# CHAPTER 2: THE BIOLOGICAL BASIS OF EFFECTIVE COLLOIDAL DRUG DELIVERY SYSTEMS

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## 2.1 Introduction

As early as the 1970s, the delivery of water soluble anticancer drugs, such as 5-fluorouracil (5-FU), was shown to be enhanced by formulating the drug in various emulsions. The lipid-

absorbing capacity of lymphatic capillaries resulted in increased and rapid uptake of these water soluble drugs in the regional lymph nodes (Agellon *et al.*, 2002) and in longer residence of the drug in local nodes. After intra-testicular injection of 5-FU into rats, the order of lymphatic uptake that was observed was: water in oil in water (w/o/w) emulsion > water in oil (w/o) emulsion > oil in water (o/w) emulsion > free aqueous drug, with lymph node levels being 9.1 and 7.1 times higher than that of I.V. administration and aqueous levels for the w/o/w emulsion. The water soluble 5-FU was probably surrounded by the oil phase in both w/o and w/o/w emulsions, thereby mimicking lymphatic transport during normal fat transport from the intestines (Lipinski, 2000). With the advent of high-throughput screening, the increasingly potent lead compounds have been found generally to be increasingly lipophilic (Trevaskis *et al.*, 2008). A wide array of drug delivery or drug carrier systems, based on conventional emulsion/colloidal systems have been designed, analysed, described, optimized and re-designed during the last few decades and an overview of these systems falls outside the scope of this thesis. More sophisticated lipid-based and non-lipid delivery systems have been developed for vaccine and peptide delivery. The non-lipid delivery systems are usually polymeric in nature and include the cyanoacrylates and polymers of lactic acid and glycolic acid (PLG) (Pandey *et al.*, 2005). These polymers are generally fairly stable, non-toxic, efficient at drug loading, and bio-soluble or biodegradable.

Simple and complex polymers and lipopolymers have been incubated with preassembled supported lipid bilayers (Boxer, 2000) containing peptides or proteins to mimic cell membrane assembly and characteristics, such as lateral mobility (Boxer, 2000; Tanaka, 2005), membrane tethering (Miller, 2007), and biosensor capability (Reimhult and Kumar, 2008). These studies showed that lipid bilayers can be regarded as a biomaterial that can be generated through the self assembly of lipid molecules into mono-, bi- or multi-layered membranes, be it in the form of vesicles, lamellae or on solid support. In such artificial membranes, the lateral fluidity of the leaflets of the bi- or multi-layer found in cellular membranes is generally retained through a combination of van der Waals, electrostatic and hydration forces (Borst *et al*,, 2000). These membranes are typically indefinitely stable, as long as the balance between lipid and water phases remains constant. Appropriate molecules may be entrapped within or adsorbed onto the artificial membrane to further mimic the cell membrane.

A relationship exists between the bioavailability of a drug, the efficient transport of such a drug (directly into the systemic circulation or indirectly *via* the lymphatic system) and the formulation and properties of the drug. The physicochemical characteristics (typically log $P > 5$, long chain triglyceride (TG) solubility > 50mg/g) of some drugs or the properties of the excipients or of the formulation of such drugs make them suitable for lymphatic transport (Trevaskis *et al.*, 2008; Charman and Stella, 1986). Several literature reviews by a number of authors have appeared in recent years (Trevaskis *et al.*, 2008; O'Driscoll, 2002; Porter and

Charman, 2001; Porter *et al.,* 2007). The intestinal lymphatics will be addressed only in so far as it is relevant to the studies described here. The mechanisms of cellular uptake and intracellular lipid trafficking will be addressed in so far as it relates to the uptake of drugs entrapped within lipid-based colloidal systems.

Whatever the system chosen as a carrier, the purpose of such a carrier stays the same: To enhance the efficacy of the administered drug whilst reducing the unwanted side-effects. To fulfill this function, a number of basic requirements need to be met:

- The carrying system must transport the drug from the site of administration to the site of action of the drug.
- At the desired site of action, the system must release the drug so as to achieve its chemotherapeutic or pharmacological effect in the required time frame. Release of the active pharmaceutical ingredient (API) can be aspecific or very specific. Specific, targeted delivery generally requires a more sophisticated and expensive delivery system. The system must be stable before administration, i.e. on the shelf and after administration under *in vivo* conditions.
- The carrier system should be non-toxic, biodegradable and non-antigenic (Agellon *et al.,* 2002).
- Preferably, the delivery system should accumulate selectively in the desired tissue or cell population, through some well-defined and predictable physiological, biochemical or immunological process.

## 2.2 Colloidal systems

The majority of carrier/delivery systems are colloidal in nature. The biodistribution of colloidal particles is dependent on the route of administration of the colloid and the physicochemical properties of the colloid. These include properties such as particle size, and surface characteristics such as surface charge and surface affinity. The impact of these properties is discussed below in reference to the biological milieu of delivery.

A summary of the different types of colloids most often used in drug delivery is described in Chapter 4. Several types of pharmaceutical carriers are listed in Table 2.1. The original hopes expected of lipid based pharmaceutical carriers fell short; these carriers are phagocytozed rapidly by macrophages. The most investigated lipid-based carrier is the liposome. Liposomes have been tested for the delivery of various drugs such as antitumour agents, antibiotics, hormones, proteins and DNA (Porter *et al.,* 2007, Kostarelos, 2003). Liposomes, so named by Bangham *et al.* in 1967 (Bangham *et al.,* 1967), are versatile, non-toxic and biodegradable lipid vesicles, containing one or more concentric lipid bilayers enclosing an equal number of

aqueous compartments in which water soluble substances can be entrapped. Lipid soluble compounds can be carried in the bilayers (Porter *et al.*, 2007).

The Pheroid™ is a hybrid between at least two of these, as discussed in Chapters 3 and 4. In this chapter, the structure/function correlation of delivery systems in general and as it pertains to the Pheroid™ system will be addressed. In order to do so, a closer look will be taken at the biology underlying efficient drug delivery at physiological or macromolecular (see sections 2.3 and 2.4) and cellular level (see section 2.5). In addition, some attempt will be made to look at desired modifications of lipid-based colloids that may contribute to the efficacy of the drug to be delivered (see section 2.6).

### 2.2.2 Factors affecting lipid-based colloidal delivery

The type and nature of both the active pharmaceutical ingredient (API) and the carrying entity must be taken into account during formulation. In this thesis, unless otherwise specified, micro-encapsulation will mean the complete inclusion of a compound in the interior hydrophilic space of a particle of less than 50 µm in size; complexation will mean the association between a compound and the wall or membrane of the colloidal carrier of less than 50 µm in size; and entrapment will mean either and will be regarded as inclusive of both micro-encapsulation and complexation; encapsulation will mean the packaging of a formulation in capsule, be it soft gel or liquid capsules. In general, compounds of a hydrophilic nature will be micro-encapsulated in carrier entities while hydrophobic compounds will be complexed to and within the membrane, depending on the architecture of the particle wall or membrane. In the list below, some of the factors that may affect the efficacy of lipid-based drug delivery are addressed:

- The chemical nature of the active will determine whether a hydrophilic or hydrophobic environment is needed for entrapment, i.e. whether the drug will be micro-encapsulated or complexed to the carrier. This will in turn have consequences for the fate of the drug once it becomes exposed to the biological environment. Micro-encapsulated drugs may be protected against enzymatic degradation to a significant degree while hydrophobic compounds attached to the outside membrane will be more prone to biological attack. Complexation within the small interior spaces within micro-sponges may protect hydrophobic compounds nearly completely. Large molecules, such as proteins, have typically both hydrophilic and hydrophobic domains, the packaging of which may be fairly unpredictable, but some protection will at least be afforded.

Delivery system types. common delivery systems from each type and most widespread biomedical and pharmaceutical uses

| Delivery system types and typical mean particle diameter | Representative systems of each type | Characteristic applications |
|---|---|---|
|  0.5-20 μm | Microspheres hydrogels | alginate, gelatin, chitosan, PLGA microspheres<br>Synthetic, biodegradable, polymer hydrogels | • sustained release of therapeutics<br>• scaffolds for cell delivery in tissue engineering |
|  0.2-5 μm | microparticles | Polystyrene. microspheres | targeted delivery of therapeutics |
|  0.15-5 μm | Emulsions, microemulsions | o/w, w/o/w, lipid. emulsions<br>o/w microemulsions | controlled and targeted delivery of therapeutics |
|  30 - 1000 nm | liposomes | phospholid and polymer-based bilayer vesicles | targeted delivery of therapeutics |
|  5 - 30 nm | micelles | natural and synthetic surfactant micelles | targeted delivery of therapeutics |
|  2 - 100 nm | nanoparticles | lipid, polymer, inorganic nanoparticles | • targeted delivery of therapeutics<br>• in vivo navigation devices |
|  2 - 100 nm | nanocrystals | quantum dots | imaging agents |

Table 2.1. Delivery system types, common delivery systems of each type and its most widespread biomedical and pharmaceutical uses. Modified and reprinted from Kostarelos (2003) with permission from Elsevier.

- Size: the effect of size is controversial and its effect will be dependent on the particular biological barriers it has to traverse, and on its interaction with the specific biological milieu to which it is exposed. The impact of size as it relates to the biological environment, with reference to the lymphatic and reticulo-endothelial systems is discussed further below (see section 2.4).
- Surface charge and interaction with opsonins and dysopsonins based on surface charge, as discussed in section 2.4 below.
- Components of the delivery system: the specific lipid molecules used in the formation of the particle or vesicle may contribute to the structure, the texture, the therapeutic application and the effect of a formulation (Trevaskis *et al.*, 2008; Porter *et al.,* 2007). Some modifications to lipid-based systems that may enhance therapeutic efficacy are discussed in section 2.6 below. In Chapter 3, the specific molecules used in the formulation of the Pheroid™, the long chain fatty acid esters, will receive some attention. The properties of conventional classes of polymers, such as their high polydispersity, limit their application. Polydispersity is a measure of the distribution of molecular weights in a given polymer (Berchane *et al,* 2007). For pharmaceutical purposes, it is generally desirable to have a well-defined polymer with a low polydispersity value, and thus a narrow range of molecular weights. Dendrimers represent a new class of polymer, having well-defined structures with precise control of size and shape as well as terminal group functionality that have found numerous applications including pharmaceutical use, catalysis and electronics (Cheng and Chu, 2008; Pan *et al.,* 2005).
- Entrapment efficiency: an effective formulation of a drug can only be developed if therapeutic doses can be delivered in a reasonable dose of carrier. The entrapment efficiency must be such that the carrier itself does not cause adverse events or toxicity; the delivery system should therefore not cause non-linear (saturable) pharmacokinetics of the drug formulation (Ranger, 1990; Alper, 2008; Walsh *et al.,* 1989).
- Physical and chemical stability: chemical instability in lipid based carriers may be caused by hydrolysis of an ester bond and/or oxidation of unsaturated acyl chains of lipids. Physical instability may be caused by leakage and change of particle size and/or aggregation or fusion of vesicles to form larger particles. Both of these influence *in vivo* performance of the drug formulation (therapeutic index). Physical instability may also occur due to partitioning of a hydrophobic drug out of the bilayer into the solvent in the long term (Nacka *et al.*, 2001; Zuidam *et al.,* 1995; Daoud-Mohammed, 2006; Flaten *et al.,* 2006; Bawarski *et al.*, 2009; Sharma, 1997; Olson *et al.*, 1979; Szoka and Papahadjopoulus, 1980; Mimms, *et al.,* 1981; Hauser and Gains, 1982; Gruner et al., 1985; Hope *et al.*, 1985; Mayer *et al.*, 1985; Mayer *et al.,* 1986).

- Site-specific targeting will be addressed in section 2.6 below.
- Compatibility with a wide variety of drugs and excipients (Kostarelos, 2003; Cametti, 2008; Barry, 2002).
- The potential to be formulated into a number of different dosage forms (Barry, 2002; Chen, 2008).
- Compliance with the desired bio-distribution of the entrapped drug, both in terms of physical location and effective time frame (see Section 2.2.3 below; Kostarelos, 2003; Barry, 2002).
- Ease and requirements of preparation: Lipid-based carriers have great potential in drug delivery but have not entered the market in great numbers so far. Some of the problems limiting the use of these carriers are related to manufacturing and include batch-to-batch reproducibility, sterilisation method, low drug entrapment, particle size control, and production of large batch sizes. A number of these problems have been partially or completely resolved, resulting in an increased number of clinical trials. Filtration is still the preferred method of sterilization as heat, radiation or chemical sterilization may be damaging to delivery system. Filtration, however, is not suitable for large vesicles (Bawarski *et al.*, 2009; Sharma, 1997; Olson *et al.*, 1979; Szoka and Papahadjopoulus, 1980; Mimms, *et al.,* 1981; Hauser and Gains, 1982; Gruner et al., 1985; Hope *et al.*, 1985; Mayer *et al.*, 1985; Mayer *et al.,* 2000).

Some requirements are dosage form specific; these will be referred to where applicable in subsequent chapters. Most of these factors will be addressed in more detail in Chapter 3.

### 2.2.3 Biodistribution, mode of delivery and release of entrapped drugs

The entrapment of a drug in lipid based delivery systems nearly always changes its biodistribution (Trevaskis *et al.,* 2008; Charman and Stella, 1986; Porter and Charman, 2001; Hawley *et al.,* 1995; Charrios and Allen, 2003). For example, IV-injected liposome-entrapped drugs mostly accumulate in the liver, spleen, lungs, bone marrow and lymph nodes. In a process referred to as passive targeting, such entrapped drugs also accumulate preferentially at sites of inflammation and infection and in some solid tumours (Trevaskis *et al.,* 2008). A number of attempts have been made to manipulate the biodistribution of particles *in vivo*, with specific reference to avoidance of the reticuloendothelial system (RES) after IV administration (Charrios and Allen, 2003). Delivery vehicles may be actively targeted by attaching a suitable receptor-interacting ligand to them (Vyas *et al.*, 2001). 'Sterically stabilized' or 'stealth' liposomes can stay in the circulation and can, at least in principle, accumulate passively at a selected site *via* the enhanced permeation and retention effect (Petrak and Goddard, 1989; Petrak, 2005).

The pharmacokinetic profile of doxorubicin (DXR) is altered by its entrapment in pegylated liposomes and follows a dose-dependent clearance saturation curve (Gabizon *et al.,* 2002). Furthermore, the entrapment led to a marked improvement in the toxicity profile, compared with nonliposomal DXR (Ranson *et al.,* 2001). Liposome-entrapped DXR seems to impair the phagocytic function of liver macrophages, thus reducing clearance. A dose increase of the entrapped DXR leads to saturation of clearance, and an increased accumulation of DXR in tumours.

