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Review

Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles

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Abstract

The process of opsonization is one of the most important biological barriers to controlled drug delivery. Injectable polymeric nanoparticle carriers have the ability to revolutionize disease treatment via spatially and temporally controlled drug delivery. However, opsonin proteins present in the blood serum quickly bind to conventional non-stealth nanoparticles, allowing macrophages of the mononuclear phagocytic system (MPS) to easily recognize and remove these drug delivery devices before they can perform their designed therapeutic function. To address these limitations, several methods have been developed to mask or camouflage nanoparticles from the MPS. Of these methods, the most preferred is the adsorption or grafting of poly(ethylene glycol) (PEG) to the surface of nanoparticles. Addition of PEG and PEG-containing copolymers to the surface of nanoparticles results in an increase in the blood circulation half-life of the particles by several orders of magnitude. This method creates a hydrophilic protective layer around the nanoparticles that is able to repel the absorption of opsonin proteins via steric repulsion forces, thereby blocking and delaying the first step in the opsonization process.

Keywords: Opsonization; Poloxamer; Poloxamine; Poly(ethylene glycol); PEGylation; Stealth nanoparticles

Contents

1. Introduction

Through spatial and temporal controlled drug delivery, injectable nanoparticle carriers have the ability to revolutionize disease treatment. Spatially localizing the release of toxic and other potent drugs only at specific therapeutic sites can lower the overall systemic dose and damage that these drugs would otherwise produce. Temporally controlling the release of a drug can also help decrease unwanted side effects that might otherwise occur due to the natural circadian fluctuations of chemical levels throughout the body (Hermida et al., 2001). The overall benefit of these improvements in disease treatment would be an increase in patient compliance and quality of life. In order for a drug delivery device to achieve these desired benefits it must be present in the bloodstream long enough to reach or recognize its therapeutic site of action. However, the opsonization or removal of nanoparticulate drug carriers from the body

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by the mononuclear phagocytic system (MPS), also known as the reticuloendothelial system (RES), is a major obstacle to the realization of these goals.

The macrophages of the MPS have the ability to remove unprotected nanoparticles from the bloodstream within seconds of intravenous administration, rendering them ineffective as site-specific drug delivery devices (Gref et al., 1994). These macrophages, which are typically Kupffer cells, or macrophages of the liver, cannot directly identify the nanoparticles themselves, but rather recognize specific opsonin proteins bound to the surface of the particles (Frank and Fries, 1991). Broadly speaking, opsonins are any blood serum component that aids in the process of phagocytic recognition, but complement proteins such as C3, C4, and C5 and immunoglobulins are typically the most common. Several methods of camouflaging or masking nanoparticles have been developed, which allow them to temporarily bypass recognition by the MPS and increase their blood circulation half-life (Illum and Davis, 1984; Gref et al., 1994; Kaul and Amiji, 2002). Many of these systems make use of surface treatments that interfere with the binding of opsonin proteins to the particle surface as a means of imparting stealth, or MPS-avoidance characteristics to nanoparticles. This review focuses on those systems that utilize poly(ethylene glycol) and PEG-containing surface treatments because these systems seem to hold the most promise and show the lowest occurrence of harmful effects in vivo.

2. Opsonization and phagocytosis

Opsonization is the process by which a foreign organism or particle becomes covered with opsonin proteins, thereby making it more visible to phagocytic cells. After opsonization, phagocytosis can occur, which is the engulfing and eventual destruction or removal of foreign materials from the bloodstream. Together these two processes form the main clearance mechanism for the removal of undesirable components larger than the renal threshold limit from the blood. In the case of polymeric nanoparticles, which cannot normally be destroyed by the phagocytes, sequestration in the MPS organs typically occurs. If the polymeric nanoparticle is non-biodegradable, then accumulation of particles in these organs, most commonly the liver and spleen, can occur leading to toxicity and other negative side effects (Illum et al., 1986; Peracchia et al., 1999a; Plard and Bazile, 1999).

