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Review

Formulation of multifunctional oil-in-water nanosized emulsions for active and passive targeting of drugs to otherwise inaccessible internal organs of the human body

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Dedicated to Prof. Simon Benita of Hebrew University of Jerusalem, Israel.

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ABSTRACT

Oil-in-water (o/w) type nanosized emulsions (NE) have been widely investigated as vehicles/carrier for the formulation and delivery of drugs with a broad range of applications. A comprehensive summary is presented on how to formulate the multifunctional o/w NE for active and passive targeting of drugs to otherwise inaccessible internal organs of the human body. The NE is classified into three generations based on its development over the last couple of decades to make ultimately a better colloidal carrier for a target site within the internal and external organs/parts of the body, thus allowing site-specific drug delivery and/or enhanced drug absorption. The third generation NE has tremendous application for drug absorption enhancement and for 'ferrying' compounds across cell membranes in comparison to its first and second generation counterparts. Furthermore, the third generation NE provides an interesting opportunity for use as drug delivery vehicles for numerous therapeutics that can range in size from small molecules to macromolecules.

Contents

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1. Introduction

An otherwise inaccessible internal (lung, liver, kidney heart, brain, etc.) and an easily accessible external (eye, nose, ear, penis, vagina, anus, etc.) organs of the human body always consist of several different types of physiological barriers. The majority of these barriers block or prevent the entry of any foreign materials including drug and drug delivery system (DDS) into both the internal and external organs of human body. Therefore, it becomes necessary for a successful DDS to overcome several different types of barriers that originate from the complexity of human organism (Sofou, 2007). Indeed, specific molecular responses are required for each barrier from the DDS and thus demand the integration of diverse molecular and supramolecular responsive designs within a single drug delivery structure. On the other hand, it has been estimated that anywhere from 40 to as much as 70% of all new chemical entities (NCE) entering drug development programs possess insufficient aqueous solubility to allow consistent gastrointestinal absorption of a magnitude sufficient to ensure therapeutic efficacy (Gursoy and Benita, 2004). Hence, the DDS should have the potential to overcome the major problems of currently available drugs or NCE, which include not only poor aqueous solubility but also toxic side effects and lack of selectivity for the diseased tissue.

Indeed, depending on the immediate requirements, the DDS should simultaneously carry on its surface various moieties capable of functioning in a certain orchestrated order for demonstrating sequentially the following properties (Torchilin, 2007): (a) circulate long in the blood or, more generally, stay long in the body, (b) specifically target the site of the disease through different mechanisms, like enhanced permeability and retention effect (EPR) and ligand-mediated recognition, (c) respond to local stimuli characteristic of the pathological site, such as abnormal pH values or temperature or respond to externally applied stimuli, such as heat, magnetic field, or ultrasound, by, for example, releasing an entrapped drug or facilitating the contact between drug-loaded nanocarriers and target cells, (d) provide an enhanced intracellular delivery of an entrapped drug in case the drug is expected to exert its action inside the cell, and (e) afford a real-time information about the carrier (and drug) biodistribution and target accumulation as well as about the outcome of the therapy due to the presence within the structure of the carrier of a certain reporter moiety.

To address all the above-said issues, DDS have initially been designed to take advantage of the enhanced vascular permeability present at disease sites (Fig. 1). DDS can easily extravasate at these sites, in contrast to nontarget tissues. This in combination with the decreased clearance and enhanced blood residence time of a DDS-associated drug will actually promote the drug concentrations in the diseased tissues, increasing the therapeutic efficacy of the incorporated drug molecules. If the DDS is so designed to possess particle sizes in submicron or nanometric level, then, the use of nanometric DDS results in a reduced volume of distribution for the entrapped drug, entailing further diminished extravasation in non-target tissues, with resultant reduction of toxic side effects. The selectivity of DDS can be even further enhanced by including targeting ligands that allow for the recognition of specific markers expressed at the diseased site.