Some of the factors that influences *in vivo* behaviour and biodistribution of lipid-based carriers can be summarized as follows:

- Small lipid-based carriers are cleared more easily that large ones (Trevaskis *et al.*, 2008; Kostarelos, 2003; Petrak, 2005);
- The half-life of a carrying particle increases as lipid dose increases (Trevaskis, 2008; Conrad *et al.,* 1996; Lundberg and Mortimer, 1996);
- Charged lipid-based systems are cleared more rapidly than uncharged systems (Trevaskis *et al.*, 2008; Kostarelos, 2003; MacKay *et al.,* 2005);
- Carriers, such as liposomes tend to leak if cholesterol is not included in the vesicle membrane (Trevaskis *et al.*, 2008; Kostarelos, 2003; McIntosh, 2004; Bhattacjarya and Haldar, 2000).

## 2.3 Anatomical and biological factors influencing drug delivery

### 2.3.1 Route/site of administration

The route of administration is to some extent determined by the class of drug. For instance, drainage into the lymphatic system is desirable for vaccines but not for pain medication. Figure 1 is a schematic illustration of the typical administration routes used for drug administration, as well as the biological distribution system into which the drugs are transported or drained. The schematic illustration is a compilation from a number of sources (Trevaskis *et al.*, 2008; Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.,* 2007; Kostarelos, 2003; Chen, 2008; Petrak 2005; Gabizon *et al.,* 2002; Ranson et al., 2001; Oja *et al.,* 1996; Lundberg and Mortimer, 1996).

The design of a pharmaceutical carrier and its associated dosage form depends primarily on the route of administration and on the drug target and/or indication. For instance, in topical administration the diffusion coefficient and partition coefficient is important and the requirements of both drug and formulation are linked to a biological factor, *viz.* the skin, with a nearly impermeable stratum corneum (Barry, 2002).

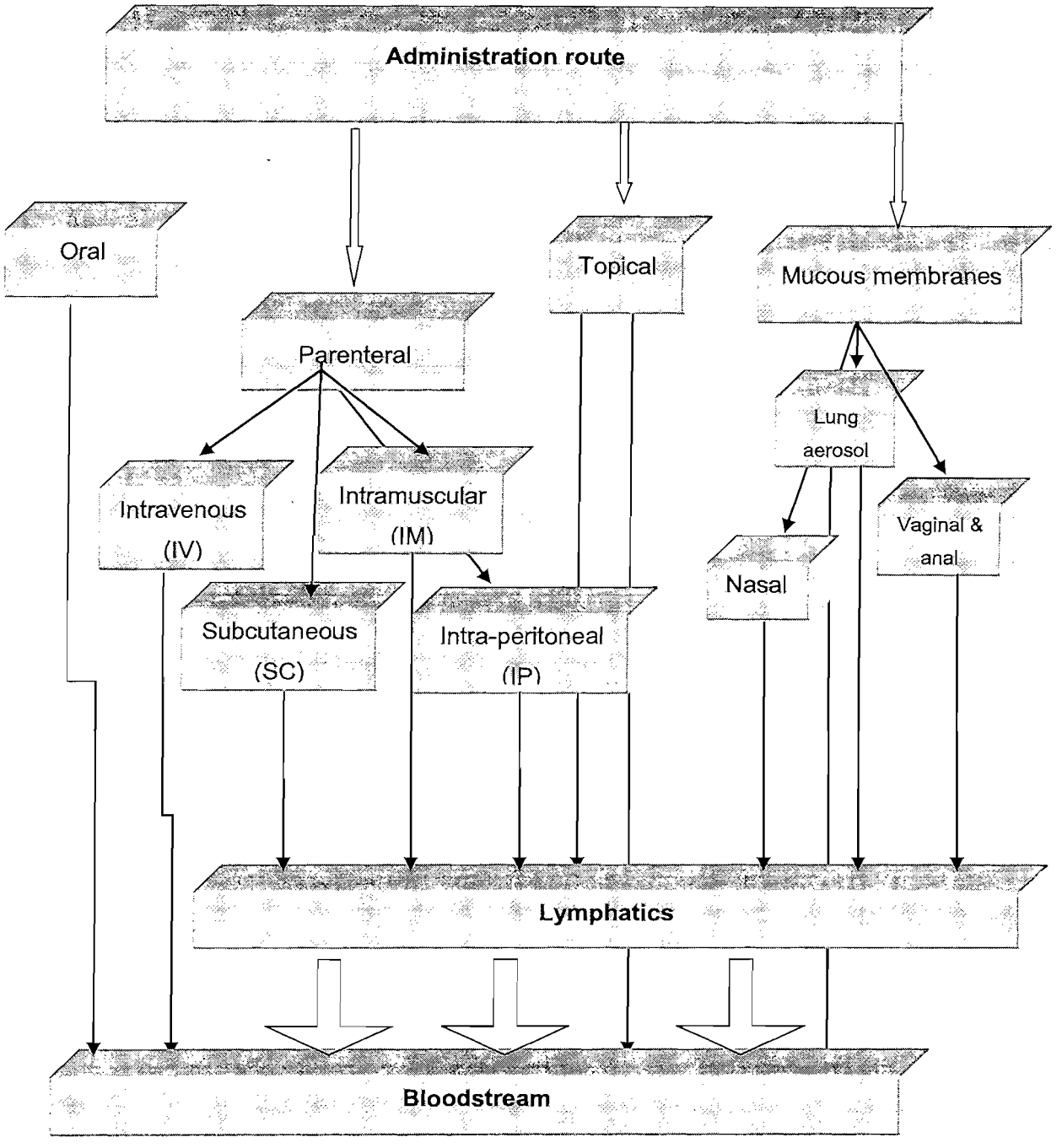


*Figure 2.1. Schematic illustration of typical administration routes used during drug delivery as well as the macromolecular catchment system into which the drugs collect (Trevaskis et al., 2008; Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter et al., 2007; Kostarelos, 2003, Chen, 2008; Petrak 2005; Gabizon et al., 2002; Ranson et al., 2001; Oja et al., 1996; Lundberg and Mortimer, 1996).*

In oral administration on the other hand, the endothelial cells or enterocytes of the intestinum is selectively permeable but the drug is exposed to a varying range of conditions that may cause its degradation or inactivation (Trevaskis *et al.*, 2008; Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.*, 2007; Kostarelos, 2003). Carriers may protect labile drugs while it is being transported to its intended target. In most administration routes, be it oral, transmucosal or parenteral, only a fraction of the administered drug is effectively delivered to its intended target.

### 2.3.2 Parenteral administration routes

The largest proportion of a dose administered parenterally by the subcutaneous, intraperitoneal or intramuscular routes is retained at the site of injection (Trevaskis *et al.*, 2008; Kostarelos, 2003; Hawley *et al.*, 1995; Charrios and Allen, 2003; Vyas *et al.*, 2001; Petrak, 2005). Depending upon the size and composition of the particles, a proportion of such administered colloids drain into the lymphatic system and may be attacked by macrophages within the lymph nodes draining the injection site (Trevaskis *et al.*, 2008; Kostarelos, 2003; Hawley *et al.*, 1995; Charrios and Allen, 2003; Vyas *et al.*, 2001; Petrak, 2005). From the lymphatic system, the colloids are transported to the circulation as described below. Once in the bloodstream, the fate of the particles is similar to those administered by the IV route, but uptake by the liver, spleen and bone marrow is reduced compared to the intravenous route. Within the lymphatic system, size becomes a factor, with the smallest liposomes being collected in the lymph, and the larger liposomes being trapped within the nodes (Trevaskis *et al.*, 2008). Formulation of drugs such as mitomycin-C into gelatin-containing emulsions has been shown to result in increased localization to regional lymph nodes (Trevaskis, 2008). It is uncertain whether the increase was due to the increased droplet size of the formulation or to an interaction between the oil phase of the emulsion and the drug or both. The parenteral administration routes have been used in the studies described in Chapters 6 and 7 and include intramuscular, intraperitoneal and subcutaneous administration, with IV administration mainly used as a control of absolute bioavailability in Chapter 7. The intravenous route has also been used to try to determine the body distribution of the Pheroid™, which will be addressed in Chapter 3. Table 2.2 summarizes some of the characteristics of the parenteral administration routes. The table is compiled from a number of references (Trevaskis *et al.*, 2008, Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.*, 2007; Kostarelos, 2003, Hawley *et al.*, 1995; Charrios and Allen, 2003; Vyas *et al.*, 2001; Petrak, 2005, Gabizon *et al.*, 2002; Ranson *et al.*, 2001; Nishiyama *et al.*, 2003; David, *et al.*, 2004; Lukyanov *et al*, 2004; Iakoubov and Torchilin, 1998, Jelinkova *et al.*, 2003; Volkel *et al*, 2004, Oussoren and Storm, 2001; Oussoren and Storm, 1998; Storm and Crommelin, 1998; Storm and Woodle, 2003).

**Table 2.2: Characteristics of parenteral administration routes**

| Intramuscular | Intraperitoneal administration | Subcutaneous administration | Intravenous administration |
|---|---|---|---|
| The effect of size, surface area and amount of injected lipid of colloids has been described. In mice IM injected liposomes containing insulin showed that smaller liposomes gave the highest lymphatic levels of insulin (Storm and Crommelin, 1998). The lower the total amount of injected lipid, the greater was the uptake of insulin *via* the lymphatic system. Regions of high muscular activity showed increased lymphatic permeability. The toxicity of bleomycin and peplomycin was reduced by IP and IM administration compared to IV administration in mice (Lipinski, 2008). | Liposome size (48 nm to 700 nm) had no effect on lymphatic uptake of $^{14}$C-labelled sucrose entrapped in negatively charged liposomes in rats (Storm *et al.,* 1995). Increased uptake of adriamycin in the lymph of rats 24 hours after IP administration was observed when the drug was formulated in liposomes (Storm *et al.,* 1995). To target the mediastinal lymph nodes, large nanoparticles of polyhexylcyano-acrylate (PHCA; 543 nm) or poly-methylmethacrylate (PMMA) were administered IP to rats, but the % dose recovered in the lymph was low; less than 1% of the administered dose was present in the lymph itself. In contrast, the presence of the drug was higher by between 118 and 226% /g in the mediastinal | Of the three average sizes (33, 98 and 58 nm) of neutral liposomes investigated, the smaller liposomes drained more rapidly from the injection site over a 24 h period, with optimal levels being reached after 6 h in rats (Lipinski, 2003; Trevaskis *et al.,* 2008). Liposomes of sizes 30-60 nm accumulated in the popliteal nodes to a greater extent when compared to larger liposomes (400 nm), with higher circulation levels and faster drainage from the injection site. The presence of negatively charged liposomes with a diameter of 43 nm was increased 5-50-fold in popliteal lymph nodes 3 h after administration, compared to free label (Lipinski, 2003; Trevaskis *et al.,* 2008). Using radio-labelled markers, the rate of localization to lymph nodes were shown to be: negatively charged liposomes > positively charged liposomes > neutral liposomes in some studies, while others | Less likely as colloids generally have difficulty with transcapillary passage because of their size (Iakoubov and Torchilin, 1998; Jelinkova *et al.,* 2003); lymphatic uptake in terms of administered dose is generally low. **Large particles** (>7 micron) are retained in the pulmonary region, while **small particles** (<7 micron) are rapidly phagocytosed by macrophages of the reticuloendothelial system (RES) residing in the liver and spleen (Lipinski, 2003; Trevaskis *et al.,* 2008). |

| | | | |
|---|---|---|---|
| | nodes. Size ranges of between 10 and 50nm PLG microspheres, containing drugs such as aclarubicin, was shown to be optimal for lymphatic uptake. The IP route led to greater localization of mitomycin-C and bleomycin in the lymph nodes than the IM route with o/w delivery being more efficient than that of w/o or free drug formulations (Storm *et al.,* 1995). | showed that neutral and positively charged liposomes were found in lymph nodes or lymphatic metastases soon after administration, but negatively charged liposomes were poorly localised (Storm and Crommelin, 1998). The attachment of ligands, e.g., non-specific human antibodies to the surface of liposomes increased the rate of drainage from the injection site and localization in the efferent lymphatic system. The charge of the particle had an effect on the degree of localization (Storm and Crommelin, 1998). The type of component attached to liposomes determines the nature and rate of change in lymphatic uptake. More mannosyl liposomes were localized in lymph nodes compared to aminomannosyl and control liposomes; liposomes with saccharide enhanced retention at the injection site; dextran or PEG enhanced localization to lymph nodes after injection (Storm and Crommelin, 1998). Immune responses to particulate colloids differ. | |

### 2.3.3 Oral and transmucosal administration routes

Dietary fats are metabolized and become incorporated into chylomicrons in the small intestine from where it enters the blood circulation *via* the thoracic duct of the lymphatic system (Trevaskis *et al.*, 2008; Paine *et al.*, 1996). Lipoprotein lipase (LPL) present on the endothelial cell surfaces hydrolyzes the triglycerides during circulation, resulting in the breakdown products of chylomicrons. These remnants are cleared by the LDL- and remnant receptors present in the liver cells.

After oral administration of a drug, lipid based drug carriers are mainly absorbed across the small intestine and are transported to the systemic circulation *via* the portal vein and the liver. A drug carrier and its drug absorbed after oral administration are generally exposed to the first pass metabolism in the liver. The oral bioavailability of drugs that are highly metabolized on first pass through the liver can be enhanced by transport *via* the lymphatic system. Lipophilic colloids and/ or drugs with a log $P$ >5 and a lipid solubility > 50mg/g may interact with lymph lipoproteins in the enterocyte (Trevaskis *et al.*, 2008). Such interaction may result in drainage into the intestinal lymph from where it is transported to the systemic circulation by the normal lymphatic vessels and ducts. In this case the first-pass metabolism is circumnavigated and the drug concentration in the lymph is significantly higher than that in the cardiovascular system (Trevaskis *et al.*, 2008).

A number of lipophilic drugs and xenobiotics are transported in the lymphatic system after oral administration (Trevaskis *et al.*, 2008). These include cyclosporine, halofantrine, MK-386 (a 5α-reductase inhibitor), moxidectin, mepitiostane, naftifine, penclomedine, probucol, retinoids, testosterone derivatives, fat soluble vitamins and their derivatives, lycopene, DDT and analogs, benzopyrene, PCBs (polychlorinated biphenyls) and a number of lipophilic prodrugs (Trevaskis *et al.*, 2008). Small quantities of hydrophilic drugs such as salicylic acid, isoniazid and caffeine have also been recovered from lymph after oral administration (Trevaskis *et al.*, 2008).