Opsonization typically takes place in the blood circulation and can take anywhere from a matter of seconds to many days to complete. The exact mechanism through which this process is activated is very complicated and not yet full understood, but the important components involved are, for the most part, well known. Immunoglobulins and components of the complement system such as C3, C4, and C5 are known to be common opsonins as well as other blood serum proteins such as laminin, fibronectin, C-reactive protein, type I collagen and many others (Frank and Fries, 1991; Johnson, 2004). The importance of these proteins in the clearance process has been indirectly demonstrated in many in vivo animal studies of inherited and induced C3 deficient animal models. For instance, research has shown that these animal models are often times more susceptible to certain diseases which are easily controlled by phagocytosis in non-C3 deficient animal models (Singer et al., 1994). The opsonins, which are present throughout the blood, are thought to come into contact with injected polymeric nanoparticles typically by random Brownian motion. However, once sufficiently close to the surface of a particle, any of several attractive forces including van der Walls, electrostatic, ionic, hydrophobic/hydrophilic, and others can be involved in the binding of opsonins to the surface of the nanoparticle.

After opsonization has occurred, the next step in the clearance process is the attachment of the phagocyte to the nanoparticle via surface bound opsonins. Without the presence of surface bound or adsorbed opsonin proteins, the phagocytes will typically not be able to bind or recognize the foreign particles. One method of attachment occurs when the bound opsonin proteins undergo conformational changes from an inactive protein present in the blood serum to an activated protein structure that can be recognized by phagocytes. Phagocytic cell surfaces contain specialized receptors that interact with the modified conformation of these various opsonins thus alerting them to the presence of a foreign material.

A second method of phagocyte attachment is the non-specific adherence of phagocytes to surface adsorbed blood serum proteins which can result in the stimulation of phagocytosis as well (Frank and Fries, 1991). This process is typically due to the association of opsonin proteins with a more hydrophobic particle surface. The third significant method of phagocyte attachment is complement activation. The complement system can be activated by one of several mechanisms including the classical, alternative, and lectin pathway. The exact details of these mechanisms are beyond the scope of this review, but several excellent sources are available on this subject (Frank and Fries, 1991; Singer et al., 1994; Morgan, 1995; Johnson, 2004). Regardless of the pathway of complement activation, the final result is the binding and phagocytosis of the foreign particle by the mononuclear phagocytes.

The third and final step in the clearance process is the ingestion of foreign materials by phagocytes. This step in the process typically involves the endocytosis of the particle or foreign material by a phagocyte. Following endocytosis of the particle, the phagocytes will begin to secret enzymes and other oxidative-reactive chemical factors, such as superoxides, oxyhalide molecules, nitric oxide, and hydrogen peroxide, to break down the phagocytosed material (Mitchell, 2004). Unfortunately, most non-biodegradable polymeric nanoparticles cannot be degraded significantly by this process and, depending on their relative size and molecular weight, will either be removed by the renal system or sequestered and stored in one of the MPS organs. As a first approximation, removal by the renal system occurs only for molecules with a molecular weight of around 5000 or less, but can be as high as 100,000 for more dense polymers such as dendrimers. Therefore, non-biodegradable particles and degradation molecules with a molecular weight higher than the renal threshold, typically become sequestered in the MPS organs. The final biodistribution of this sequestration depends on several factors and is discussed in more detail in the biodistribution and pharmacokinetics section of this paper.

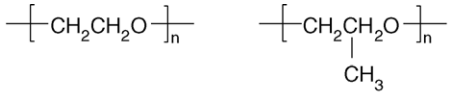
Since the initial opsonization of particles is so critical to the process of phagocytic recognition and clearance from the bloodstream, most research in the area of stealth drug delivery has focused on trying to stop or block this step of the process. There are no absolute rules or methods available to completely and effectively block the opsonization of particles, but research over the last 30 years has found some trends and methods that can be effective at slowing this process, thus increasing the blood circulation half-life and effectiveness of stealth devices. As a general rule, the opsonization of hydrophobic particles, as compared to hydrophilic particles, has been shown to occur more quickly due the enhanced adsorbability of blood serum proteins on these surfaces (Carstensen et al., 1992; Muller et al., 1992; Norman et al., 1992).