Among the different available nanocarriers, such as different polymeric and metal nanoparticles, liposomes, niosomes, solid lipid particles, micelles, quantum dots, dendrimers, microcapsules, cells, cell ghosts, lipoproteins, and different nanoassemblies (Torchilin, 2006), that have the potential to address all the described issues, an oil-in-water (o/w) type nanosized emulsion (NE) is ideal to resolve these challenges owing to its complex self-assembled nature that is intrinsically responsive to its immediate environment and the versatility of the emulsion components which can be combined to result in structures with multiple responsive functionalities. Fig. 2 shows the schematic structure of the assembly of the multifunctional NE. In the scheme, the possibilities of developing magnetic nanocarrier (Scheme 3) and contrast nanocarrier for imaging purposes (Scheme 5), cell-penetrating nanocarrier for intracellular drug delivery purposes (Scheme 6) and DNA-carrying nanocarrier such as lipoplex for correcting genetically determined diseases (Scheme 7) are not investigated yet using NE. But these four innovative avenues, if successful, should revolutionize the medical field due to their selective improvement in imaging and therapeutic efficacies.

The present review deals with a comprehensive summary on how to formulate the multifunctional o/w NE for active and passive targeting of drugs to otherwise inaccessible internal organs of the human body. This review starts by introducing a brief description regarding various nomenclatures commonly used in medical field to indicate emulsion DDS and why the term nanosized emulsion is preferred over other nomenclatures already described in medical and pharmaceutical literatures. A short note on the gen-

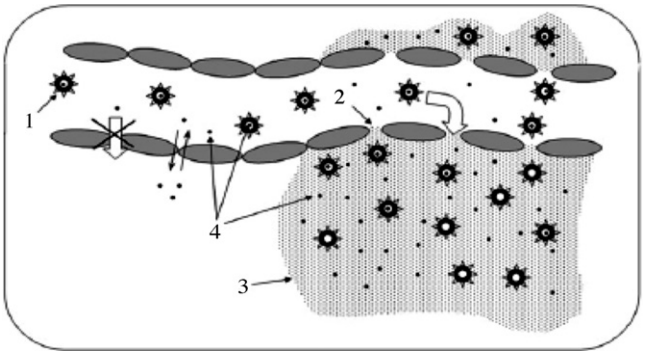

Fig. 1. Enhanced permeability and retention (EPR) effect. Key: Long-circulating drug carriers (1) penetrate through the leaky pathological vasculature (2) into the tumor interstitium (3) and degrade there, releasing a free drug (4) and creating its high local concentration.