Some drugs and their associated carriers that are applied topically may penetrate the stratum corneum (Barry, 2002). These drugs may enter either systemic or lymphatic vessels. The lymphatic system may therefore be central in the transport, bio-distribution and bioavailability of a drug, despite the fact that the drug may have been administered by different routes.

## 2.4 Physiological barriers to drug delivery

### 2.4.1 Lymphatic uptake of colloidal particles

The lymphatic system is principally involved in the absorption of particulate cellular compounds and plasma proteins from the interstitial fluid across the thin endothelium of the initial lymphatic vessels *via* intercellular junctions (Trevaskis *et al.,* 2008; Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.,* 2007; Hawley and Davis, 1995). Lymph is then returned to the blood through the lymphatic capillaries, which feed into larger collecting ducts feeding into lymph nodes and then to efferent lymphatics, which in turn feed into the *cisterna chyli* and the thoracic duct (Trevaskis *et al.,* 2008; Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.,* 2007; Hawley and Davis, 1995). The circulating blood volume is maintained in this manner. The chemical composition of lymph is very similar to that of plasma, and it contains all the proteins of plasma but at lower concentration - typically 10 - 50% of the amount of plasma proteins. Most immunoglobulins present in the lymph originate from the plasma. Enzymes pass continuously through the interstitial fluid from plasma to lymph and the level of enzyme activity is generally similar to that of the nearby plasma (Trevaskis *et al.,* 2008).

The lymphatic uptake of colloids, whether lipid or non-lipid in nature, is influenced by:

- the physiology of the lymphatic system, particularly the physiological structure of the interstitial space and lymph nodes;
- the physicochemical characteristics of the colloid in question and specifically the size and hydrophobicity of the colloid; and
- the route of administration, with the subcutaneous route generally considered to be most appropriate for targeting to the regional lymph nodes.

Most *in vivo* quantitative studies on the impact of lymphatic content, flow and drainage on drug bioavailability have been performed in the rat model, as it typically entails invasive procedures which cannot be performed on humans (Edwards *et al.*, 2001). The drug plasma profiles can also be determined in the presence or absence of an inhibitor of intestinal chylomicron flow (e.g. Pluronic-L81 or colchicine) (Dahan and Hoffman, 2006) after oral administration of a drug and should give an indication of the influence of intestinal lymphatic transport on bioavailability.

Some correlation ($r^2$ = 0.94) exists between the *ex vivo* degree of association of a number of lipophilic drugs with plasma chylomicrons and the extent of their lymphatic transport (Gershkovich and Hoffman, 2005). This *ex vivo* correlation may be used as a predictive tool of the potential for lymphatic transport of a carrier or drug as it was found to be a better indicator

of the extent of lymphatic transport when compared to the triglyceride (TG) solubility or log *P* for the drugs investigated (Holm and Hoest, 2004). The Caco-2 *in vitro* cell model has been used extensively in the assessment of intestinal epithelium permeability properties and lymphatic transport of drugs (Trevaskis *et al.,* 2008; Murota and Storch, 2005; Ho and Storch, 2001). In attempting to develop a predictive *in silico* method of the correlation between molecular structure of a drug and the extent of its intestinal lymphatic uptake, a relatively complex set of molecular descriptors was needed to predict the likelihood of lymphatic transport (Trevaskis *et al.,* 2008; Holm and Hoest, 2004).

Colloidal particles administered interstitially for therapeutic effect in the regional lymph nodes require sufficient drainage from the site of administration, and should preferentially be taken up into the lymph nodes. The principal barrier to the drainage of particles from a subcutaneous injection is the initial drainage through the interstitium into the lymphatics, with the size of a colloidal particle having the most impact on the drainage rate (Lipinski, 2000). The uptake of the active compounds or particles into the lymphatics is thus a naturally occurring passive process, with the rate of uptake similar to the rate of lymph flow (Lipinski, 2000).

Compounds absorbed from the gastrointestinal tract (GI tract) through its enterocytes can potentially drain into either blood or lymphatic capillaries as both of these are found in the *lamina propria* underlying the enterocytes. Since the rate of portal blood flow is about 500 times that of mesenteric lymph, most of the absorbed compounds are transported to the liver by the portal blood vessel. Although lymphatic capillaries are much more permeable than the corresponding blood capillaries (Leak, 1976) and high molecular weight or colloidal materials may end up in the lymphatic system, the amount of large colloids absorbed into and crossing through the enterocyte is low (Trevaskis *et al.,* 2008). Interaction with lipoproteins and subsequent drainage into the lymphatics has been illustrated by a number of studies (Sieber *et al.*, 1974; Vost and Maclean, 1984; McIntosh *et al.,* 1999). For lipid-based carriers entry into the lymphatics may be easier: The lipids are digested after absorption and following uptake into the enterocytes, triglycerides (TG) are produced from the fatty acids (FA) and monoglycerides (MG). The TG is then built into colloidal lipoproteins (LP) within the endoplasmic reticulum (Trevaskis *et al.,* 2008; Stremmel *et al.,* 2001; Stremmel, 1998; Chow and Hollander, 1979; Strauss, 1966; Endemann, 1993). The lipoproteins enter the mesenteric lymph rather than the vascular endothelium after being exocytosed and crossing the basolateral membrane underlying the enterocytes. Drugs associated with the lipid component and with an affinity for the lipoprotein, i.e. lipophilic drugs, may be transported to the mesenteric lymph by this route (Trevaskis *et al.,* 2008; Stremmel *et al.,* 2001; Stremmel, 1998; Chow and Hollander, 1979; Strauss, 1966; Endemann, 1993; Rajaraman *et al.,* 2005; Levy *et al.,* 2007; Hauser *et al.,* 1998). Trevaskis *et al.*, (2008) suggested that lipid given concomittently with a lipophilic drug

may provide a driving force for lipid digestion, solubilisation and lipoprotein assembly. The nature and amount of lipid can alter the extent of lymphatic drug transport in the following manner:

- Long chain FA and TG composed of long chain FA are more prone to lymphatic drug transport than medium and short chain FA (Palin *et al.*, 1982; Caliph *et al.*, 2000; Sheehe *et al.*, 1980; Feldman *et al.*, 1983; Green and Glickman, 1981; Cheema *et al.*, 1987; Ockner *et al.*, 1972; Bergstedt *et al.*, 1990; McDonald, *et al.*, 1980). Around 50% of the long chain fatty acids (C14 and longer) are transported to the systemic circulation *via* the intestinal lymph; the other 40–60% being transported *via* the portal vein blood. FA with chains shorter than C14 is more hydrophilic and is primarily absorbed *via* the portal blood (Lipinski, 2000; Trevaskis *et al,.* 2008; Kiyasu *et al.*, 1952; Chaikoff *et al.*, 1951).
- The degree of unsaturation of FA influences the extent of lymphatic transport, with mono- and poly-unsaturated FA (MUFA and PUFA, respectively) resulting in larger lipoproteins and a greater enhancement of lymphatic lipid and drug transport when compared with the equivalent saturated FA (Lipinski, 2000; Trevaskis *et al,.* 2008; Shiau *et al.*, 1985; Feldman *et al.*, 1983; Sheehe *et al.*, 1980; Cheema *et al,.* 1987; Holm *et al.*, 2001).
- Phospholipids (PL), such as phosphatidylcholine (PC) and its digestion product, lyso-phosphatidylcholine (LPC) enhance lymphatic lipid transport. The lymphatic transport of both halofantrine (Trevaskis *et al.*, 2006) and α-tocopherol (Koo and Noh, 2001) were shown to be enhanced by LPC.

To exploit the lipid-based absorption processes and the specific characteristics of the intestinal lymphatics, the following applications have been investigated or suggested:

- Prodrugs may be designed within which the drug molecule is covalently linked to a lipid moiety, such as a FA or phospholipid, to enable association with lipoproteins (Trevaskis *et al.*, 2008; Porter and Charman, 2001; Porter *et al.*, 20007; Nordskog *et al.*, 2001; Charman and Porter, 1996; Shackleford *et al.*, 2009). The ester bond between fatty acids and a drug molecule is thought to be unstable, resulting in relatively inefficient transport into the mesenteric lymphatics, while the process of lipoprotein association with di- and triglycerides into phospholipids is thought to follow the physiological processes and the complexes are therefore expected to be more stable (Charman and Stella, 1986; Nordskog *et al.*, 2001; Charman and Porter, 1996; Shackleford *et al.*, 2009). Alternatively, as is the case in this study, the drug may be stably entrapped in a lipid-based carrier, such as the Pheroid™.

- The lymphatic system is also the principle systemic transport pathway for B and T lymphocytes (Trevaskis *et al.,* 2008; Cense *et al.,* 2006; Arya *et al.,* 2006). There is some evidence that suggests that at least some viruses may spread *via* the lymphatic network. The viruses include hepatitis B and C (Umeda *et al.,* 2005; Kessel and Toubi, 2007), morbillivirus (von Messling *et al.,* 2006), canine distemper virus (Lan *et al.,* 2006), severe acute respiratory syndrome (SARS) associated coronavirus (Spiegel *et al.,* 2006) and HIV (Kessel and Toubi, 2007). Antiretrovirals that target AIDS may be more effective when absorbed *via* the intestinal lymphatics, as lymph and the GI tract lymphatic system are thought to be involved in development of the human immunodeficiency virus (Pantaleo *et al.,* 1994). For this reason, lipid-based prodrugs of didanosine have been designed to try to improve the treatment of AIDS (Lalanne *et al.,* 2007).
- It has been suggested that anticancer compounds may be more effective when absorbed *via* the lymphatic route (Lipinski, 2000; Trevaskis *et al.,* 2008; Garzon-Aburbeh *et al.,* 1983) as this route is the primary route of metastatic spread of a number of solid tumours.
- As the biodistribution of drugs are changed by their transportation *via* the intestinal lymph, so may the toxicological profiles also change and side effects may become more or less severe because of a change in the administration route or mode of delivery to the systemic circulation.

### 2.4.2 The lymphatic system

The interaction between proteins and particles of a delivery system depends on the charge the particles bear - either attraction or repellence. Different types and strengths of surface charges of the particles will result in different degrees and types of opsonization. With the exception of the central nervous system (CNS), the optic cornea and lens, and cartilage, all tissues contain lymphatic capillaries that originate in spaces between cells (Trevaskis *et al.,* 2008; Hawley and Davis, 1985). The capillaries (10 to 60pm in diameter) are lined with endothelial cells (0.1-0.6 pm) supported by a discontinuous and tenuous basement membrane (Hawley *et al.*, 1985; Vost and Maclean, 1984; Sieber *et al.,* 1974). The endothelial intercellular junctions are formed by intercytoplasmic connections or interdigitation of overlapping feet and are highly permeable to large molecules (Hawley *et al.,* 1985; Vost and Maclean, 1984; Sieber *et al.,* 1974). Movement of fluid and macromolecules into the lymphatic capillaries cannot normally occur as a result of the pressure differential between the interstitial fluid and the capillaries, as the pressure of the interstitial fluid is sub-atmospheric and lower than that of the average intra-lymphatic system (Hawley and Davis, 1985; Oussoren and Storm, 1998). The

formation of lymph therefore needs a mechanism that would cause the junctions to open. Three mechanisms have been proposed:

(i) The structure of the basement membrane and the presence of clefts between the endothelial cells cause lymphatic capillaries to act as a mechanical pump with the intercellular architecture providing a functional flap valve for unidirectional flow (Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.,* 2007; Kostarelos, 2003; Hawley and Davis, 1985; Oussoren and Storm, 1998; Leak, 1976). Microvascular vasomotion, arterial pulsation and tissue movement cause compression that drives the pump.

(ii) An osmotic pressure imbalance, caused by a similar movement-related external compression, may force protein-poor fluid between the endothelial cells into the capillaries. When the compression is lifted, the lower concentration of molecules in the fluid of the capillaries will generate a solvent suction that would include protein (Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.,* 2007; Kostarelos, 2003; Hawley and Davis, 1985; Oussoren and Storm, 1998).

(iii) Macromolecules may be transported within pinocytotic vesicles across the capillary wall, resulting in significant osmotic pressure (Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.,* 2007; Kostarelos, 2003; Hawley and Davis, 1985; Oussoren and Storm, 1998; Leak, 1976).

The capillaries join to form proximal collecting lymphatics with unicuspid or bicuspid valves that prevent back flow of lymph (Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.,* 2007; Kostarelos, 2003; Hawley and Davis, 1985; Leak, 1976; Sieber *et al.,* 1974; Ohhashi *et al.,* 2005). Although the collecting vessels have thicker walls with underlying connective tissue and smooth muscle, lymph may still be formed across the vessel wall. The thickness of the walls of the vessels increases progessively, with the thickest walls having a trilaminar structure similar to that of blood vessels: smooth muscle in one or two concentric layers enable vessels to contract. Lymph is propelled upstream in this manner, while blood vessels and nerves in the adventitia serves the needs of the lymphatic wall (Leak, 1976; Sieber *et al.,* 1974; Ohhashi *et al.,* 2005) and the basal lamina becomes more pronounced. From the lymph nodes, the efferent lymphatics combine to form post-nodal vessels that branch and reunite, either to the next set of lymph nodes or to larger lymphatic vessels (Sieber *et al.,* 1974; Ohhashi *et al.,* 2005). Lymph from the different parts of the body is returned to the bloodstream at the confluence of the internal jugular and subclavian veins *via* seven groups of collecting lymphatic trunks.

