	152.37	73.22	[163]
PLGA-PEG	SN-38	DCM	PVA	249.2	81.85	[184]
PEG-PLA	Rhodamine B	DCM	PVA	169.5	67.79	[185]
PLGA–PEG	Curcumin	DCM	PVA	100-200	52.2	[186]

Meanwhile, O/W is typically used for encapsulating hydrophobic drugs, The double-emulsion or water-in-oil-in-water (W/O/W) technique is useful for encapsulating both hydrophilic and hydrophobic drugs (Table 5). They are dissolved in water and emulsified in an organic phase containing polymer creating a water-in-oil (W/O) which is a primary emulsion, dispersed in water phase forming a water-in-oil-in-water emulsion. The double-emulsion technique allows a more sustained and slow release of therapeutic reagents [165].

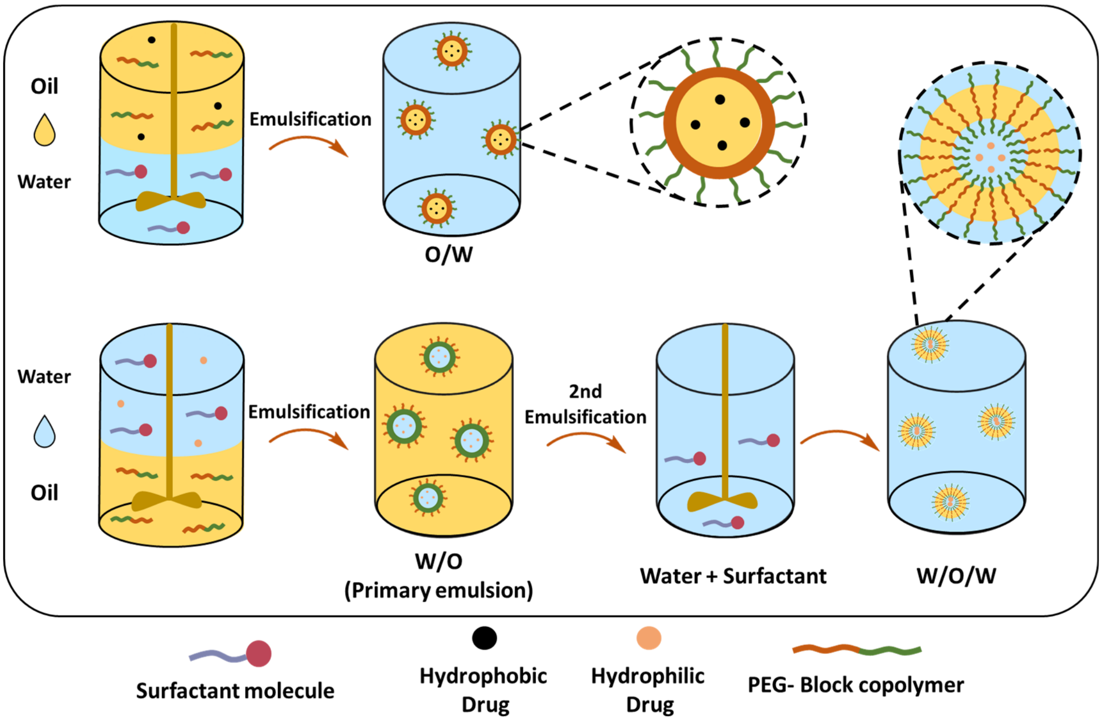

Figure 10. Single and double-emulsion solvent evaporation methods for nanoparticle preparation.

Table 5. Characteristics of PEGylated NPs prepared by double emulsion-solvent evaporation method.

Polymer	Encapsulated Drug	Organic solvent	Emulsifier	Particle size (nm)	Encapsulation Efficiency (%)	Refs.
PLA-PEG	Tetanus toxoid	Ethyl Acetate	Sodium cholate	196	33.4	[187]
PLGA-PEG	Memantine	Ethyl Acetate	PVA	193-224	77-80	[188]
PLA-PEG-PLA	Methotrexate	Chloroform	PVA	100-173	23-48	[189]
PLA-PEG	Tetanus toxoid	Ethyl Acetate	Gelatin	136.8	35.3	[190]
PLGA-PEG	BSA	DCM	PVA	198.1	48.6	[165]
PLGA-PEG	Sorafenib, Doxorubicin	DCM/Acetone	PVA	177.2	88 69	[191]
PLGA-PEG	Paclitaxel, Doxorubicin	DCM	PVA	243.63	70.13 57.5	[192]