A correlation between surface charge and opsonization has also been demonstrated in vitro, with research showing that neutrally charged particles have a much lower opsonization rate than charged particles (Roser et al., 1998). Therefore, one widely used method to slow opsonization is the use of surface adsorbed or grafted shielding groups which can block the electrostatic and hydrophobic interactions that help opsonins bind to particle surfaces. These groups tend to be long hydrophilic polymer chains and non-ionic surfactants. Some examples of polymer systems that have been tried in the literature as shielding groups include polysaccharides, polyacrylamide, poly(vinyl alcohol), poly(*N*-vinyl-2-pyrrolidone), PEG, and PEG-containing copolymers such as poloxamers, poloxamines, polysorbates, and PEG copolymers. Of all the polymers tested to date, the most effective and most commonly used are the PEG and PEG-containing copolymers. These polymers are typically very flexible and highly hydrophilic, which can help shield even hydrophobic or charged particles from blood proteins. They are also typically charge neutral, which lessens the effect of electrostatic interactions.

3. PEGylation

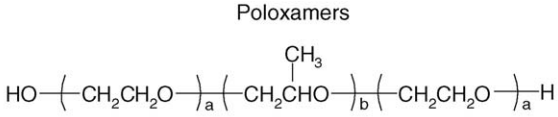
As previously mentioned, the preferred method of imparting stealth, or sterically stabilized properties to nanoparticles is through the PEGylation of these particles. PEGylation simply refers to the decoration of a particle surface by the covalently grafting, entrapping, or adsorbing of PEG chains. Also, in the case of biodegradable nanoparticles, PEG chains can be incorporated as copolymers throughout the particle so that some surface PEG chains are always available even after the degradation of surface layers. The purpose of these PEG chains is to create a barrier layer to block the adhesion of opsonins present in the blood serum, so that the particles can remain camouflaged or invisible to phagocytic cells. Experimental research using freeze-fracture transmission electron microscopy (TEM) has even been able to demonstrate visually the protein rejecting capabilities of PEGylated surfaces (Peracchia et al., 1999b).

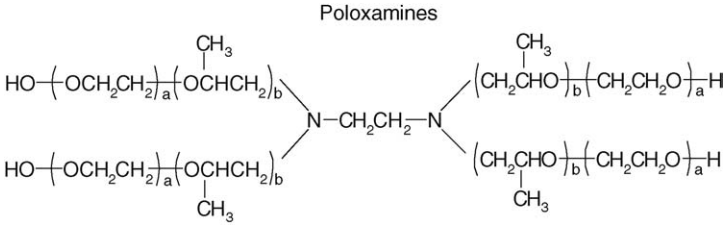
Many different types of PEG-containing polymers have been tested for their ability to impart stealth characteristic to polymeric nanoparticles. The basic repeating units of poly(ethylene glycol) and poly(propylene glycol) are shown below. Because of the chemical structure of the repeating units, these polymers are also known as poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO).



poly(ethylene glycol) poly(propylene glycol)

Tables 1 and 2 contain a representative listing of PEG-containing polymers for adsorbed and covalently attached surface coatings, (adapted from Storm et al., (1995)). From Table 1, it is evident that the vast majority of research in PEG surface coatings has involved surface adsorbed poloxamers and polaxamines.





These polymers are amphiphilic block copolymers consisting of blocks of ethylene oxide (EO) and propylene oxide (PO) monomer units, which are typically formed by anionic polymerization.

The important difference between these structures is the additional methyl group of the PO unit, which makes it more hydrophobic, while the EO unit is more hydrophilic. Therefore, the hydrophobic sections of the polymer which contain PO units can be used to adsorb and anchor the surfactant molecule to the nanoparticle surface, while the hydrophilic EO containing polymers or PEG sections can extend into solution and shield the surface of the particle. This method has the advantage of being fairly simple to achieve and can impart increased MPS-avoidance characteristics to the particles. Conversely, it has the draw back that surface adsorbed PEG polymers can also desorb, leaving holes in surface coverage where opsonins can bind (Neal et al., 1998). The situation is even worse when PEG polymers are surface adsorbed on biodegradable polymer nanoparticles.