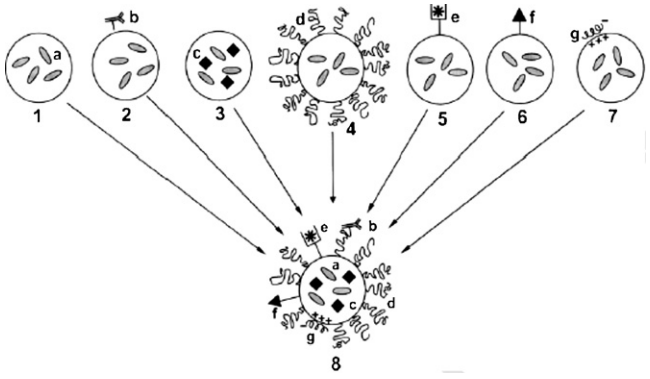

Fig. 2. Schematic structure of the assembly of the multifunctional nanosized emulsions. Key: (1) Traditional "plain" nanocarrier (a – drug loaded into the carrier); (2) targeted nanocarrier or immunocarrier (b – specific targeting ligands, usually monoclonal antibody, attached to the carrier surface); (3) magnetic nanocarrier (c – magnetic particles loaded into the carrier together with the drug and allowing for the carrier sensitivity towards the external magnetic field and its use as a contrast agent for magnetic resonance imaging); (4) long-circulating nanocarrier (d – surface-attached protecting polymer (usually PEG) allowing for prolonged circulation of the nanocarrier in the blood); (5) contrast nanocarrier for imaging purposes (e – heavy metal atom – ^{111}In, ^{99m}Tc, Gd, Mn-loaded onto the nanocarrier via the carrier-incorporated chelating moiety for gamma- or MR imaging application); (6) cell-penetrating nanocarrier (f – cell-penetrating peptide, CPP, attached to the carrier surface and allowing for the carrier enhanced uptake by the cells); (7) DNA-carrying nanocarrier such as lipoplex (g – DNA complexed by the carrier via the carrier surface positive charge); (8) hypothetical multifunctional pharmaceutical nanocarrier combining the properties of the carriers # 1–7.

erations of o/w NE is also given followed by a detailed description concerning how the modifications had been or are being proposed onto the o/w NE to extract the multifunctional activity from these nanocarriers.

2. O/w type nanosized emulsions (NE)—nomenclature and classification

Other nomenclatures are also being utilized often in the medical and pharmaceutical literatures to refer to NE, which include miniemulsions (El-Aasser and Sudol, 2004), ultrafine emulsions (Nakajima, 1997), and submicron emulsions (Benita, 1998; Klang and Benita, 1998). The term nanosized emulsion (Tadros et al., 2004) is preferred because in addition to giving an idea of the nanoscale size range of the dispersed droplets (having size distribution ranging between 50 and 1000 nm with a mean droplet size of about 250 nm), it is concise and avoids misinterpretation with the term microemulsion (which refers to thermodynamically stable systems). Hence, NE can be defined as heterogenous dispersions of two immiscible liquids [oil-in-water (o/w) or water-in-oil (w/o)], and they are subject to various instability processes such as aggregation, flocculation, coalescence, and therefore eventual phase separation according to the second law of thermodynamics. However, the physical stability of NE can substantially be improved with the help of suitable emulsifiers that are capable of forming a mono- or multi-layer coating around the dispersed liquid droplets in such a way as to reduce interfacial tension or increase droplet–droplet repulsion. Depending on the concentrations of these three components (oil–water–emulsifier) and the efficiency of the emulsification equipment/techniques used to reduce droplet size, the final NE may be in the form of o/w, w/o, macroemulsion, micrometer emulsion, submicrometer emulsion, and double or multiple emulsions (o/w/o and w/o/w). Preparation know-how, potential application, and other information pertinent to w/o emulsions (Solans et al., 2005), macroemulsions (Becher and Schick, 1987; Kabalnov, 1998; Stefan et al., 2003), microemulsions (Ceglie et al., 1987; Attwood, 1994), and multiple emulsions (Hino et al., 2000) are thoroughly covered elsewhere. In addition, some studies have compared the performance of different emulsified systems (macroemulsions, microemulsion, multiple emulsions, and gel emulsions) prepared with similar oils and surfactants for applications such as controlled drug release (Gallarate et al., 1999) or drug protection (Er Ãfnofas et al., 1998). Similarly the state of the art of the so-called oxygen carriers or perfluorocarbon emulsions, dispersions containing submicrometer/nanosized fluoroorganic particles in water, is also thoroughly covered in the literature (Lowe, 1999; Spahn, 2000; Krafft, 2001) and readers can refer to these complete and interesting articles.