Emulsion Polymerization

Emulsion polymerization **(EP)** takes place in the aqueous phase, where the PEG-block copolymers are initially presented as a “monomer droplet”, those block copolymers form micelles above the CMC. Monomers and initiators from the aqueous phase diffuse into the micelles where the polymerization process occurs. As more monomers diffuse inward, polymer chains grow inside the micelle. At the final step, chain growth is terminated and polymer particles are formed (Figure 11). Gurnani et al. synthesized biocompatible core-shell PEGylated butyl acrylate nanoparticles by RAFT emulsion polymerization technique with very small particle size, high biocompatibility, long circulation time and without a need for purification; however, authors report high NP accumulation in the liver in a CD1 mouse [193]. Colombo et al. reported the synthesis of surfactant-free PEGylated PCL NPs loaded with the anticancer drug paclitaxel (TXL) using EP without cremphor, which is widely used for NP preparation, as it increases drug solubility, however, cremophor causes serious side effects including hypersensitivity and toxicity. NPs prepared by EP showed similar antitumor activity to free TXL and the pharmacokinetic behaviour of TXL encapsulated in PCL nanoparticles. It shows no significant difference in drug recovery from urine and faeces compared to free paclitaxel. This formulation offers advantages in terms of safety and potential targeting modifications for enhanced cancer therapy compared to the free drugs [194]. Lupi et al. prepared PEGylated Rhodamine B labelled PCL NPs using an environmentally friendly two-step approach combining ring-opening polymerization and water-based semi-batch emulsion polymerization, which is a surfactant-free process and no organic solvents are required, PEGylation provided stealth properties, fast cellular uptake (within 1-2 hours) and maintained steady concentration in tumours for 120 hours [195]. Similarly, Ferrari et al. prepared PEGylated PLA NPs through surfactant-free and aided copolymerization of (hydroxyethyl)methacrylate (HEMA)-LA_4 with HEMA-PEGm macromonomers, after surfactant removal NPs with short PEG chain length of less than 8.3 nm precipitated; however, when PEG chain length was greater than 8.3, the PEGylated NPs remained stable in PBS solution, and no aggregation was observed, the surfactant addition reduced the final NP size to 30 nm. In the absence of surfactant, as the PEG average chain length increased, the diameter of the NPs tended to decrease at lower copolymer ratios, indicating that longer PEG chains give rise to smaller particles [196].

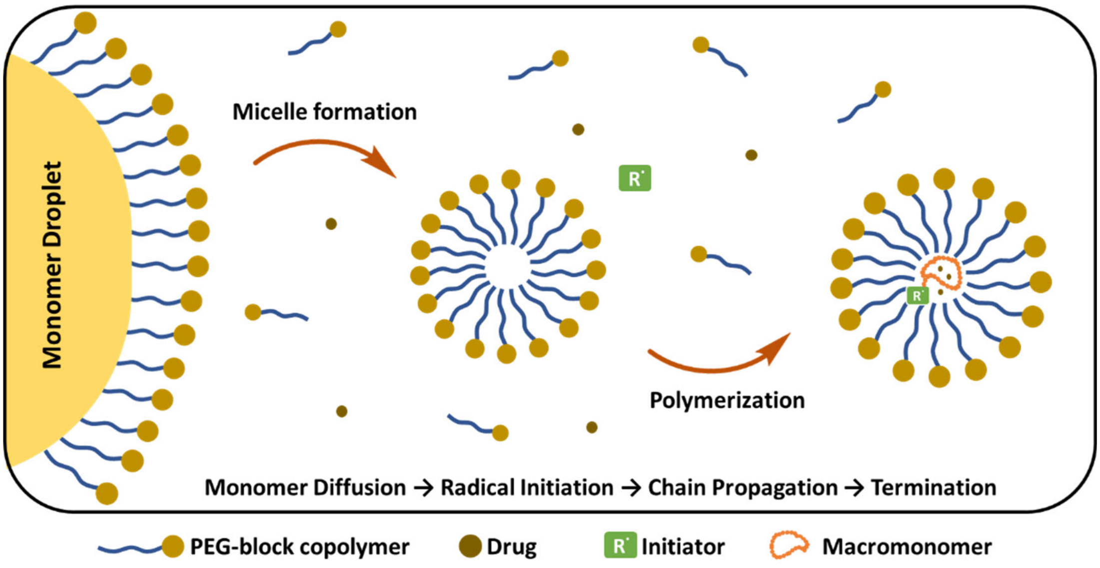

Figure 11. PEG-block copolymer micelle formation and drug encapsulation mechanism.

9. Conclusions

PEGylation technology has revolutionized drug delivery systems by overcoming some of the challenges in drug administration in cancer treatment, infectious diseases, gene therapy and so on. In this review, we examined the evolution of PEG from a simple aliphatic polymer to a sophisticated drug delivery platform, which improves pharmacokinetic properties- such as solubility, stability, and bioavailability, while reducing the toxicity of the drug. The PEGylation improves those properties by stealth effect, thus the nanocarriers evade immune system detection and reach extended circulation times while maintaining safety and biocompatibility. The molecular weight, architecture, and density of PEG chains significantly impact the behaviour of PEGylated systems. Properly optimized chain size and conformation enhance pharmacokinetics, immune evasion, and controlled drug release. Despite these advances, anti-PEG immunogenicity and the accelerated clearance of PEGylated products remain critical challenges. Current research into optimizing PEG molecular architecture, as well as developing new conjugation strategies are important to overcome these limitations.

Author Contributions: Conceptualization, D.M. and J.P.; formal analysis, D.M., L.d.V. and J.P.; writing—original draft preparation, D.M and R.K..; writing—review and editing, D.M., R.K., L.d.V. and J.P.; supervision, L.d.V. and J.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish Ministry of Science, Innovation and Universities through PID2022-140302OB-I00 and by the Generalitat de Catalunya under the project 2021-SGR-01042. This work is part of Maria de Maeztu Units of Excellence Programme CEX2023-001300-M / funded by MCIN/AEI / 10.13039/501100011033.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Not applicable.

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