Table 1
Studies of the opsonization of polymeric nanoparticles with surface adsorbed PEG and PEG containing polymer layers

Nanoparticle	Surface coating	Reference
Poly(butyl 2-cyanoacrylate) (PBCA)	Poloxamer-338	Douglas et al. (1986)
	Poloxamine-908	Douglas et al. (1986)
Poly(ε-caprolactone) (PCL)	PEG (6000, 20,000)	Leroux et al. (1995)
	Poloxamer-407	Jackson et al. (2000)
Poly(β-hydroxybutyrate) (PHB)	Poloxamer (338, 407)	Muller and Wallis (1993)
	Poloxamine-908	Muller and Wallis (1993)
Poly(lactic acid) (PLA)	PEG (6, 20 kDa)	De Jaeghere et al. (2000)
	Poloxamer-188	Vittaz et al. (1996)
	Poloxamer-338	Muller and Wallis (1993)
	Poloxamer-407	Muller and Wallis (1993); Jackson et al. (2000)
	Poloxamine-908	Muller and Wallis (1993)
Poly(lactic-co-glycolic acid) (PLGA)	PEG (2000 or 5000)-*b*-PLA	Stolnik et al. (1994)
	Poloxamer (184, 188, 388)	Muller and Wallis (1993)
	Poloxamer-407	Muller and Wallis (1993); Dunn et al. (1997); Neal et al. (1998); Park et al. (2003)
	Poloxamine-904	Muller and Wallis (1993); Dunn et al. (1997); Neal et al. (1998)
	Poloxamine-908	Stolnik et al. (1994); Dunn et al. (1997)
Poly(lactic acid): poly(ethylene-co-vinyl acetate) (PLA:EVA) 50:50	Poloxamer-407	Jackson et al. (2000)
Poly(methyl methacrylate) (PMMA)	Poloxamer-184	Troster et al. (1990)
	Poloxamer-188	Leu et al. (1984); Troster et al. (1990)
	Poloxamer-338	Troster et al. (1990); Troster and Kreuter (1992)
	Poloxamer-407	Troster et al. (1990); Jackson et al. (2000)
	Poloxamine-904	Troster and Kreuter (1992)
	Poloxamine-908	Troster et al. (1990); Troster and Kreuter (1992); Troster et al. (1992)
	Poloxamine-1508	Troster and Kreuter (1992); Troster et al. (1992)
	Polysorbate (20, 60, 80)	Troster et al. (1990)
	Polyxyethylene (23) lauryl ether (Brij 35)	Troster et al. (1990); Troster and Kreuter (1992)
Polystyrene (PS)	PEG (2000)	Harper et al. (1991)
	PEG (22,000)	Tan et al. (1993)
	PEG (550)-*b*-BSA (Bovine Serum Albumin)	Moghimi (2002)
	PEG (5000)-*b*-BSA (Bovine Serum Albumin)	Gbadamosi et al. (2002); Moghimi (2002)
	PEG (5000)-*b*-IgG (Rat)	Moghimi (2002)
	PEG (2000 or 5000)-*b*-PLA	Stolnik et al. (1994)
	Poloxamer-184	Illum et al. (1987b); Blunk et al. (1993); Muller and Wallis (1993)
	Poloxamer-188	Illum et al. (1986, 1987b); Blunk et al. (1993); Muller and Wallis (1993)
	Poloxamer-235	Norman et al. (1992)
	Poloxamer-237	Illum et al. (1987b); O'Mullane et al. (1990); Norman et al. (1992)
	Poloxamer-238	Illum et al. (1987b); Harper et al. (1991); Norman et al. (1992)
	Poloxamer-338	Illum and Davis (1983, 1984); Illum et al. (1986, 1987b); O'Mullane et al. (1990); Watrous-Peltier et al. (1992); Muller and Wallis (1993); Tan et al. (1993)
	Poloxamer (401, 402)	Moghimi (2003)
	Poloxamer-407	Davis and Illum (1988); Moghimi et al. (1991); Norman et al. (1992); Porter et al. (1992a,b); Blunk et al. (1993); Muller and Wallis (1993); Moghimi and Gray (1997); Neal et al. (1998); Stolnik et al. (2001); Moghimi (2003)
	Poloxamine-904	Muir et al. (1991)
	Poloxamine-908	Illum et al. (1987a,b); Davis and Illum (1988); Moghimi et al. (1991); Muir et al. (1991); Norman et al. (1992); Watrous-Peltier et al. (1992); Moghimi et al. (1993a,c); Muller and Wallis (1993); Tan et al. (1993); Dunn et al. (1994); Stolnik et al. (1994); Moghimi and Gray (1997); Neal et al. (1998); Moghimi et al. (2003)
	Poloxamine-1508	Muir et al. (1991); Tan et al. (1993)