Possible usefulness as carrier stems from the NEs ability to solubilize substantial amounts of hydrophilic/hydrophobic drug either at the innermost (oil or water) phase or at the o/w or w/o interfaces. While hydrophilic drugs are contained in the aqueous phase of a w/o type emulsion or at the w/o interface of the system, hydrophobic drugs could be incorporated within the inner oil phase of an o/w type emulsion or at the o/w interface of the system. It appears that the choice of the type of emulsion to be used therefore depends, to a large extent, upon the physicochemical properties of the drug. Between w/o and o/w types, the o/w type of NE would be preferred in order to successfully exploit the advantages of an emulsion carrier system. Additionally, within the o/w type, simple modifications on surface/interface structures of emulsions can be made. For instance, incorporating an emulsifier molecule alone or in a specific combination that is capable of producing either positive or negative charges over the emulsified droplets surface will lead to the formation of surface (charge)-modified emulsions. Based on these surface modifications, the o/w type NE can be divided into cationic and anionic emulsions.

The o/w nanosized emulsions have many appealing properties as drug carriers. They are biocompatible, biodegradable, physically stable, and relatively easy to produce on a large scale using proven technology (Fukushima et al., 2000). Due to their subcellular and submicrometer size, emulsions are expected to penetrate deep into tissues through fine capillaries and even cross the fenestration present in the epithelial lining in liver. This allows efficient delivery of therapeutic agents to target sites in the body. Not only considered as delivery carriers for lipophilic and hydrophobic drugs, nanosized emulsions can also be viewed nowadays as adjuvants to enhance the potency of deoxyribonucleic acid (DNA) vaccine. For instance, Ott et al. (2002) prepared a cationic o/w emulsion based on MF59 (commercially termed Fluad®), a potent squalene in water and a cationic lipid, 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium propane (DOTAP). It was shown that an interaction of cationic emulsion droplets with DNA and the formed DNA-adsorbed emulsion had a higher antibody response in mice *in vivo* while maintaining the cellular responses equivalent to that seen with naked DNA at the same doses. Another example of o/w emulsion-based adjuvants resulting from U.S. patent literature is the Ribi adjuvant system (RAS) (Ribi, 1984; Ribi et al., 1984; Myers and Truchot, 1990). Depending on the animal species used, RAS can be classified into two types: one for use in mice, termed monophosphoryl-lipid A + trehalose dicorynomycolate emulsion (MPL + TDM emulsion), and another for use in rabbits, goats, and larger animals, called monophosphoryl-lipid A + trehalose dicorynomycolate + cell wall skeleton emulsion (MPL + TDM + CWS emulsion). Strikingly, the MPL + TDM and MPL + TDM + CWS emulsions are prepared based on 2% oil (squalene)–Tween 80–water. These adjuvants are derived from bacterial and mycobacterial cell wall components that have been prepared to reduce the undesirable side effects of toxicity and allergenicity but still provide potent stimulus to the immune system. Another example is the syntex adjuvant formulation (SAF) that contains a preformed o/w emulsion stabilized by Tween 80 and Pluronic L121 (Allison and Byars, 1986).

Furthermore, based on the performances in previous and present decades, o/w type NE can conveniently be classified into three generations (see Fig. 3). While the use of surface (charge)-modified o/w NE (both anionic and cationic emulsions) in improving ocular efficacy of lipophilic drugs were overviewed elsewhere by Tamilvanan and Benita (2004), the implications of lipid emulsions in both ocular and parenteral delivering systems were elaborated in an another review by Tamilvanan (2004). Table 1 lists some of the selected marketed medical and non-medical emulsions available for various human consumption purposes.

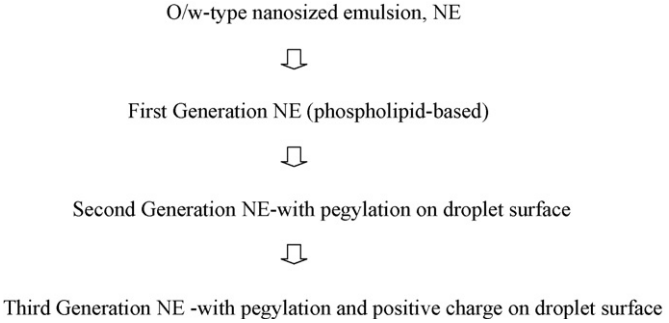

Fig. 3. Flow chart of three generations of emulsion.