### 2.4.2.1 The lymph nodes

Lymph nodes (2 mm to 20 mm) are composed of lymphoreticular tissue, surrounded by a capsule of dense collagenous fibres and smooth muscle fibers. The principal groups of nodes lie close to blood vessels (Ohhashi *et al.,* 2005). Differentiation and proliferation of B-lymphocytes take place in the germinal centres, and the nodes also form sites for the recirculation of both B- and T-lymphocytes (Cense *et al.,* 2006). Lymph nodes are organized into the three zones populated by different types of lymphocytes, accessory cells and stromal cells (Swartz, 2001). The three main functions of the lymph nodes and its correlating zones are shown in Table 2.3:

Table 2.3: Main functions and correlating zones of the lymph node (Ohhashi *et al.,* 2005; Swartz, 2001)

| Main function | Correlating zone |
|---|---|
| Lymphopoiesis | The cortex with its lymphoid follicles represents the bursa dependent, B-cell area mainly associated with mechanisms of humoral immunity. |
| Filtration of lymph, with lymph nodes acting as mechanical filters that retain particulate matter. The nodes provide at least two types of filtration:<br>(i) a simple mechanical reticular meshwork which traverses the sinuses, and<br>(ii) a more sophisticated biological filter based on reactions of macrophages and reticular cells, aided by the slow passage of the lymph through the channels of the sinuses of lymph nodes (Hawley *et al.*, 1995; Sieber *et al.,* 1974; Ohhashi *et al.,* 2005; Swartz, 2001). | The paracortex, populated by numerous T-lymphocytes, is the main site of cellular immunity. It is also the site for antibody production and cellular immunity responses to regional antigens.<br><br>Particulate antigens are ingested by the reticuloendothelial cells, whereas soluble antigens may be taken up to a very small extent (Hawley *et al.*, 1995). |
| Processing of antigens (Swartz, 2001). | The sinuses present in the medulla vary in size and cell composition according to functional demands. The parenchyma or medullary cords contain lymphocytes, plasmacytoid lymphocytes and mature plasma cells and are the main site of plasma cell proliferation and production of antibodies. The sinuses contain a system of mononuclear phagocytes (Swartz, 2001), with both fixed and mobile macrophages: The flat elongated cells that line the sinuses function as actively phagocytic macrophages and remove any particulate matter and degenerate cells draining into the lymph nodes. These fixed cells are functionally important as the first phagocytic cells in lymph nodes to come into contact with antigen arriving in the lymph. The mobile macrophages are conspicuous during the |

| | |
|---|---|
| | reactive process of histiocytosis, when the sinuses become progressively dilated and filled with macrophages and histiocytes. The sinusoidal macrophages:<br>a) function as a filter in removing, neutralising and degrading antigen and non-antigenic particulate matter (Hawley *et al.*, 1995);<br>b) in collaboration with lymphocytes, initiate an immune response; and<br>c) may function as antigen-presenting cells. |

### 2.4.3 The interstitium as drug delivery barrier

Substances injected interstitially must traverse the interstitial space on its way to the lymphatic system. In addition to the uptake of colloidal particles through the process described above, colloids may also gain access to the lymphatic system through intercellular clefts of patent junctions (Hawley *et al.*, 1995). Interstitial fluid, similar in composition to plasma with the exception of its protein content, is regarded as the precursor of lymph, which forms as fluid crosses from the blood capillary to the initial lymphatic, *via* the interstitial space. The interstitial space consists of fibres embedded in an interstitial fluid containing ground substance. The mechanical structure or platform of the interstitial space is formed by three types of fibers: collagenous and reticular fibers, and elastin (Hawley *et al.*, 1995). At physiological pH, few of these sites are charged, and their behaviour is thus non-ionic. The ground substance is negatively charged at physiological pH as a result of the presence of tissue-specific mucopolysaccharides with low isoelectric points (Hawley *et al.*, 1995).

The ground substance contains regions with a high degree of aggregation resulting in a colloid-rich, water-poor phase and regions with a low degree of aggregation, resulting in a water-rich, colloid poor phase (Hawley *et al.*, 1995; Sieber *et al.,* 1974; Ohhashi *et al.,* 2005; Swartz, 2001). This aggregate distribution causes the formation of narrow aqueous tissue channels of approximately 100 nm in diameter that allow the passage of macromolecules by diffusion (Casley-Smith, 1980). The size of administered particles should theoretically be less than 100 nm in diameter to achieve good drainage from the injection site through the aqueous channels. The major pathway of colloidal uptake is intercellular, *via* the open junctions, although some intracellular vesicular transport may occur (Hawley *et al.,* 1995; Sieber *et al.,* 1974; Ohhashi *et al.,* 2005; Swartz, 2001; Casley-Smith, 1980).

The movement of administered colloids or particles in the lymphatic vessels is inhibited by the lymph nodes and the cells they contain during passage through lymphatic vessels. The propulsion of lymph and the colloids it contains toward the thoracic duct is regulated by both extrinsic and intrinsic factors (Casley-Smith, 1980; O'Driscoll, 1992; Leak *et al.,* 1995). The

extrinsic factors include muscle contraction, respiratory movements, movements of the intestine, venous pressure, gravity, pulsation of blood vessels and local temperature. The most important intrinsic factor is thought to be the rhythmic smooth muscle contractions of the valved lymphatic vessels. Humoral and neural mediators and the vessel distension influence the contractions (Hawley *et al.,* 1995; Casley-Smith, 1980; O'Driscoll, 1992; Leak *et al.,* 1995).

### 2.4.4 Opsonization of colloidal particles

Effective drug delivery may be influenced by the following processes or molecular structures:

(i) On exposure to blood, most particulate colloids become coated with plasma or serum components. IM, IP or SC injected particles are in contact with the interstitial fluid or sera and may interact with the proteins present. Such interaction may prevent or retard particle lymphatic drainage and localization in the lymph nodes (Hawley *et al.,* 1995).

(ii) Colloids are filtrated when passing through the sinuses of the lymph nodes. Because of a combination of Brownian motion and changes in lymph flow rate, colloids may be immobilized within nodal structures by mutually attractive forces.

(iii) Specific and non-specific opsonins may associate with administered particles. Such opsonization may occur in the interstitial fluid before the particle enters the lymphatic vessels, or in the initial lymphatics (Hawley *et al.,* 1995).

(iv) Membrane receptors on the macrophages present in the lymph nodes recognize opsonized particles and will ingest them. Specific opsonins interact specifically with receptors on macrophages, and include the immunoglobulins, such as heat-stable IgG antibodies and the complement system (Hawley *et al.,* 1995; O'Driscoll, 1992; Leak *et al.,* 1995; Charrios and Allen, 2004). In macrophages, only the free Fc portion of IgG, opsonized onto the outermost surface of a foreign body or colloidal particle, interacts with its receptor to cause ingestion of the IgG coated foreign body or colloidal particle. Since IgM antibodies lack the Fc domain, it does not in itself have opsonic activity but opsonization with IgM may activate the complement system to form IgM and C3b (see complement below) complexes, which will initiate phagocytoses. IgG antibodies opsonize colloids, including bacteria, by increasing the hydrophobicity of the particle surface. Macrophages may also recognize the surface type of a particle itself (Hawley *et al.,* 1995; Charrios and Allen, 2004), and this is often the fate of lipid-based drug carriers. As in the case of IgG antibodies, non-specific opsonins may also enhance the adhesivity of particles by altering the surface properties of the foreign particle, the phagocyte, or both (Hawley *et al.,*

1995). Similarly, dysopsonins (serum components that retard or inhibit phagocytosis) affect adherence of foreign bodies to the cell by altering the surface properties of the foreign body or the phagocytes or both. IgA and secretory IgA prevent adherence of microorganisms to epithelial cell surfaces and ingestion by phagocytes (Hawley *et al.,* 1995; Charrios and Allen, 2004). The dysopsonic effect of IgA may be due to the hydrophilic carbohydrate in the molecule. No role has as yet been described for either IgD or IgE in opsonization.

(v) A number of immunological and non-immunological stimuli can activate the effector molecules of the humoral immune system, the heat-labile opsonins that form part of the complement system. C1 through to C5 seem to have opsonic activity, while no phagocytic effect has been shown for C6 through to C9 (Hawley *et al.,* 1995; Carrstensen *et al.,* 1992). The effector molecules and C1-C5 opsonins are present in lymph at concentrations significantly lower than that of blood.

(vi) Complement molecules are normally required for internalization and ingestion of the opsonized particle and include fibronectin, C-reactive protein (CRP) and tuftsin. The effect of fibronectin is limited as particles need to be coated with gelatine for it to bind. CRP activates the complement system as part of the inflammatory response and causes complement molecules to bind with a high affinity to the membrane of specific phagocytes. The phagocytic activity of neutrophils and macrophages is enhanced by the positively charged tetrapeptide consisting of Thr-Lys-Pro-Arg, named tuftsin. Tuftsin may also trigger internalization of particles attached to other cell receptors (Hawley *et al.,* 1995; Carrstensen *et al.,* 1992).

### 2.4.5 Factors influencing colloidal particle uptake

The rate of particle uptake from an interstitial injection site or by ingestion or by transmucosal uptake is influenced by:

(i) The injected or ingested dose and site of uptake;

(ii) Blood or lymphatic flow;

(iii) In the case of parenteral administration, the anatomical site of injection: the barrier to uptake from the peritoneal cavity into the lymphatics after colloids are administered intraperitoneally is the open junctions of the initial lymphatic walls. In this case the size range is of less importance, as the colloids simply drain from the cavity into the initial lymphatics, with no diffusion through the interstitial space (Trevaskis *et al.,* 2008; Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.,* 2007; Kostarelos, 1983; Hawley and Davis, 1985).

(iv) The reticuloendothelial component of the lymphatic system: particles may be recognized by the receptors on both macrophages and reticular cells lining the sinuses

of the lymph nodes, with resultant phagocytosis of the particles (Hawley and Davis, 1995).

(v) Local tissue damage and other pathologies: drug delivery usually occurs because of pathological conditions and the lymphatic system may not be functioning normally. Or the flow rate, distribution and cellular content of the lymphatic system may be altered. A decrease in lymph flow as a result of oedema or nodal obstruction present in malignant disease will hinder drug biodistribution.

(vi) Interaction of particles with serum proteins: the adsorption of serum components onto the particles affects their biodistribution patterns (Hawley and Davis, 1995). Such adsorption depends on the physicochemical properties of particles.

### 2.4.6 Impact of colloidal physicochemical properties on biodistribution

The interaction between colloidal particles referred to above is to a large extent dependent on the physicochemical properties of the particles. Kostarelos (2003) summarized the biological applications achieved by engineering the colloid and interface characteristics of delivery systems. Some of the characteristics are surface charge, phase behaviour, steric stabilization, endosomal escape and mean particle size. A few of these factors are addressed below. Kostarelos did not, however, include biologically based factors, a few of which is included in the factors discussed below:

(i) **Size of colloidal particle:** The size dependence of interstitial particle absorption has been verified in both animal and human studies, and with the use of standard scintigraphic radiocolloids (Trevaskis *et al.,* 2008; Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.,* 2007; Kostarelos, 2003; Hawley *et al.*, 1995; Vost and Maclean, 1984). Drainage into the lymphatics is generally slower with increasing particle diameter: Particles with diameters less than a few nanometres would be exchanged mainly through the blood capillaries, particles with diameters up to a few hundred nanometres would be absorbed into the lymph capillaries and particles larger than few hundred nanometres would be trapped in the interstitial space for a period of time. The size of the gaps in vasculature is one of the determining factors in extravasation of colloids and macromolecules, with the chances of a particle passing through a gap or pore inversely proportionate to the diameter of the gap or pore. This has been found to be true in both normal and in tumour tissue (Trevaskis *et al.,* 2008; Hawley *et al.*, 1995). Altering the size of liposomes has been shown to change their biodistribution. Liposomal doxorubicin (Caelyx, STEALTH® or Doxil®), is approved for use in AIDS-related Kaposi's sarcoma and refractory ovarian cancer, and is in clinical trials for other indications as it seems to be generally effective in treating a variety of solid tumours (Trevaskis *et al.,* 2008; Charrios and Allen. 2003; Petrak, 2005). Entrapment of the doxorubicin (DXR) in STEALTH® liposomes changes the

pharmacokinetics and biodistribution of the drug, with DXR released from the liposomes over several days or weeks. Patients seem to suffer less from doxorubicin-associated side effects although some the subcutaneous side effects may be more severe. Dose-limiting toxicities for various anticancer agents include paraesthesias, oedema, and erythema (PPE) with areas of necrosis in the basal layers of the skin when given as prolonged infusions (Trevaskis *et al.,* 2008; Kostarelos, 2003; Charrios and Allen, 2003; Petrak, 2005). A correlation exists between the development of PPE and the dose intensity of Caelyx therapy and between the development of PPE and the plasma half-life of the drug. This may be the result of the long circulation time and small size (100 nm diameter) of the particle. For instance, the development of PPE correlated with the plasma $t_{1/2}$ of DXR in patients with metastatic breast cancer (Charrios and Allen, 2003). Caelyx may mimic prolonged infusions due to its extended circulating time; the plasma half-life ($t_{1/2}$) of Caelyx is approximately 48 hours in humans. The extended circulation time could allow extravascation and accumulation in pressure areas and/or micro-traumatized cutaneous tissues, such as skin (Charrios and Allen, 2003). This possibility is supported by the fact that a different liposomal formulation of DXR, Myocet™, does not produce PPE and has a different dose-limiting toxicity (leucopoenia). The liposomes of Myocet is larger than that of Caelyx (160 versus 100 nm), its half-life is about one seventh of that of Caelyx, it has a significantly larger volume of distribution and the drug is released more rapidly (Charrios and Allen, 2003).

Tumours are known to have a defective endothelium with gaps in the range of 380–780 nm between the cells, and the blood vessels in tumours are reported to be leakier than normal capillaries (Charrios and Allen, 2003). Tumours therefore seem to be permeable to macromolecules of the appropriate diameter. In view of the dose toxicities, Charrois and Allen (2003) hypothesized that skin accumulation of liposomes may be reduced without compromising tumour accumulation by increasing liposomal mean diameter. They tested whether changes in the diameter of the liposomes led to differential accumulation of liposomes into tumour relative to skin and/or paws. Unexpectedly, an increase in diameter concomitantly reduced liposome accumulation in normal tissues and in tumours. Similarly, the therapeutic efficacy of various sizes of DXR-loaded STEALTH® liposomes in a murine mammary carcinoma model showed that $T_{max}$ (time to peak levels) was not dependent on liposome size. $T_{max}$ of liposome accumulation was twice that in cutaneous tissues relative to tumour tissue and was thought to be influenced by the exertion of external pressure or an increase in circulation, such as movement (Charrios and Allen, 2003). Even though pore size range is heterogeneous in any given tumour, the sizes of the gaps or so-called pores in tumour blood vessels were found to be dependent on the anatomical location of the implanted

tumour in the test animals and on the tumour model used (Charrios and Allen, 2003; 2004). Most authors now suggest that the diameter of a colloidal particle should be smaller than that of the pore to transfer through the pore. However, as will be shown below and in Chapter 3, the surface properties of the membrane of the colloid and its elasticity may be an important contributing factor to its biodistribution.

(ii) **Surface charge:** Size was originally thought to be the major determining factor in the behaviour of particulates after injection. However, the surface charge of colloids affect their lymphatic uptake from both SC and IP injection sites, with the order of colloid localization in the lymph nodes negative > positive > neutral (Hawley and Davis, 1995).