Table 2
Studies of the opsonization of polymeric nanoparticles with covalently bonded or entangled surface PEG and PEG containing polymer layers

Nanoparticle	Surface coating	Reference
Albumin (BSA)	PEG (1750)	Ayhan et al. (2003)
Gelatin (Type-B)	PEG (5000)	Kaul and Amiji, 2002 (2004)
Polyalkylcyanoacrylate (PACA)	PEG (2000)-*b*-polyhexa decylcyanoacrylate	Peracchia et al. (1999a,b)
Poly(ε-caprolactone) (PCL)	PEG (5000)-*b*-PCL	Gref et al. (1994, 2000); Mosqueira et al. (2001); Ameller et al. (2003a)
	PEG (12,000, 20,000)-*b*-PCL	Gref et al. (1994)
	Poloxamer-188	Chawla and Amiji (2002); Shenoy and Amiji (2005)
	Poloxamer-338	Shenoy and Amiji (2005)
	Poloxamer (188, 237, 238, 407)-*b*-PCL	Ha et al. (1999)
Poly(isobutyl 2-cyanoacrylate) (PIBCA)	PEG (4500)-PIBCA	Peracchia et al. (1997)
Poly(lactic acid) (PLA)	PEG (2000)-*b*-PLA	Bazile et al. (1995); Vittaz et al. (1996); De Jaeghere et al. (2000); Gref et al. (2000)
	PEG (5000)-*b*-PLA	Bazile et al. (1995); De Jaeghere et al. (2000); Gref et al. (2000); Mosqueira et al. (2001); Ameller et al. (2003a,b)
	PEG (10,000 or 15,000)-*b*-PLA	Gref et al. (2000)
	PEG (20,000)-*b*-PLA	Gref et al. (2000); Zambaux et al. (2000); Mosqueira et al. (2001); Ameller et al. (2003a,b)
	PLA-*b*-PEG (6000 or 20,000)-*b*-PLA	De Jaeghere et al. (2000)
	Poloxamer-188	Bazile et al. (1995)
Poly(lactic-co-glycolic acid) (PLGA)	PEG (2000 or 5000)-*b*-PLA	Stolnik et al. (1994)
	PEG (5000)-*b*-PLGA	Gref et al. (1994, 2000); Mosqueira et al. (2001); Panagi et al. (2001); Ameller et al. (2003a); Avgoustakis et al. (2003)
	PEG (12,000 or 20,000)-*b*-PLGA	Gref et al. (1994)
	Poloxamer-407	Dunn et al., (1997)
	Poloxamine-904	Dunn et al. (1997)
	Poloxamine-908	Stolnik et al. (1994); Dunn et al. (1997)
Polystyrene (PS)	PEG (1500)-PS	Meng et al. (2004b)
	PEG (3400 or 5000)-PS	Meng et al. (2004a,b)
	PEG (2000)-PS	Harper et al. (1991); Dunn et al. (1994)
	PS—NH—CH_2—$(CHOH)_2$—PEG (linear 250, 500, 1000, 1500, 4000, 19,000)	Bergstrom et al. (1994)
	PS—NH—CH_2—$(CHOH)_2$—PEG (branched 1000, 1700, 6000)	Bergstrom et al. (1994)

In this case, not only can desorption occur, but biodegradation of the particle can also increase the loss of surface bound PEG moieties. Because of these issues, several different methods have been developed in the literature, see Table 2, to covalently attach PEG chains to the surface of nanoparticles. Some research has directly shown that particles with covalently bound PEG chains achieve longer blood circulation half-lives than similar particles with only surface adsorbed PEG (Harper et al., 1991; Bazile et al., 1995). Nevertheless, there are some disadvantages to this method as well. It is sometimes hard to ensure that covalently binding of the PEG chains occurs at the surface and not in the bulk of the material, if surface coverage is the goal. Also, as a result of this, it can be much more difficult to control and optimize the surface coverage density and conformation. On the other hand, the covalent bonding of PEG chains throughout the particle maybe preferred for biodegradable particles, due to the availability of surface exposed PEG chains during the entire degradation and erosion process.