Table 1
Non-exhaustive or selected list of marketed medical and non-medical emulsions (modified from Tamilvanan, 2008).

Parenteral fat emulsions (o/w type) for nutrition		Registered emulsions (o/w type) containing drugs			
Product	Producer	Product	Drug	Producer	Application
Abbolipid/Liposyn	Abbott Diazepam-Lipuro	Diazepam	Braun Melsungen	IV	
Intralipid	Pharmacia-Upjohn	Diprivan	Propofol	AstraZeneca	IV
Lipofundin N/Endolipide	B.Braun	Etomidat-Lipuro	Etomidate	Braun Melsungen	IV
Lipofundin MCT/LCT/		Lipotalon	Dexamethasone		
Medialipide/Vasolipid	B.Braun	(Limethason)	palmitate	Merckle	Intra-arthr.
Medianut	B. Braun	Stesolid	Diazepam	Dumex	IV
Lipovenos	Fresenius	Gengraf	Cyclosporin A	Abbott	Oral
Ivelip/Salvilipid	Clintec/Baxter	Norvir	Ritonavir	Abbott	Oral
Clinoleic	Clintec/Baxter	Restasis	Cyclosporin A	Allergan	Ocular topical use
Intralipos	Green Gross	Refresh Endura	Drug-free	Allergan	Ocular topical use
Kabimix	Pharmacia-Upjohn	Fluad (MF59)	Adjuvant	Chiron Parenteral use	
Trivè 1000	Baxter SA				

Perfluorocarbon emulsions (fluorocarbon-in-water emulsions)		Selected topical formulations based on o/w or w/o emulsion		
Product	Producer	Application	Product	Producer
Fluosol DA	Green Gross, Osaka	Blood supplement or O_2 carrier	Daivonex cream and ointment	Laboratoire Leo
Imagent	Alliance Pharmaceutical Corp	Contrast agent to image heart	Voltaren emulgel	Ciba-Geigy
Oxygent	Alliance Pharmaceutical Corp	Blood supplement or O_2 carrier	EMLA cream	Astra, Sweden
			Physiogel®	Stiefel Lab, Germany

2.1. First generation NE

To be healthy with quality life style is every human's desire. According to documented Indian scriptures, dating back to 5000 BC, nutritional status has always been associated with health (Chandra and Grace, 1985). Because any kind of nutritional depletion due to either changes in the quality and amount of dietary fat intake or abnormalities in lipid metabolism results in immunosuppression and therefore host defense impairment, favoring increased infection and mortality rates.

Traditionally depletion in dietary fats in malnourished or hypercatabolic patient is compensated through intravenous feeding using a solution containing amino acids, glucose, electrolytes and vitamins as well as NEs. Structurally, NE (o/w type emulsion) is triglyceride droplets enveloped with a stabilizing superficial layer of phospholipids (Shils, 1998). NE for parenteral use are complex nutrient sources composed of not only fatty acids but also including substances other than triglycerides, such as phosphatidylcholine, glycerol and α-tocopherol in variable amounts. The NE also had a complex inner structure and consisted of particles with different structures, namely, oil droplets covered by an emulsifier monolayer, oil droplets covered by emulsifier oligolayers, double-emulsion droplets and possibly small unilamellar vesicles. Commercially available NE used as intravenous high-calorie nutrient fluids have particle size normally around 160–400 nm in diameter and typically, their surfaces are normally negatively charged. The triglycerides used to prepare NE may be presented structurally in long- or medium-chain forms such as long-chain triglycerides (LCT) and medium-chain triglycerides (MCT). LCT contains fatty acid chains with 14, 16, 18, 20 and 22 carbon atoms and sometimes with double bonds. The number of double bonds present defines the fatty acids in LCT as saturated, mono or polyunsaturated. If the first double bond is on carbon number 3, 6 or 9 from the methyl end of the carbon chain then the fatty acid is *n*-3, *n*-6 or *n*-9, respectively. Purified soybean or safflower oil contains LCT with a high proportion of *n*-6 polyunsaturated fatty acids (PUFA) whereas olive oil has LCT with *n*-9 monounsaturated fatty acids (MUFA). Fish oil includes LCT with 20 or more carbon atoms where the first double bond is located between the third and the fourth carbons from the methyl terminal of the fatty acids chain (omega-3 or *n*-3). On the other hand, MCT is derived from coconut oil and has saturated fatty acids (SFA) with chains containing carbon atoms at 6, 8, 10 or 12 positions. Fatty acids that are important in parenteral nutrition and their sources are listed in Table 2. Fatty acids have common names (Table 2) and systematic names. They are also referred to by a shorthand nomenclature that denotes the number of C in the chain, the number of double bonds and the position of the first double bond relative to the methyl-C (*n*; also termed ω; Table 2).