(iii) **Molecular weight:** Compounds with MW < 1000 Da are hardly absorbed by the lymphatic vessels, gaining access to the blood capillaries, whereas molecules with MW > 16000 Da are absorbed mainly by the lymphatics, i.e. as the molecular weight of the macromolecule increases, exchange across blood capillaries decreases, and lymphatic drainage increases. When targeting colloids such as vaccines to the lymphatic system, the effect of molecular weight becomes negligible, as the molecular weight of a colloidal carrier is unlikely to be under 1000 Da. On the other hand, a linear relationship has been shown to exist between the molecular weight of a macromolecule and the proportion of the dose absorbed by the lymphatics draining at a SC injection site (Hawley and Davis, 1995).

(iv) **Particle concentration:** An inverse relationship has also been shown to exist between the number of injected particles and the rate of drainage from the injection site. At particle concentrations above a certain critical saturation value, lymphatic drainage is decreased, owing to increased hindrance of their diffusion through the interstitial space (Hawley and Davis, 1995; Charrios and Allen, 2004). The exception seems to be colloidal oils - researchers at Nottingham found that an increase in the volume of oil (sesame in this case) of subcutaneous (SC) injections led to accelerated oil transport into the lymphatic system (Hawley and Davis, 1995; Charrios and Allen, 2004). Increasing the aqueous volume had no significant effect.

(v) **Particle surface:** The surface properties and interfacial interactions of the colloids with the biological milieu may determine the pharmacological profiles of the delivered therapeutic. Alteration of the particle surface may be generated artificially during the production process or may occur *in vivo* through phenomena such as opsonization. Different arrays of opsonins and dysopsonins are attracted to specific surface characteristics and their attachment will change the surface properties and may lead to a change in conformation of the particle. Such changes will in turn lead to changes in

both the biodistribution and the rate and site(s) of particle clearance (Traveskis *et al.,* 2008; Hawley and Davis, 1995; Charrios and Allen, 2004).

(vi) **Hydrophobicity:** The hydrophobicity of a colloid is one of the major determinants of the phagocytic response. The adsorption of hydrophilic block co-polymer surfactants onto colloids has been shown to reduce the phagocytosis of hydrophobic polystyrene nanospheres by Kupffer liver cells (Carrstensen *et al.,* 1992). This adsorption results in actual fact in the so-called steric stabilization described for lipid based colloids (see below) and is a result of the orientation of the hydrophilic portions of the polymers at the surface so that they protrude into the surrounding environment, forming a hydrophilic 'skin'. Because opsonization is prevented, the sequestration of the particles by the reticuloendothelial system (RES) is avoided, and the circulation times of the colloid and its associated drug is extended. Opsonization can be minimised by a number of factors (Hawley *et al.,* 1995; Charrios and Allen, 2004; Carrstensen *et al.,* 1992). Opsonins preferentially associate with hydrophobic surfaces and it is possible to give the particle a hydrophilic coating by changing the molecular conformation of the surface of the particle or by increasing the surface mobility. A correlation exists between lymph node uptake and the length of the hydrophilic chains of the co-polymers adsorbed onto the surface of a particle. Drainage from the injection site has been shown to be enhanced by the adsorption of long chain block co-polymers such as poloxamine 908 and with block co-polymers with intermediate lengths of polyoxyethylene (Trevaskis *et al.,* 2008; Charman and Stella, 1986; O'Driscoll, 2002; Porter and Charman, 2001; Porter *et al.,* 2007; Kostarelos, 2003; Hawley and Davis, 1995). Work with polyethylene glycol (PEG) polymers showed the correlation between molecular motion of the tails of the polymers and adsorption rates (Kostarelos, 2003; Hawley and Davis, 1995). The effect on adsorption of the ever changing molecular conformation caused by chain mobility is also known as mobile steric hindrance.

All of the above factors must be considered if effective delivery of colloids and their drugs is to be achieved. By manipulating the physicochemical properties, the biopharmaceutical characteristics (e.g. drug release mechanisms) of the carrier systems can be enhanced in addition to overcoming the main physiological barriers.

The delivery of therapeutics requires definition of the stages involved in a delivery event and determination of all critical parameters. Some of the characteristics not yet addressed are the stability in the digestive system, the mechanism of cellular uptake, the mechanism of release of the entrapped drug molecule, the metabolism of the delivery system, the body distribution of the delivery system and the clearance of the delivery system from the body. The section below deals with some of these aspects.

## 2.5 Cellular barriers to drug delivery

In the previous section, the physiological barriers or membranes formed by (a) layer(s) of cells, connecting fibres and biological "glue" were discussed. This section will deal with the basic cellular and sub-cellular barriers or membranes that:

(a) underlie the physical and macromolecular physiological barriers,
(b) generally act as final targets for drug delivery,
(c) direct and screen cellular and sub-cellular uptake of compounds, including colloids and APIs,
(d) generally form the site for the release of an API from its carrier, and
(e) may contain a mechanism for the active release and/or activation of an API.

### 2.5.1 General principles

Cells and sub-cellular organelles or structures are defined by the biological membranes surrounding them, the basic structure of which is the lipid bilayer. This lipid bilayer membrane can essentially be regarded as a disperse system or at the very least as a two-dimensional fluidic system, as it contains different components, mostly proteinaceous in nature, that may diffuse laterally through the membrane or over the membrane surface within the plane of the membrane or it may be transiently or permanently attached to or anchored in the membrane through a lipid based tether (Stolnik *et al.*, 2000). Though the lipid foundation of membranes is relatively simple, the organization of the membranes into three-dimensional functional domains underlies complex functions, such as intercellular contact and communication, the generation and maintenance of electric potential across the membrane, energy transduction as in the cytochrome oxidase and photosynthetic systems, active and directed transport and/or processing of compounds and elements, ligand absorption and directed adsorption, such as tethering of fatty acids and proteins, the creation of signalling pathways, and defensive functions in terms of the immune system (Boxer, 2000; Stolnik *et al.*, 2000).

This section does not aim to describe the basic structure of cell membranes and their functions, but to briefly discuss those structural components relevant to cellular and sub-cellular delivery and release as it pertains to the Pheroid™.

### 2.5.2 Transporters and binding proteins

Dietary lipids may be endocytosed from the small intestine in the form of mixed micelles or vesicles. Clathrin- or caveolae-mediated endocytosis of vesicles containing lipids has been observed across the plasma membrane of hepatocytes, adipocytes and endothelial cell lines (Trevaskis *et al.*, 2008; Stremmel, *et al.*, 2001; Stolnik *et al.*, 2000; Ring *et al.*, 2002; Pohl *et al.*, 2002, 2004). Several families of intracellular lipid binding proteins influence the absorption and intracellular transport of endogenous and dietary lipids and lipid digestion products either

positively or negatively. Lipid transport proteins have been identified on both the apical and basolateral membranes of enterocytes (Trevaskis *et al.,* 2008). Lipid absorption from the lumen of the GI tract across the apical membrane of enterocytes may occur by passive diffusion (Strauss, 1966) or active transport (Stremmel *et al.,* 2001; Stremmel, 1988; Stolnick *et al.,* 2000; Ring *et al.,* 2002). In the case of active transport, protein mediated absorption and transfer has been shown for FA, cholesterol, phospholipids and MG. The proteins involved in the uptake of FA are CD36/FAT (cluster determinant 36/fatty acid translocase) (Endemann *et al.,* 1993; Pohl *et al.,* 2002), scavenger receptor BI (SR-BI) (Febbraio *et al.,* 2001; Krieger, 2001), caveolin-1 and $FABP_{pm}$ (plasma membrane fatty acid binding protein) [Trevaskis *et al.,* 2006; Besnard *et al.,* 2002] while that of cholesterol are CD36/FAT (Rajaraman *et al.,* 2005; Levy *et al.,* 2007), SR-BI (Poirier *et al.,* 1996; Hauser *et al.,* 1998), and caveolin-1 (Bietrix *et al.,* 2006), but also aminopeptidase N (Trevaskis *et al.*, 2008; Rajaraman *et al.*, 2005; Murata *et al.,* 1995), caveolin-1/annexin 2 complex (Rajaraman, 2006), NPC1L1 (Niemann Pick C1-Like 1) (Trevaskis *et al.,* 2008; Kramer *et al.,* 2005; Davis *et al.,* 2004) and possibly the ABC (ATP-binding cassette) transporter P-glycoprotein, which also seems to influence intestinal lipoprotein formation (Altmann *et al,* 2004; Seeballuck *et al.,* 2003; Schmitz *et al.,* 2001). ABC efflux transporters are involved in the transport of metabolised or digested lipids and excess cholesterol and sterol back into the intestinal lumen (ABCG5 and ABCG8), with the exsorption of cholesterol across the basolateral membrane as well as lipid uptake and intracellular lipid trafficking at sites other than the small intestine (Trevaskis *et al.,* 2008; Seeballuck *et al.,* 2003; Schmitz *et al.,* 2001; Borst *et al.,* 2000; Sarkadi *et al,* 2006). Transport across the cytoplasm towards the basolateral membrane is mediated by intracellular lipid binding proteins, including I-FABP (intestinal fatty acid binding protein), L-FABP (liver fatty acid binding protein), SCP (sterol carrier protein) and ABCA1 (Trevaskis *et al.,* 2008).

Drug absorption can be decreased by efflux transporters such as P-glycoproteins (Wakabayashi *et al.,* 2006). The P-glycoproteins effectively transport a diverse range of compounds ranging in size from 250 Da (e.g. cimetidine) to about 1900 Da (e.g. gramicidin) with no obvious structural similarities except that they are amphipathic in nature (Constantino *et al;* 2007). Efflux transporter systems, such as P-glycoproteins MDR1 and MDR2, are found not only in the apical membrane of epithelial cells of the small intestine, but also in various other tissues throughout the body (Schinkel, 1997; Poirier *et al.*, 1996; Davis *et al.,* 2004), including the subepithelial lymphatics and vascular endothelium, and the oropharyngeal and intranasal mucosa, where they actively pump drugs and other compounds out of the cells. One of the functions of MDR1 is the transport of peptides, such as growth factors (Constantino *et al.,* 2007). Efflux transporters could also act as additional barriers to drug transport. The presence of P-glycoprotein was shown histologically in the apical regions of the ciliated epithelial cells and in the submucosal vessels (Wakabayashi *et al.,* 2006). MDR1 (and other transporter

systems such as the multidrug resistance associated protein, MRP1) has been found in the human nasal respiratory and olfactory mucosa, where it causes the efflux of a variety of hydrophobic and amphiphilic compounds from the cells. This efflux may contribute to cell detoxification of xenobiotics (Schinkel, 1997). P-glycoprotein is thought to attenuate the transport of a range of drugs that act as substrates for P-glycoprotein from the intranasal cavity to brain tissue following nasal administration (Wieland *et al.,* 2000). When drugs such as verapamil and quinidine were co-administered with rifampicin (also see Chapter 5), a P-glycoprotein efflux inhibitor, uptake of the drugs into the brain was enhanced.

### 2.5.3 Intracellular trafficking

Caco-2 cells have been used extensively as an *in vitro* model to investigate intracellular lipoprotein assembly and the influence of lipids and lipidic excipients on drug incorporation into lipoproteins and lymphatic transport (Trevaskis *et al.,* 2008; Dahan and Hoffman, 2005; Stremmel *et al.,* 2001; Altmann *et al.,* 2004; Graff and Pollack, 2005; Levy *et al.,* 1995). Lipid digestion products such as FA, Ch, MG and LPC appear to cross the enterocyte cytoplasm by passive diffusion. Interaction between these lipid based products and intracellular lipid binding proteins (ILBPs) leads to intracellular solubilization of lipids and may as such impact on the uptake and intracellular trafficking of drug molecules either by direct interaction between the ILBPs and the drug, or indirectly by altering the pattern of lipid disposition. The intestine and liver fatty acid binding proteins (I-FABP and L-FABP respectively) (Stremmel *et al.,* 2001; Seeballuck *et al.,* 2004; Bass, 1998), sterol carrier protein (SCP) (Besnard *et al.,* 2002), retinol and retinoic acid binding proteins and ileal bile acid binding protein (I-BABP) (Bass, 1988) are amongst the proteins involved in cytoplasmic trafficking of lipid-based carriers and/or drugs.

A number of proteins have been shown to be involved in the acute intestinal response to lipid ingestion. These proteins are coordinately regulated and influence the rate and extent of intestinal lymphatic lipid and drug transport. In a hierarchical cascade, proteins such as the nuclear peroxisome proliferator activated receptors (PPAR) (Murphy, 2002; Harrison, 2002; Poirier *et al.,* 2001) regulate the transcription of a number of proteins involved in lipid absorption. One of these families of proteins is the FABP that are upregulated in response to lipid ingestion (Willson *et al.,* 2000). Several findings described in the literature suggest a significant role for L-FABP and I-FABP in the lymphatic transport of lipids and their associated drugs, be it directly or indirectly. One of the rate limiting steps during lipid transport into the lymph is the budding of vesicles from the endoplasmic reticulum (ER) to the Golgi. Both I-FABP and L-FABP are capable of initiating such vesicle budding (Chawla *et al.,* 2001). Drugs with structural similarities to FA, the endogenous ligand of I-FABP and L-FABP, showed a high affinity for these two binding proteins (Neeli *et al.,* 2007).

Drug binding affinity to I-FABP and transport of lipophilic drug molecules were found to be related to drug lipophilicity (Porter *et al.,* 2007). The expression of I-FABP and L-FABP mRNA (messenger ribonucleic acid) in small intestinal epithelial cells seems to be regulated by the presence of relatively small quantities of lipid (Porter *et al.,* 2007; Velkov *et al.,* 2005) and transcriptional up-regulation of I-FABP and L-FABP mRNA was described after a chronic diet of high fat in both rats and mice (Bass, 1988; Trevaskis *et al.,* 2006; Poirier *et al.,* 2001). The mRNA status was related to the rate of uptake and transport of lipids into the intestinal lymph in the presence or absence of the model drug halofantrine (Velkov *et al.,* 2005).

Several pathways exist for

- the uptake of lipid digestion products from the intestinal lumen,
- the intracellular processing of the digestion products in the enterocyte, and
- the transport of the products to the systemic circulation *via* either the intestinal lymphatic system or portal vein blood.