To create these types of nanoparticle systems, most researchers use a copolymer of PEG with another biodegradable polymer, such as poly(lactic acid), poly(lactic acid-*co*-glycolic acid), or poly(alkylcyanoacrylates). In this case, a surface PEG layer is typically created by addition of PEG containing copolymers to the reaction mixture prior to polymerization. Since these reactions typically employ an emulsion, precipitation or dispersion polymerization in aqueous media, the PEG portion of the copolymer is able to orient itself within the non-reacting water phase, while the biodegradable portion of the copolymer is covalently bonded or physically entangled inside the polymerizing nanoparticle matrix. Alternatively, PEG moieties might also be

covalently bonded to fully formed nanoparticles after polymerization by various "living" polymerization techniques, such as ATRP and iniferter, or through traditional surface functional group chemistry. However, their has only been a small number of stealth nanoparticle systems studied that utilize these more difficult methods of PEGylation (Bergstrom et al., 1994; Dunn et al., 1994).

Several theories have been proposed to explain the apparent protein resistance and stealth characteristics imparted to materials by the incorporation of surface bound PEG. Alternatively, some theories have implied that PEGylated nanoparticles, added in excess, simply overload the opsonization and clearance systems of the body, thereby giving the particles the false appearance of stealth properties (Moghimi and Szebeni, 2003). However, the most widely accepted of these theories is one based on the interactions between proteins and PEGylated surfaces, which supports the hypothesis that PEGylation can add protein resistant (i.e. opsonization resistant) properties to materials (Jeon et al., 1991).

This theory makes the argument that the hydrophilic and flexible nature of the surface PEG chains allows them to take on a more extended conformation when free in solution. Therefore, when opsonins and other proteins are attracted to the surface of the particle, by van der Waals and other forces, they encounter the extended surface PEG chains and begin to compress them. This compression then forces the PEG chains into a more condensed and higher energy conformation. This change in conformation creates an opposing repulsive force that, when great enough, can completely balance and/or over power the attractive force between the opsonin and the particle surface. It is important to note that for effective blocking or repulsion of opsonins to occur, the surface coating layer needs to exceed a minimum layer thickness. The exact thickness of the layer required can vary depending on the situation and is sometimes hard to control. Therefore, layer thickness is usually correlated to other factors such as PEG molecular weight, surface chain density, and conformation.

Most research indicates that a surface PEG chain molecular weight of 2000 or greater is required to achieve increased MPS-avoidance characteristics. This minimum MW is most likely due to the loss in flexibility of shorter PEG chains. Also, it has been shown that as molecular weight is increased above 2000, the blood circulation half-life of the PEGylated particles is also increased, which may be due in part to the increased chain flexibility of higher MW PEG polymers (Gref et al., 1994; Leroux et al., 1995; Peracchia et al., 1997; Peracchia, 2003). In addition to chain molecular weight, surface chain density and conformation are also critical factors to achieving improved stealth characteristics, although these two aspects are much more interrelated. For instance, at low surface coverage, the PEG chains have a larger range of motion and will typically take on what is termed a "mushroom" configuration, where on average they will be located closer to the surface of the particle. Very low surface coverage can also lead to gaps in the PEG protective layer where opsonin proteins can freely bind to the nanoparticle surface. On the other hand, at high surface coverage the PEG chains range of motion will be greatly restricted and they will most often exhibit a semi-linear or "brush" configuration. Although a high surface coverage ensures that the entire surface of nanoparticle is covered, this method also decreases the mobility of the PEG chains and thus decreases the steric hindrance properties of the PEG layer (Storm et al., 1995). A 3D schematic diagram of the PEG "brush" and "mushroom" configurations is illustrated in Fig. 1.