The simplest *n*-6 fatty acid is linoleic acid (18:2*n*-6) and the simplest *n*-3 fatty acid is α-linolenic acid (18:3*n*-3). Although mammalian cells cannot synthesize linoleic and α-linolenic acids, they can metabolize them by further desaturation and elongation. Linoleic acid can be converted to γ-linolenic (18:3*n*-6), then to dihomo-γ-linolenic acid (20:3*n*-6) and then to arachidonic acid (20:4*n*-6). Using the same series of enzymes α-linolenic acid is converted to eicosapentaenoic acid (EPA) (20:5*n*-3). A complex pathway for further conversion of EPA to docosahexaenoic acid (DHA) (22:6*n*-3) exists (Calder and Burdge, 2004; Gurr et al., 2002; Sprecher, 2002).

Lipids were first introduced into parenteral nutrition formulas in the 1960s in order to provide a more balanced supply of energy, along with glucose (Edgren and Wrtelind, 1963; Hallberg et al., 1966; Wretlind, 1972). The lipid typically used in parenteral nutrition is soybean oil, in which linoleic acid comprises about 50% of the fatty acids present. Commercially available soybean oil-based NEs include: Intralipid® (Fresenius Kabi, Bad Homberg, Germany); Lipovenoes® (Fresenius Kabi); Lipofundin® (B Braun, Melsugen, Germany); Ivelip® (Baxter Healthcare, Maurepas, France). A study in patients following major gastrointestinal surgery has identified

Table 2
Common names, shorthand nomenclature and sources of fatty acids used in parenteral lipid emulsions.

Common name	Shorthand nomenclature	Typical source
Caprylic acid	8:0	Coconut oil
Capric acid	10.0	Coconut oil
Myristic acid	14.0	Coconut oil
Palmitic acid	16.0	Olive oil, soybean oil, fish oil
Oleic acid	18:1*n*-9	Olive oil, soybean oil
Linoleic acid	18:2*n*-6	Soybean oil
α-Linolenic acid	18:3*n*-3	Soybean oil
Eicosapentaenoic acid (EPA)	20:5*n*-3	Fish oil
Docosahexaenoic acid (DHA)	22:6*n*-3	Fish oil