These pathways are extensively described and can be found in a number of sources (Trevaskis *et al.,* 2008; Kostarelos, 2003; Feldman *et al.,* 1983; Nordskog *et al.,* 2001; Poirier *et al.,* 1997; Nilsson, 1968; Sato, 1970; Scow *et al.,* 1967; Le Kim and Bezing, 1976; Ottolenghi, 1964; Clark and Tercyak, 1984; Gallo *et al.*, 1977).The size and density of lipoproteins formed in the intestine are dependent on the mass and type of lipid ingested. The type of complex formed - very low density lipoproteins (VLDL) or chylomicrons (CM) – depends on both the ingested lipids and the size and the density of the lipoprotein complexes. The lipid component of these complexes has its origin in a number of sources (Trevaskis *et al.,* 2008; Ockner *et al.,* 1972; Shiau *et al.,* 1985; Pool *et al.,* 1991; Ockner *et al.,* 1969; Baxter, 1966):

- Exogenous lipid is absorbed from the intestinal lumen after a fatty meal;
- An endogenous lipid flux from enterocytes to the mesenteric lymph is maintained even in the fasting state, with lipid sources being the intestinal blood supply;
- *De novo* synthesis in the enterocyte; and
- Bile and desquamated enterocytes. In rats, about 50% of the lipids are derived from bile.

### 2.5.4 Long chain fatty acids and intestinal drug absorption

The extent of lymphatic transport of the lipophilic antimalarial, halofantrine (Hf) in the presence of an acceptable dosage of a long chain lipid-based formulation, was found to be substantially greater (28.3%) than the 1.3% recovered in lymph after administration of a lipid free formulation of halofantrine in the fasted state but was less than the 54% recovered in lymph when the drug was administered after a lipid meal (Tso and Balint, 1986; Khoo *et al.,* 2001). The amount of TG recovered from the lymph over a 10 hour period was more than the

sum of the exogenously supplied long chain lipids supplied and the normal fasting TG transport into the lymph. It would thus seem that dosing with the long chain lipid formulation resulted in the recruitment of endogenous lipid into the lymph. Similar results were obtained in rats: continuous infusions of oleic acid increased both endogenous and exogenous lipid transport into the lymph in a dose dependent manner (Pool *et al.,* 1991; Ockner *et al.,* 1969). The administration of oleic acid containing halofantrine in lymph cannulated and bile-duct cannulated rats increased both endogenous and exogenous lipid transport into the lymph when compared with a positive control containing normal saline with a similar dosage of halofantrine. The results also indicate that the endogenous lipid recruitment was specifically dependent on the lipid dose (Khoo *et al.,* 2001).

The lipid droplets that form within the endogenous cytosolic lipid pools and that are transported into the lymph, range in size from a diameter of around 60 nm (VLDL) to large drops with a diameter of up to 400 nm (Trevaskis *et al.,* 2005; Hussain, 2000; Mansbach and Nevin, 1998; Tso *et al.,* 1987). Two lipid precursor pools exist in the cytosol of the enterocyte: a portal and a lymphatic precursor pool. The portal lipid precursor pool is distributed throughout the cytoplasm in the form of discrete lipid droplets, while the lipid droplets of the lymph lipid precursor pool are typically located in the lipoprotein assembly pathways in the ER and Golgi. The portal lipid precursor pool lipids may be redirected to the lymph lipid precursor pool *via* hydrolysis and resynthesis. Lipids may in fact be interchangeable between the two pools as the size of the two pools has been shown to be inter-related in the fasting state and after administration of exogenous lipids (Davidson *et al.,* 1986; Sabesin and Frase, 1977; Tipton *et al.,* 1989). The size of the portal lipid precursor pool may be inversely related to the efficiency of lymphatic lipid output. Trevaskis *et al.* (2005) studied FA and halofantrine transport from the lymph lipid precursor pool into the lymph in a steady state lymph-cannulated rat model (Traveskis *et al.,* 2008; Trevaskis *et al.,* 2006; Velkov et al., 2005; Khoo *et al.,* 2001). The rate of transport of radioactive FA and halofantrine into lymph and the rate constants and the mass of FA and drug in the lymph lipid precursor pool were calculated from the washout profiles. An investigation into the source of the lipid found in the lymph showed that exogenous FA (oleic acid) was the major lipid source in the lymph as well as the lymph lipid pool after administration of oleic acid dose formulations of 20 mg FA/h. In this case drug and lipid transport was found to be proportional to the sum of the exogenous and endogenous FA in the lipid pool after administration but the first order turnover rate constants describing halofantrine turnover were lower than the rate constants for oleic acid. The exogenous oleic acid was found to be the primary driver of lymphatic drug transport (Khoo *et al.,* 2001) but the observed increase in the rate of lymphatic FA transport was not in direct proportion with the increase in size of the lymph lipid pool, again suggesting that the transport of FA from the lymph lipid precursor pool into the lymph may be saturated as the lymph lipid pool expands. The rate limiting factor is probably

the rate at which a lipoprotein transport vesicle buds off from the ER membrane (Chawla *et al.,* 2001).

Enterocyte-based drug metabolism can also influence drug kinetics]. Drug absorption from the intestines may be limited as a result of the coordinated action of intestinal enzymes and efflux transporters (Mansbach and Dowell, 2000; Benet *et al.,* 1996; 2004). One such enzyme, cytochrome P450 (CYP) 3A4, has been shown to contribute to the intestinal first-pass metabolism of drugs (Wakabayashi *et al.,* 2007; Wacher *et al.,* 1996). Indeed, again using the steady state lymph cannulated rat model, Trevaskis *et al.* (2008) showed with the help of the CYP3A inhibitor ketoconazole, that halofantrine is susceptible to first-pass metabolism to desbutylhalofantrine by CYP3A. A dynamic interplay seems to exist between CYP3A and P-glycoproteins in the intestine and the liver, and P-glycoprotein efflux transport can enhance or impede metabolism by CYP3A (Benet *et al.*, 1996).

Since the delivery system under discussion in this thesis is composed partly of modified unsaturated fatty acids, some attention is paid to the role of these molecules in lymphatic uptake. The difference between the observed first order rate constants of halofantrine and long chain fatty acid mobilization may be an indicator of enterocyte-based metabolism, as a reduction in the rate of enterocyte-based metabolism of halofantrine was shown when the lymph lipid precursor pool was expanded by the FA. The FA seems to contribute in negating the influence of both enterocyte-based and hepatic first-pass metabolism. The hepatic first pass metabolism has been well reviewed and will be referred to in Chapter 5. In summary, the rate and extent of lymphatic drug transport is proportionate to the size and turnover kinetics of the lymph lipid precursor pool. This precursor pool may be expanded by formulation excipients or colloidal lipids to enhance lymphatic drug transport. Results described in the literature suggests that lipid doses, and more specifically long chain FA of acceptable oral dosage size, do have an impact on intracellular drug disposition.

## 2.6 Modifications of lipid-based delivery systems for drug delivery

As can be discerned above, there are several physiological advantages to the use of lipid-based colloids for drug delivery. These formulations can include oils, surfactant dispersions, emulsions, self-emulsifying drug delivery systems (SEDDS), solid lipid nanoparticles and liposomes. Liposomes are the most used colloid to date, but amounts reaching the drug target sites have generally been low. The delivery of drugs may be improved by modifying the basic colloidal system. A more extensive description of the classification of the Pheroid™ as poly-disperse or complex disperse system may be found in Chapter 4 in the incorporated book chapter and in the dissertation of Uys (2006). In this chapter some manipulation of colloidal

systems will be discussed as it pertains to the pharmaceutical applications of the Pheroid™ system.

Colloids can be classed as either lyophobic or lyophilic. The lyophilic colloids include micelles and microemulsions and are thermodynamically stable whereas the phases of the lyophobic colloids tend to separate. In order for colloidal dispersions to be stable, a repulsive force comparable in range and magnitude to van der Waals forces, which is relatively long range in character and may extend over a distance of a few nanometres, is required. Van der Waals attraction is only one of the forces to be considered when designing a particle-based delivery system. The stability of many colloidal systems, including emulsions, nano- and microparticles, and self-aggregating colloids, can be described by the classic DLVO colloid theory. This abbreviation has its origin in the surnames of the authors of the theory: Derjaguin, Landau, Verwey and Overbeek. The so-called DLVO potential is based on two components: van der Waals attraction and electrostatic repulsion. To explain the stability of several uncharged colloidal systems such as lecithin liposomes of high purity, additional forces were added to the attraction and repulsion components. For unstable systems, forces such as repulsive hydration, protrusion and undulation forces were introduced (Flaten *et al.,* 2006; Mayer *et al.,* 1985; Lukyanov *et al.*, 2004; Chow and Hollander, 1979), while attractive hydrophobic and electrodynamic forces were added when too strong stability was observed.

Some surfaces are responsive to stimuli. This is the case with cell membranes that show patterns of physical behaviour based on surface equilibration dynamics when the surface possesses reciprocal mobility (Paine *et al.,*1996; Keszei *et al.,* 2005). Another possibility is a bilayer system that can be reversibly switched from the liquid crystalline to the gel phase within the range of room temperature. A polymer may be adsorbed to the surface without changing the chemical composition and without the bilayer being penetrated or destroyed. Local changes in the physical properties of bilayers have been shown to allow bilayer deformation, and vesicle division and fusion may take place (Paine *et al.,*1996).

### 2.6.1 SEDDS/SMEDDS and all the other EDDSs

Self-emulsifying drug delivery systems (SEDDS) belong to the lipid-based class of delivery formulations. In these delivery systems, the rate-limiting dissolution that may hinder the absorption of hydrophobic drugs in the crystalline state (Zie and Granick *et al.,* 2002; Tang *et al.,* 2008) is avoided as the drug to be delivered remains in solution in the intestines. Formulation of poorly water-soluble and lipophilic drugs in SEDDS has been shown to improve the oral bioavailability of these drugs in general. The improved bioavailability may in part be due to the fact that lipids often alter biopharmaceutical properties. These alterations include an increase in the dissolution rate and solubility in the intestinal fluid, protection of the drug from

enzymatic attack and chemical degradation and improved lymphatic transport of highly lipophilic drugs as a result of lipoprotein involvement (Zie and Granick *et al.,* 2002).

Self emulsifying formulations are usually isotropic mixtures of oil/lipid and a suitable solubilizing agent that can be a surfactant or a surfactant in combination with a cosurfactant, and within which the desired drug is dissolved. In the preparation, fine emulsion/lipid droplets are formed. Emulsions with particles of approximately 50 nm and less diameter on dilution with physiological fluid, are referred to as self-microemulsifying drug delivery systems (SMEDDS) (Zie and Granick *et al.,* 2002).

The lipid component is generally a fatty acid ester or a medium or long chain fatty acid that may be saturated, partially unsaturated or unsaturated. The lipid component can be in a liquid, semisolid or solid form at room temperature, depending on the level of saturation. The raw materials containing such lipid component include mineral oil, vegetable oil, silicon oil, lanolin, refined animal oil, fatty acids, fatty alcohols, and mono-/di-/tri-glycerides (Xie and Granick *et al.,* 2002a and 2002b; Hauss, 2007). The solubilizing agents are typically non-ionic surfactants with a relatively high hydrophilic–lipophilic balance (HLB) value. For stable SEDDS, the surfactant concentration generally has to be between 30% and 60% (w/w) (Xie and Granick *et al.,* 2002a and 2002b). The self-emulsification process is dependent on:

- the nature of the lipid;
- the nature and concentration of the surfactant;
- the oil/surfactant combination;
- the oil/surfactant ratio; and
- the temperature at which self-emulsification occurs.

Efficient self-emulsifying systems (SES) require very specific combinations of pharmaceutical excipient and oils or lipids (Friedman, 2007). In addition, the physicochemical compatibility between the SES and the drug will in turn determine the efficiency of drug entrapment.

As described in section 2.5.4, both the nature of the fatty acid or triglyceride, including the chain length and degree of saturation, as well as the dosage of administered lipid, have an impact on drug absorption and blood/lymph distribution. The presence of lipids may similarly promote the absorption of lipophilic drugs into the portal blood (Caliph *et al.,* 2000). Some of the disadvantages of SEDDS/SMEDDS are the high production costs, the low drug loading and the possibility of irreversible drugs/excipients precipitation. Furthermore, the systems may suffer from low stability and few dosage forms are applicable for these systems (Zie and Granick *et al.,* 2002; Constantinides, 1995). However, the most serious drawback of self-

emulsification systems is that the large quantity (30-60%) of surfactants in the formulations can induce gastrointestinal (GI) irritation.

The essence of SEDDS and SMEDDS are self-emulsification. As such, the Pheroid™ drug delivery system can be classified as a SEDDS and/or SMEDDS except that the concentration of the surfactant is much much lower and the co-surfactant is in fact part of the lipid component, as will be described in Chapter 3.

### 2.6.2 Drug targeting strategies

When a drug is administered, it is absorbed and then distributed in the body. Only a portion of the administered dose reaches the pharmacological site(s) of action. The remaining fraction acts on non-pharmacological sites(s) and may result in an undesirable adverse reaction. Several publications describe and mathematically analyze targeted site-selective delivery kinetics (Prajapati and Patel, 2007; Petrak, 2005; Stella and Himmelstein, 1985; Hunt *et al.*, 1986).

Targeting drugs to specific pharmacological sites(s) enhances the therapeutic index and/or bioavailability of the drug while reducing the toxic side-effects without loss in drug potency. Site-specific drug delivery or, in other words, drug targeting can be classified into three different levels of selectivity:

(i) organ/tissue targeting - delivery to specific organs or tissues;
(ii) cellular targeting - delivery to specific cells within the target organs;
(iii) sub-cellular targeting - delivery to specified sub-cellular compartments in the target cell.

Despite significant efforts over the past 40 years, an effective site-specific drug delivery system has yet to be developed. Various efforts have been made to define a minimum set of requirements for site-specific delivery (Prajapati and Patel, 2007). The so-called 'magic bullet' - a drug that is aimed precisely at a disease site and that would not harm healthy tissues - has yet to materialize. Very few drugs bind solely to their intended therapeutic target.

In a drug-carrier approach, a drug is attached to or packaged within a macromolecular carrier *via* a chemically labile linker or through the use of chemical attractions and affinities. The carrier then transports the drug to the biological site preferred by the carrier and not by the drug, thus changing the pharmacokinetics of the drug. The site of delivery by the carrier would preferably be the site of action of the drug and the drug is released at the target site through some mechanism. Drug delivery by a drug carrier thus entails that several sequential steps (e.g. drug binding or packaging, drug transport and release) are correctly executed and that a number of conditions are met (e.g. drug suitability, nonspecific interactions, and target site access). Some of these steps and/or conditions are under the control of the drug manufacturer

and some steps are regulated by the body itself. For a drug to exert its desired effect it needs to be in physical contact with its pharmacological or physiological target, such as an organism or receptor of a specific cell respectively. In principle, every drug may therefore benefit from selective delivery. At least two types of selectivity should be looked at: site-selective drug delivery relates to a specific anatomical location of the body whereas function-selective drug delivery relates to specific cell type or organism. Most published research is limited to an anatomical target site, while function-selective drug delivery has received very little attention.