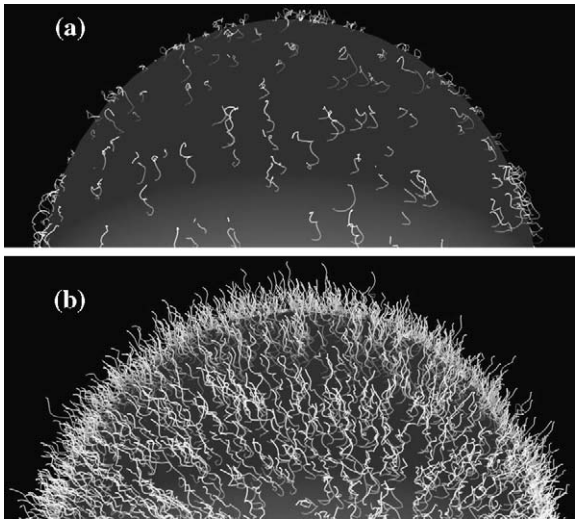


Fig. 1. Schematic diagrams of PEG configurations on the upper hemisphere of a polymeric nanoparticle. In (a), the low surface coverage of PEG chains leads to the "mushroom" configuration where most of the chains are located closer to the particles surface. In (b), the high surface coverage and lack of mobility of the PEG chains leads to the "brush" configuration where most of the chains are extended away from the surface.

Therefore, the optimal surface coverage is located somewhere in between the "mushroom" and "brush" configurations, where most chains are in a slightly constricted configuration, but are present at a high enough density to ensure that no gaps or spaces on the particle surface are left uncovered. As a general guideline, researchers have pointed to a minimum effective hydrodynamic layer thickness of roughly 5% of the particle's diameter, or one that is greater than twice the hydrodynamic radius of the polymer coil in its dilution solution conformation (Stolnik et al., 1995; Storm et al., 1995). It should also be noted that this analysis of surface coverage was developed primarily for solid surfaces, which is not always the case in drug delivery systems. For instance, when the surface PEG chains of swollen hydrogel materials are compressed, there is a finite probability that these chains will penetrate back into the hydrogel matrix itself, instead of being compressed into a higher energy conformation, thereby making the surface coating layer less effective (Huang et al., 2001). Currently, this effect has not been fully studied in stealth nanoparticles and should therefore be taken into consideration when designing stealth hydrogel systems.

4. Biodistribution and pharmacokinetics

Typically once a polymeric nanoparticle is opsonized and removed from the bloodstream, it is sequestered in one of the MPS organs. In the case of "naked" nanoparticles, or nanoparticles that have not been PEGylated and lack stealth properties,

sequestration in the MPS organs is very rapid, typically a matter of minutes, and usually concentrates in the liver and spleen (Illum et al., 1987a; Gref et al., 1995; Panagi et al., 2001). However, for PEGylated stealth nanoparticles the speed of clearance and final biodistribution is dependant on many factors.

Research has shown that particle size plays a key role in the final biodistribution and blood clearance of stealth particles. As discussed earlier, molecules that have a molecular weight less than 5000, or even higher for dense polymers such as dendrimers, can be removed from the body via the renal system. For large molecules and particles that can not be removed by the renal system, research has shown that particles with hydrodynamic radii of over 200 nm typically exhibit a more rapid rate of clearance than particles with radii under 200 nm, regardless of whether they are PEGylated or not (Moghimi et al., 1993b). In other words, a 250 nm PEGylated nanoparticle would be cleared from the blood stream much more rapidly than a 70 nm PEGylated particle. Likewise a 250 nm "naked" nanoparticle would be removed more quickly than a 70 nm "naked" nanoparticle, but both "naked" nanoparticles and the 250 nm PEGylated particle would be removed orders of magnitude more quickly than the 70 nm PEGylated nanoparticle. Besides blood clearance rate, the final biodistribution is also affected by particle size. In the case of PEGylated nanoparticles, a hydrodynamic radius of less than 150 nm was shown to produce an increased uptake of particles in the bone marrow of rabbits, where as particles of 250 nm in diameter where mostly sequestered in the spleen and liver, with only a small fraction of uptake by the bone marrow (Porter et al., 1992b).

Researchers have hypothesized that differences in the uptake and biodistribution of stealth particles indicates the presence of opsonins that are specific to only a certain type of phagocyte. For instance, Moghimi and Patel (1988) hypothesized that an increased accumulation of cholesterol-rich liposomes in the spleen was due to the presence of opsonins specific to splenic phagocytes, which exhibited stronger binding on cholesterol-rich surfaces than Kupffer cell specific opsonins. Also, opsonins specific to the Kupffer cells may have large binding regimes that require the presence of larger particles in order to achieve binding, thus leading to the preferential sequestration of large particles in the liver. Another possible explanation is that size-dependent biodistribution might have more to do with a simple filtering effect, whereby larger particles are removed by the spleen and liver more rapidly while smaller particles are directed to the bone marrow (Moghimi et al., 1993b). Though the exact reason for these size dependencies has not yet been fully elucidated.