that the amount of *n*-6 PUFA (i.e. linoleic acid) infused is one of the two predictors of the length of hospital stay (increased by 1.6 days/100 g *n*-6 PUFA infused), the other being the delay in the onset of initiating nutritional support (Koch and Heller, 2005). A number of *in vitro* experiments have shown that soybean oil-based NEs can exert immunosuppressive effects [for references, see Calder et al. (1994)], which would clearly be detrimental in patients at risk of infection and sepsis. Clinical trials with soybean oil-based NEs provide conflicting evidence, with some showing selective immunosuppressive effects (Monson et al., 1988; Battistella et al., 1997; Furukawa et al., 2002), perhaps linked to poorer patient outcomes (Battistella et al., 1997). However, other studies do not show such effects on the immune system (Dionigi et al., 1985; Gogos et al., 1990; Sedman et al., 1991) or on clinical outcomes (Lenssen et al., 1998). Details of these studies are reported by Calder (2006). Despite the inconsistencies in the outcomes of such studies, there is a view developing that the use of NEs based entirely on soybean oil may not be optimal or may even be harmful. The concern about potential harm, based mainly on the notion that *n*-6 PUFA might be 'proinflammatory, immunosuppressive and pro-coagulatory', has led to the development of alternative first generation NEs for parenteral applications. Two alternative philosophies to reducing the amount of linoleic acid have been adopted. The first has been to simply dilute soybean oil with another oil that is fairly inert. Examples of this strategy include the use of the so-called MCT and the use of olive oil. With MCT/LCT combinations in a specific ratio, NE appears to provide a more readily metabolizable source of energy (Rubin et al., 1991). Moreover, for drug solubilization purpose, MCT is reported to be 100 times more soluble in water than LCT and thus to have an enhanced solubilizing capability. The second approach has been to partially replace soybean oil with another oil that is believed to exert benefits in its own right. An example of this strategy is the use of fish oil. Soybean oil is often referred to as 'LCT', but this nomenclature is an incorrect use of this term since the lipids found in olive oil, fish oil and other oils not used in parenteral nutrition also contain LCT. The following FDA-approved NEs are available as alternatives to pure soybean oil emulsions: Lipofundin MCT/LCT® (B Braun), a 50:50 (v/v) mixture of MCT (in the form of coconut oil) and soybean oil; Lipovenoes MCT® (Fresenius Kabi), a 50:50 (v/v) mixture of MCT (in the form of coconut oil) and soybean oil; Structolipid® (Fresenius Kabi), produced by inter-esterification of a 50:50 (v/v) mixture of MCT (in the form of coconut oil) and soybean oil; ClinOleic® (Baxter Healthcare), an 80:20 (v/v) mixture of olive and soybean oils; Lipoplus® (also known as Lipidem®; B Braun), a 50:40:10 (by vol.) mixture of coconut, soybean and fish oils; SMOFLipid® (Fresenius Kabi), a 30:30:25:15 (by vol.) mixture of coconut, soybean, olive and fish oils. In addition, the product Omegaven® (Fresenius Kabi), which is 100% fish oil, is available for use as a supplement to be diluted with another lipid emulsion of choice. In Europe NE containing LCT/MCT enriched with fish oil became available for research. Fish oil consists of two major omega-3 fatty acids, namely EPA and DHA. Both EPA and DHA have been shown to be active in a number of biological processes, including retinal and brain development, immune function, blood clotting, and prevention of cardiac arrhythmias (Horrocks and Yoe, 1999; Harbige, 1998; Leaf et al., 1998). Thus, omega-3 fatty acids laden NEs made from fish oil are likely to be increasingly used not only for nutrition support but also for the modification of a number of biological and pathological processes.

2.1.1. Advantages of MCT/LCT combination in parenteral NE—a case study

Emulsions containing MCT mixed with soybean oil are well established, having been introduced in the 1980s (Ulrich et al., 1996; Adolph, 1999). Medium-chain fatty acids are: more soluble than long-chain fatty acids and readily cleared from the circulation; easily oxidized and not stored as triacylglycerides; may be protein sparing because they are ketogenic; do not impair liver function and do not interfere with pulmonary hydrodynamics or gas exchange; resistant to peroxidation (Ulrich et al., 1996; Adolph, 1999).