Drug targeting can be either a passive or an active process. Passive targeting exploits the natural distribution of colloidal particles *in vivo*. The basic structure of the colloidal particles may be either unmodified or if modified, the modification is not aimed at altering the bio-distribution. In active targeting the bio-distribution is altered by a modification of the particles aimed specifically at redirecting the particle to a site different to the site of natural distribution. In this manner, drugs can be directed to specific cells, tissues or organs.

Physical targeting falls somewhere in between passive and active targeting: the relative increased permeability of some tissues and malignancies may be used as an advantage and has in fact been used successfully in the development of more effective cancer therapies. This type of targeting may be relevant to the studies described in this thesis and can be achieved by:

- the entrapment of well-characterized pharmaceutically active agents in an effort to improve the physical properties of the agent (Chapter 5);
- the addition of any specific chemical moiety that might alter the bio-distribution of the particles (Chapter 3);
- a prodrug or pro-delivery system strategy where activation of these complexes to pharmaceutically active compounds occurs predominately in the target cells (Chapter 3 and 5).

In a similar approach, the phenotypic differences between tumour and normal tissues may be employed for drug targeting (Petrak, 2005). Such phenotypic differences can include a change in the type of receptors or an increased number of receptors or antigens and elevated protease activity at the cell surface. Changes in these types and numbers of molecules are not necessarily correlated with genetic changes. Neither the physical nor the phenotypic targeting approach meets all of the requirements for pharmaceutical targeting. Attempts at inhibition of specific unwanted cellular processes in cancer therapy have been only partially successful in pre-clinical and clinical studies (Boddy *et al.,* 1989).

A biochemical process inherent to the body itself may be used in selective targeting. The mechanism of action of Rituxan®, the first therapeutic antibody approved by the FDA (1997) for

the treatment of cancer, is based on the binding between the Rituxan® antibody and the CD20 antigen located on the surface of normal and malignant B cells (Petrak, 2005). Binding leads to the killing of the marked or tagged B cells by the body's own defence mechanisms. Healthy B cells can regenerate after treatment since B cell progenitors or bone marrow stem cells lack the CD20 antigen, thus escaping the triggered immune response. The B cell population usually returns to normal levels within months, but serious side effects are still associated with the treatment (Petrak 2005).

The phagocytic system of the liver and spleen remains a major site for passive targeting of particles. However, passive carriers can also respond to the conditions in the body, i.e. it may be biologically responsive. Acid and salt triggered multifunctional poly(propylene imine) dendrimers containing guanidium groups for targeting as well as poly(ethylene glycol) chains for stability and protection, have been described (Cheng and Xu, 2008; Pan *et al.,* 2005). Release of drugs from the dendrimers is proposed to be through a change in pH in the body. The clinical applicability of this system remains to be demonstrated. Poly(vinylpyrrolidone-co-dimethyl maleic anhydride) modified superoxide dismutase has been shown to accumulate in the kidneys after intravenous administration in a mouse model. It may be possible to use this natural route by which macromolecules is eliminated from the body to target drugs conjugated to such a macromolecule to the kidneys (Prajapati and Patel, 2007). Coating of polystyrene and poly(methyl methacrylate) nanoparticles with certain surfactants led to enhanced but inexplicable accumulation of the particles in the lung (Lipinski, 2000; Nishiyama *et al.,* 2003; Prajapati and Patel, 2007). Enhanced uptake of surfactant coated particles has also been illustrated in heart, brain and kidneys. These organs contain relatively few macrophages, and the accumulation may be the result of adherence to the capillary endothelium rather than phagocytosis (Petrak and Goddard, 1989; Satchi-Fainaro *et al.,* 2004). A surfactant coat has been shown to reduce hepatosplenic uptake by 40-60% in the short term but not in the long term (Trevaski *et al.*, 2008; Shackelford *et al.,* 2009; Wu and Ojima, 2004). Partial removal of coating from the particle surface in the circulation may be responsible for loss of the ability to avoid phagocytic cells in the long term. The polymer poloxamer-188 has been reported to be relatively easily displaced by plasma components (Trevaski *et al.,* 2008; Petrak, 2005, 2009; Wu and Ojima, 2004).

In active targeting, a drug carrier with targeting properties should have structural characteristics that specifically interact with the target. However, the ligand-receptor interaction upon which targeting to disease sites is generally based, often relies on the differential expression of receptors at the disease site, or on the existence of a patho-physical difference between the targeted site and the normal tissue. Angiogenesis inhibitor TNP-470 is used for

treatment of patients with metastatic cancer (Petrak, 2005; Huang and Allen, 2001). Using this drug, at least two different carriers were tried in an effort to target the drug:

- Immunoliposomes that target dividing endothelial cells were produced by chemically linking a single-chain variable fragment (scFv A5) directed against human endoglin to the surface of liposomes (Volkel *et al.,* 204). Although tumour growth slowed, patients suffered from neurotoxicity at the doses required (Volkel *et al.,* 204), generating some doubt as to the efficiency of targeting.
- A water-soluble conjugate of TNP-470, chemically linked to the HPMA copolymer, Gly-Phe-Leu-Gly, was shown to selectively accumulate in tumour vessels (Huang and Allen, 2001). The activity of TNP-470 was both enhanced and prolonged by this linkage in an *in vivo* hepatectomy and murine tumour model.

In another approach, acetylated dendrimers conjugated to folic acid targeting agents, were coupled to the API methotrexate (Stolnick, 1995). These modified dendritic polymers were administered intravenously into immunodeficient mice that suffered from human malignancies and that over-expressed the folic acid receptor. The folate conjugated nanoparticles specifically accumulated in the tumour and the liver tissue, the methotrexate antitumour activity was increased and the toxicity was decreased. Polymer-directed enzyme prodrug therapy (PDEPT) was also based on this synthetic polymer (Kukowska-Latallo *et al.,* 2005). This treatment consists of a polymeric prodrug and a polymer-enzyme conjugate that allows the rapid generation of cytotoxic drug at the tumour site. This enzymatic based therapy significantly decreased tumour growth in comparison to the free drug and polymer.

The number of targeted receptors expressed and accessible at the disease site, compared with the total number of this receptor expressed and accessible in the rest of the body, also referred to as the 'overall mass balance' by Petrak (2005), should determine the relative efficacy and toxicity ratio. Thus the efficacy of a site-selective delivery system will reflect the relative distribution ratio for the physical characteristic used for targeting. Because antibodies recognize their specific antigen, they are increasingly used as drugs, as drug carriers and as targeting moieties for other drug carriers (Vyas *et al.,* 2001; Lukyanov *et al.,* 2004; Jelinkova *et al.,* 2003), but their use may not always result in sufficient benefit, as was shown with a series of paclitaxel-monoclonal antibody conjugates (Petrak, 2005; Satchi-Fainaro *et al.,* 2003). On the other hand, the same group of researchers conjugated epidermal growth factor receptor (EGFR) to a cytotoxic C-10 methyldisulfanyl-propanoyl taxoid and this led to complete inhibition of tumour growth in the treated mice (Wu and Ojima, 2004). Similarly, DXR conjugated to an anti-B-cell antibody that targets CD74 resulted in successful treatment of SCID mice that were challenged with lymphoma cells (Ojima *et al.,* 2002).

Not all drugs require targeting or are suitable for selective delivery. At least three scenarios exist where targeting may not be advantageous:

(a) Some drugs, such as therapeutic antibodies, have an inherently high specificity for their target and do not require targeting.

(b) The therapeutic action of drugs that have the same anatomical or biological site of action and toxicity may not be improved by selective delivery as the efficacy : side effect ratio may be prohibitive.

(c) The time available for interaction and release of drugs may not justify targeting, i.e. drugs have to be retained at the site of action for a period (which will differ for various drugs) that will allow specific release of a targeted drug.

### 2.6.3 Bioavailability *versus* therapeutic efficacy

Bioavailability cannot summararily be equated with therapeutic concentration. A number of factors will contribute to the therapeutic concentration at any time. Although perhaps oversimplified, the formulas (i) to (iii) below reflect some of the factors that will determine the efficacy of a drug delivered by a delivery system:

(i) The circulating therapeutic concentration at any time T (generally termed bioavailability) = the amount delivered to systemic circulation – the amount cleared from systemic circulation.

(ii) The therapeutic concentration at the site of action at any time T = the therapeutic concentration determined above – the amount not at the therapeutic site.

(iii) The effective therapeutic concentration available at the site of action at any time T = the amount of drug released at the target site available and available for a sufficient time period to allow effective drug action.

The circulating therapeutic concentration at any time T will be dependent on the initial dosage and the amount absorbed from the GI tract and transported to systemic circulation and this will determine the rate of delivery of a drug-carrier conjugate to the target site. Sufficient amounts of the drug conjugate should reach the target within a specific time period to maintain a pharmacologically and therapeutically effective concentration at the site. However, it does not follow automatically that the drug delivered at the target is available – the drug needs to be released from its carrier. In addition, a therapeutic level of the drug has to be maintained at the site for a time period that will allow drug action to take place. This efficacy time profile is therefore dependent on both the characteristics of the API and the release mechanism of the API from its carrier. The rate of such release is determined by the interaction between the drug and its carrier within the specific biological milieu of the target site, the manner and the efficiency of release of the drug from its carrier. For instance, a drug may be conjugated to a

carrier by different linkers and a change in the specific linker may determine drug efficacy. In this regard, the use of a succinate linker for a paclitaxel-antibody conjugate was compared with a glutamate linker, with the glutamate-linked product showing a 16-fold increase in the half-life of the release profile of the drug (Woodle *et al.,* 1994). The antitumour activity of the glutamate linked product was also higher in a mouse test model than the succinate linked product.

If the systemic clearance of the drug-carrier conjugate is too rapid, the delivered API concentration at the target site might not be therapeutically effective. The benefit : cost or risk ratios of using site-selective targeted delivery should be sufficient. A drug that is released at sites other than the target may become toxic at the non-targeted sites, forcing the administration of concentrations that are not therapeutically effective. The complete elimination of both the API and its carrier from the body is preferable. Since the carriers and the drug-carrier conjugates are often too large to be cleared through the kidneys, the conjugates are removed from the circulation by the liver (Petrak and Goddard, 1989).

The drug loading capacity of delivery systems differs. The system selected needs to be suitable for the amount and type of API to be delivered and released and the biological processes at the target site to which it is exposed. The amount of API released at non-target sites but reaching target sites through the circulation may be greater than the amount of API actually being delivered site-selectively. In addition, APIs may be exposed to higher enzymatic activity at the target site or to escape mechanisms developed by organisms to be killed at target sites. Claims of preferential or non-preferential site-selective delivery should therefore not be deduced from apparent efficacy, but should be supported by quantitative measurement of the actual amount of API delivered at the target site.

Delivery of the API-carrier conjugate to the target organ might not guarantee an adequate amount of the free drug at the actual target, particularly if that target is intracellular. Processes to be taken cognizance of include release from carriers, transport across the cellular and/or intracellular membranes to the targeted organelles, drug release, drug metabolism and further processing. Permeability of cell membranes and body-fluid components must be considered in the design and application of drug carriers. Drug delivery at the cellular level is subject to the same pharmacokinetics and pharmacodynamics as that applied to the compartmentalized body.

### 2.6.4 Steric stabilization of bilayers – the use of polymers

The particles in colloidal systems generally need to be stabilized to retain their stability in biological environments. Stabilization may be required because the adsorption of various blood components onto the surface of the particles has to be prevented. For example, the interactions between opsonin ligands and one or more receptors on the macrophage cell

surface may be prevented. There is some speculation that a contributing factor to steric stabilization is the fact that the surface of the particles starts to mimic the cell surface and confers a relative 'invisibility' to the colloidal particles (Petrak, 2005). The hepatic first pass metabolism is diminished and the MPS (mononuclear phagocyte system) avoided to some extent. Stabilization can be achieved through electrostatic or steric repulsion between the particle and its surroundings. Colloidal particles of delivery systems, which often belong to the lyophobic class, can be sterically stabilized by the adsorption or chemical attachment of polymer molecules to the surface of the colloidal particles - the attractive forces between particles can be counterbalanced by either electrostatic or steric repulsion. Polymer adsorption can modify the following macroscopic properties:

- Membrane leakiness can be modified (Keszei *et al.,* 2005; Griffiths *et al.,* 2003; Mayer *et al.,* 2000);
- The size and geometry of lipid-based particles can change owing to the adsorption of polymer (Mayer *et al.,* 1985; Mayer *et al.,* 1986; Keszei *et al.,* 2005; Vogt and Bechinger, 1999);
- Membrane stiffness can be altered (Cates, 1991).

A number of lipid-based colloidal systems has been sterically stabilized by surface adsorption or grafting of polymers. Steric stabilization is in essence based on the additional repulsive force of the surface associated polymeric compounds, which are typically flexible carbon backbones with a hydrophobic anchor and hydrophilic tails or buoys. In practice, steric stabilization has the following advantages over electrostatic stabilization (Storm and Crommelin, 1998; Storm and Woodle, 2003; Storm *et al.,* 1995; Proffit *et al.,* 1983):

(i) Electrolytes do have an influence on steric stabilization and may be used to manipulate the surface of the particle;
(ii) Steric stabilization seems to be effective over a wide particle concentration range, including high particle concentrations;
(iii) If the surface bound polymer have solvation properties, the particles should be equally effective in polar and nonpolar media;
(iv) Some stabilized particles show dry-rehydrate stability;
(v) Flocculated particles can be redispersed;
(vi) Sterically stabilized particle formulations may be subjected to freeze-thaw cycles.

The generation of a repulsive steric barrier to the adsorption of biological components such as plasma proteins that may promote opsonization, contributes to the stability of sterically stabilized particles in the biological environment. Theoretically, neither the nature of colloid or polymer nor the mode of polymer binding should matter, but in practice the strength of polymer association with the particle surface can make a big difference. The polymer layer bound to

one particle must repel that bound to the other particle if steric stabilization is to be achieved. It has been shown that the adsorbed or surface-bound molecules are under stress and that the stress can be relieved by desorption from the particle surface. To prevent desorption, and to optimize steric stabilization by flexible non-ionic polymers the following is usually required:

(i) strong surface anchoring of the stabilizing moieties in the lipid bilayer;
(ii) the lipid surface should be completely coated with polymer to prevent a lateral movement of the polymer chains over the surface, as the resultant Brownian collisions may result in desorption or so-called 'thinning' of the polymer coat; and
(iii) the polymer coat should be of sufficient thickness.