Besides particle size, another important factor in determining the final biodistribution and clearance rate of nanoparticles is the PEG layer itself. The characteristics of this layer such as its thickness, charge, surface density, functional groups, and conformation all impact the way in which it interacts with opsonins. Failure to adequately address and optimize anyone of these variables can lead to a dramatic increase in the rate of opsonization and clearance of nanoparticles from the bloodstream. However, the final biodistribution of these opsonized nanoparticles is not as easily predicted. Currently, there is a large amount of conflicting data in the literature, and due to the lack of a comprehensive study of these factors across various animal models, intra- and inter-species variations in animal models, and the variability observed in the raw materials and polymers used to perform these studies, very few definite trends can be developed concerning how these parameters affect the final biodistribution of sequestered particles (Porter et al., 1992a; Hunter and Moghimi, 2002).

Despite these discrepancies, there seems to be at least two general trends that are consistent throughout most biodistribution studies. First, most researchers found that the use of larger molecular weight PEG polymers led to longer blood circulation half-lives for the particles in vivo (Gref et al., 1995). The second common trend showed that uncoated nanoparticles concentrated most heavily in the liver and spleen, but with the addition of PEGylation this final biodistribution was shift toward the spleen. One specific example showed that 24 h after injection 40% of PEGylated particles were found in the liver, while 90% were found in the liver after only 3 min for "naked" particles. On the other hand, after 60 min of blood circulation the concentration of PEGylated particles in the spleen was 12% while it was only 2% for "naked" particles (Peracchia et al., 1999a).

Several different methods are available to study the final biodistribution and clearance rates of these particles both in vivo and in vitro. In vitro methods typically make use of techniques such as flow cytometry and cell-associated fluorescence measurements (De Jaeghere et al., 2000; Jaulin et al., 2000). The typical cell lines used for these studies are human monocytes, murine macrophages, non-parenchymal liver cells, and neutrophilic granulocytes (Neal et al., 1998; De Jaeghere et al., 2000; Jaulin et al., 2000; Zambaux et al., 2000). For in vivo studies, the most popular methods for tracking particle uptake involve the use of radio labeled ^{14}C or encapsulated indium histology studies (Leu et al., 1984; Troster and Kreuter, 1992; Troster et al., 1992; Gref et al., 1994; Bazile et al., 1995). In these studies, the animal model used is typically broken into several groups and injected with radio labeled particles. Then each group is sacrificed at a different predetermined times and histology slices of each animal are prepared and examined using autoradiography. The intensity of radiation form various sections of the histology slice are then correlated to the final biodistribution and rate of particle clearance. The most popular animal models for these studies are rat, rabbit, and guinea pig.

5. Conclusions

The summarized work above demonstrates that the study of stealth nanoparticles and their opsonization by the mononuclear phagocytic system remains a very active and developing area of research. Although the proteins and blood serum components involved in this process are fairly well known, the mechanism by which they activate specific cellular responses and interact with stealth nanoparticles is still not fully understood. Also, the lack of a comprehensive study of these responses across multiple cell lines and animal models and the inherent variability in these systems has hindered our understanding of these mechanisms and produced conflicting results. Furthermore, there are still several

major factors that have not been adequately addressed in this area of research. They include the effect of molecular weight polydispersity and particle size distribution in polymeric systems and intermediate polymer degradation products on stealth properties and biocompatibility. Until comprehensive and systematic studies can be conducted to account for all of these critical factors, there will be some difficulty in achieving truly exceptional stealth properties in polymeric nanoparticle systems. However, despite these issues, great strides have been made over the past several decades at improving the overall MPS-avoidance characteristics and stealth properties of PEGylated polymeric carriers. While this and other research has led to exciting discoveries in the field of stealth nanoparticles, a significant amount of work remains before these systems can be considered safe for use in humans. Hopefully, with more characterization and understanding of the factors that affect stealth materials, long circulating stealth nanoparticle drug delivery in humans will soon become a reality.

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