Complete coating of the surface and strong anchoring should prevent bridge flocculation between coated particles and maintain the dispersion state by the repulsion between the coated surfaces. Repulsion has been shown to be dependent on the chain length of the polymer and again on the coating or grafting density. Two models have been proposed (Storm and Crommelin, 1998; Storm and Woodle, 2003; Storm *et al.,* 1995, Kostarelos, 2003; Trevaskis *et al.,* 2008):

- The mushroom model is applicable to situations with low surface coverage so that the polymer chains cannot interact among themselves. In this model, protein adsorption onto the surface is prevented, but not the adsorption of smaller macromolecules;
- The brush model where interaction between polymer chains cause them to extend from the surface because of the higher density of their packing. In this situation, lateral pressure between extended polymer chains may allow exposure of hydrophobic sites and therefore weak protein adsorption.

An explanation of the calculation of the steric repulsion between particles for both models falls outside the scope of this thesis, but these calculations give an indication of the relevant factors contributing to steric repulsion, and therefore to the stability of the system. According to the original calculations of DeGennes, the following is of importance: steric repulsion is based on the distance of the extension of a polymer above the surface, which is determined by the segment size of the polymer and the degree of polymerization, which in turn is related to the average distance between grafting or anchoring points, i.e. the density of packing (Storm and Woodle, 2003; Keszei *et al.,* 2005; Jakobs *et al.,* 1999).

The brush model is reminiscent of the brush model of hyaluronic acid in the interstitium. Looking at that model, the above models may suffer from some deficiencies, as the interbilayer spacings and the flexibility of the surface-attached polymer chains may not be receiving sufficient attention. Fairly strong compression may be exerted on the particles during extravasation or drainage into the lymphatics and the steric stabilization should allow for such

compression - unmodified bilayers have been known to collapse and shattering owing to compression may occur (Keszei *et al.,* 2005). This led to the development of elastic particles such as transferosomes. Such elastic particles have not appeared in commercially viable pharmaceutical products yet, and its success is therefore not really known. The compressibility and elasticity of particles will be addressed again in Chapter 3.

Since a summation of the small adsorption energies per segment of polymer adds up to a large adsorption energy per molecule (Keszei *et al.,* 2005), a high degree of adsorption of polymers onto bilayers from solution may take place. The exposure of a supported lipid bilayer to dilute polymer chains in solution results in high affinity and very rapid adsorption. The driving force for adsorption is thought to be electrostatic attraction between the negative charges on the polymer chains and the dipoles in the headgroups of the lipid bilayer, supported by hydrophobic attraction. On the other hand, interactions with polymers may also disrupt the membrane structure and make them leaky, as in gene delivery using phospholipid membranes: the outflow of drugs from vesicles or the inflow of encapsulated DNA into cells becomes possible (Keszei *et al.,* 2005). Disruption of phospholipid membranes by polymers may even result in bactericidal action (Mayer *et al.,* 2000).

In the case of an unresponsive underlying material, the rate of polymer adsorption is not necessarily proportionate to the concentration of polymer available for coating or to the molecular weight, provided that the chains are long enough for adsorption. However, the acidic groups on a weak polyelectrolyte such as carboxylic acid groups dissociate to an extent that responds to the local environment, and the dissociation can vary between nearly no dissociation to nearly complete dissociation (Keszei *et al.,* 2005; Allen *et al.,* 1999; Xie and Granick, 2001). The proportion of charged to uncharged adsorbed carboxylic groups depends on the physiological milieu, i.e. the surface is now responsive. Adsorption seems to be more rapid at higher concentrations. By measuring the vibration characteristics and orientation of lipid headgroups, Feng Xie *et al.* (Keszei *et al.,* 2005; Allen *et al.,* 1999; Xie and Granick, 2001) showed direct coupling between adsorption and surface reconstruction. The surface reconstruction process was found to be rate-limiting. In the specific experimental conditions used by them, an average of five lipid headgroups were influenced by each charged unit of the polymer in equilibrated solutions. As this influence is cumulative, the result is a surface reorganization process that involves changes of local curvature and local stiffness. Thus, polymer adsorption stiffens a membrane locally, and during the surface reorganization process, an increasing number of lipid headgroups becomes involved.

Polymers are adsorbed onto lipid membranes in the formulation of many cosmetics and pharmaceutical products (Xie and Granick, 2001). For optimal stabilization of uncharged colloidal particles the surface has to be fully covered with an inert, non-ionic solvent-compatible

and flexible polymer. A polymer-containing fatty acid is used in Pheroid™ formulations. The factors that may influence the adsorption of the polymers and the spatial self-organization of adsorbates on fluid bilayers therefore become important. These factors include:

- The amount of polymer adsorbed/degree of polymerization;
- The ratio of polymer to bilayer;
- The ratio of the charged to uncharged polymeric groups adsorbed within the formulations firstly and secondly, the applicable biological milieu;
- The packing density of the polymer;
- The length and the molecular weight of the polymer;
- The flexibility of the polymer; and
- The physical state of the formulation, i.e. gel phase versus liquid crystalline phase at the relevant temperature.

Polymers that have been used to sterically stabilize particles include polystyrene, poly(methyl methacrylate), poly(butyl 2-cyanoacrylate), poly(ß-malic acid-co-benzyl malate, poly(p-hydroxybutyrate), poly(lactic acid), poly(lactic acid-co-glycolic acid, poly(ethylene glycol) (PEG), and o/w (oil/water) emulsions (soybean 10%). The approximate size of these particles ranges from 60nm to 1µm (Storm *et al.,* 1995). Of these materials, PEG is relevant to the studies described in the next few chapters and will be discussed in more detail in Chapter 3. Other polymers will be referred to as and when necessary or explanatory.

### 2.6.5 The structure/function relationship of modifications

The establishment of a 'steric barrier' with the aim of modifying the pharmacokinetics and biodistribution of colloidal particles has to be tested in terms of its impact on *in vivo* behaviour. Volume 391 of Methods in Enzymology (2005) is dedicated to the various therapeutic applications of liposomes. Most of the approaches currently under clinical investigation are related to the therapeutic potential of long-circulating liposomes or nanoparticles. Independent of the type of modification used in a colloidal particle, the modification is aimed at giving the system the following general capabilities:

(i) To act as circulating microreservoir. By extending the circulation time of colloidal particles, a reservoir may be established. This characteristic is especially useful for drugs that are rapidly degraded or cleared, such as the artemisinin-based antimalarials or peptide drugs (also see Chapter 7). Non-ionic polymers, such as the homopolymer PEG and the amphipathic copolymers can impart prolonged circulation (Griffiths *et al.,* 2003). Because of the anatomical blood distribution, the majority of colloidal particles smaller than 5 µm will collect in the liver and spleen where they will be processed by the ubiquitous macrophages. Targeting of drug particles to organs other than the liver or spleen and its associated

macrophages requires a mechanism whereby the distribution of the particles can be changed. A number of approaches have been described by various authors, some of which are not clinically acceptable. Examples of these are the use of phagocytosis depressants like dextrane sulphate, methyl palmitate and gadolinium chloride and the saturation of the MPS (mononuclear phagocyte system) with a 'dummy' dose of colloidal particles (e.g. polystyrene nanoparticles or liposomes). A clinically acceptable approach is the optimization of particle characteristics such as surface size, surface charge and surface hydrophobic and hydrophilic texture. An extension of this approach is the engineering of the surface of particles by the attachment of polymers such as PEG to the particles. PEG has been found to increase the stability of the particles in the biological environment. Polymers with polarizability close to water may be covalently attached to the surface of the particles or more loosely attached through electrostatic interactions (Storm and Woodle, 2003).

(ii) Adsorption of the polymers polyethylene glycol (PEG), poloxamer-407, poloxamine-908, and poloxamine-1508 onto colloidal particles seems to efficiently reduce blood clearance and fast liver uptake.(Storm and Woodle, 2003; Frederik and Hubert, 2005; Torchilin and Trubetskoy, 1995).

(iii) To promote bio-distribution to non-MPS sites: The phagocytosis of circulating colloidal particles, and the drugs entrapped in the particles, by the MPS and more specifically the macrophages, is one of the determinants of drug biodistribution and clearance kinetics. The forces coming into play during circulation are the interfacial physicochemical interaction between foreign particles and cells or circulating body proteins. In this regard, hydrophobic particles have been shown to be much more rapidly removed from the circulation than hydrophilic particles, probably because of the rapid adsorption of certain plasma proteins or opsonins on hydrophobic surfaces. The hydrophilic/hydrophobic balance (HLB) of the surface coatings is therefore important and should ideally be above 15, according to Storm *et al.* (1995). The surfactant used during the emulsification procedure of the particles may contribute to the HLB and should be selected with some care. Surface and size are two of the major determinants of the clearance kinetics and biodistribution of colloidal particles (Storm *et al.*, 1995). Sterically stabilized particles may be able to localize preferentially at diseased sites, such as tumours and sites of infection and inflammation (Storm *et al.*, 1995; Trevaskis *et al.,* 2008; Kostarelos, 2003; Petrak, 2005). This passive localization or targeting is probably the result of the prolonged circulation and release of a drug based on the above described microreservoir function, and the increased capillary permeability at the pathological site. The size of the colloidal particles may be of relevance. Passive targeting has been shown with drugs for the treatment of cancer (Fenske and Cullis, 2005; Bally *et al.,* 2005; Schwendener and Schott,

2005; ten Hagen, 2005; Oku and Namba, 2005), infectious diseases, including cytokines (de Kroon *et al.,* 2005), antibiotics/bactericides (Reszka *et al.,* 2005; Jones, 2005; Bakker-Woudenberg *et al.,* 2005; Gupta and Haq, 2005), antivirals (Zarif, 2005; Désormeaux *et al.,* 2005), nucleic acid based drugs (Dűzgűnes and Gregoriadis, 2005), and drugs for chronic inflammatory conditions such as asthma and arthritis (Dűzgűnes *et al.,* 2005; Sethi *et al.,* 2005; Eugenia *et al.,* 2005). The accumulation of micelles prepared from PEG and/or phosphatidyl-ethanolamine conjugates was found to be eight times higher in the infarction zone than in a nondamaged part of the heart muscle (Petrak, 2005). Surface modification of polystyrene and poly(methyl methacrylate) nanoparticles with non-ionic polymeric surfactants or of lipid-based colloidal systems with PEG has led to systems that partially evade the MPS and in particular the Kupffer cell surveillance.

(iv) To generate kinetically favourable *in vivo* drug release at the dedicated site from the colloidal particles – the long circulating microreservoir function should not hinder drug release (Storm *et al.,* 1995; Frederik and Hubert, 2005; Torchilin and Trubetskoy, 1995).

(v) To enable immunospecific targeting: the conjugation of antibodies and other proteinaceous 'homing devices' (Storm *et al.,* 1995) to the surface of the PEG polymeric layer may result in target site binding.

PEG, specifically PEG polymers in the size range below 5000 Kda, has been extensively used during the last decade to both prolong the circulation time of liposomes, also referred to as Stealth® liposomes, and to avoid the MPS (Trevaskis *et al.,* 2008; Pandey *et al.,* 2005; Lukyanov *et al.,* 2004; Iakoubov and Torchilin, 1998; Trevaskis *et al.,* 2006; Shackleford *et al.,* in press). The PEG polymers have generally been linked to distearoyl phosphatidyl ethanolamine (PEG-DSPE) to allow anchoring of the polymer in the lipid bilayer of colloidal particles, mostly liposomes. PEG may be anchored in lipid bilayers stronger than polymeric surfactants or can be physically adsorbed onto particle surfaces and has been covalently attached to particles other than liposomes with great success in terms of an inhibition of the uptake of the particles by Kupffer cells in *in vitro* systems. The efficiency of covalently attached PEG-PLGA poly(lactic-coglycolic acid) copolymers in altering the biodistribution of nanoparticles in mice showed that the blood circulation time increased as the molecular weight of PEG increases from 5000 up to 20000 Da (Petrak, 2005; Storm *et al,* 1995; Frederik and Huber, 2005; Torchilin and Trubetskoy, 1995). Few polymers seem to combine the hydrophilicity, flexibility and relative non-toxicity of PEG (Storm *et al.,* 1995; Griffiths *et al.,* 2003). Derivatives of polyoxazoline have been reported to be as effective as PEG in prolonging circulation time and minimizing hepatosplenic uptake (Trubetskoy and Torchilin, 1995). The prolongation of blood circulation achieved by PEG colloids was reported to be better than that of oligo- and polysaccharides such as glucuronic acid, cellulose derivatives, dextrans, and

copolymers of glucuronic acid with simple sugars conjugated to the colloidal particle (Storm *et al.,* 1995).

## 2.7 Conclusion

From the above it becomes clear that the design of effective carriers of therapeutic molecules is complex and specific to the API, the disease and the target site. Controlled changes in the characteristics of the biomaterials owing to environmental changes are useful in applications such as site selective delivery (e.g. the environmental difference between the stomach and colon). Some applications require additional characteristics, such as pH or temperature dependent phase or volume transformations. For ophthalmological and vaginal applications polymers with strong bioadhesive properties such as adhesive gels and membranes are preferred. In this case, the polymers selected would probably be hydrophilic and/or amphiphilic. The structure of the carrier determines properties such as adhesion, environmental responsiveness to stimuli (such as pH) and degradation rate. The preferential accumulation of APIs at specific sites in the body is dependent upon a complex array of biological interactions, physiological defence mechanisms, physicochemical and biomechanical factors (electrostatic interactions, flow dynamics and hydrostatic pressures).

An important required property of colloidal carrier systems is lack of toxicity. Polymers used for coating are in general regarded to be of low toxicity. Long term administration of the nonbiodegradable carriers such as polystyrene and poly(methyl methacrylate) particles does not seem to cause toxicity, but dose-dependent systemic hypotension and myocardial depression has been reported for polystyrene particles (Petrak, 2005). Biodegradability is a requirement for the application of colloidal carriers in humans. For a potential clinical application, the toxic effects of each sterically stabilized carrier system will have to be investigated and biodegradable materials should preferably be used in its design.

The literature describes impressive and sophisticated drug delivery systems and carriers with multifunctional properties, made from responsive biomaterials. The components include sterically stabilizing lipids, targeting ligands, pH and temperature sensitive lipid components and components that induce intracellular delivery. However, delivery systems and carriers ultimately need to be shown to work in humans. The choice of materials dedicated to a specific treatment is based on its mechanical, physicochemical, interfacial and biomimetic properties, and include traditional properties such as elasticity, compressibility, impact strength, and permeability.

## 2.8 References

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