Studies have directly compared the effects of soybean oil and a mixture of MCT and soybean oil on immune function (Gogos et al., 1990; Sedman et al., 1991). In critically ill patients there is no difference in numbers of various immune cells in the bloodstream but $CD4^+$:$CD8^+$ cells is maintained in the MCT–soybean oil group whereas it declines in the soybean oil group (Gogos et al., 1990). This finding is indicative of better maintenance of immune function in the former group. In patients post-gastrointestinal surgery there are no differences in lymphocyte proliferation or interleukin (IL)-2 production between soybean oil and MCT–soybean oil groups (Sedman et al., 1991). However, natural killer cell activity is increased in the MCT–soybean oil group. Again, this finding is suggestive of better immune function in the MCT–soybean oil group.

In a randomized study in preterm infants who received total parenteral nutrition for 8 days, Lehner et al. (2006) compared the effects of a MCT/LCT-based emulsion and of a LCT emulsion on the fatty acid composition of plasma phospholipids and triacylglycerols. The MCT/LCT emulsion provides less polyunsaturated fatty acid (PUFA) than an LCT emulsion and thus has been associated with a lower risk of lipid peroxidation and fewer alterations of membrane structures (Halliwell and Chirico, 1993). High amounts of linoleic acid (LA; C18:2*n*-6) and α-linolenic acid (ALA; C18:3*n*-3) were reported to inhibit Δ6 desaturation, the initial step in the formation of long-chain polyunsaturated fatty acids (LC-PUFAs) (Spielmann et al., 1988). Thus, the authors hypothesized that a reduced supply of LA and ALA with the MCT/LCT emulsion might enhance LC-PUFA formation. Because of the fast growth of brain and retina during the perinatal period, an inadequate supply of LC-PUFAs, mainly arachidonic acid (AA; C20:4*n*-6) and docosahexaenoic acid (DHA; C22:6*n*-3), may have profound effects on the development of brain and visual function in preterm infants (O'Connor Hall et al., 2001; SanGiovanni et al., 2000). Although infants are able to synthesize LC-PUFAs from LA (C18:2*n*-6) and ALA (C18:3*n*-3) by desaturation and elongation from the first postnatal week onward, the rate of synthesis is rather low relative to the requirements for tissue incorporation (Salem et al., 1996; Szitanyi et al., 1999). In human adults, infused MCTs are oxidized faster and to a greater extent than LCTs (Metges and Wolfram, 1991), but data on their metabolism in infants are scarce (Koletzko, 2002; Rubin et al., 1994). Therefore, the authors also hypothesized that in preterm infants the supply of a MCT/LCT emulsion would result in predominant oxidation of MCT, rather than LCT, as a major energy source. Thus, via decreased LCT oxidation, the lower LCT intake might be partly compensated for by a higher availability of LCT for structural functions and for conversion into LC-PUFAs. The results of this clinical study indicate that the use of the MCT/LCT emulsion in parenteral nutrition of preterm infants for a period of 8 days is well tolerated and provides equivalent carnitine, vitamin E, and EFA status compared with the LCT emulsion. The concentration of the functionally important omega-3 fatty acid, DHA (C22:6*n*-3), was higher in plasma triglycerides of the MCT/LCT group, and there is also a trend towards higher levels of other LC-PUFAs in triglycerides and phospholipids. Because the availability of LC-PUFAs, and particularly of DHA (C22:6*n*-3), was shown to be of great functional importance in early life for the development of visual acuity (SanGiovanni et al., 2000) and cognitive development (O'Connor Hall et al., 2001), the use of the MCT/LCT emulsion might provide important clinical benefits over the use of a standard soybean oil emulsion in these patients.

The rationale for using newer first generation NEs, prepared from olive oil and fish oil, as parenteral nutrition and the clini-

cal trials performed using these NEs in adult patients post-surgery (mainly gastrointestinal) or critically ill adults was reviewed recently by Calder (2009).

2.1.2. Pharmacopoeial and safety issues for first generation NE

First generation, lipid-based NE can be given as a separate infusion or added into total parenteral nutrition admixtures. Despite such broad use, no pharmacopoeial standards exist with respect to the optimal pharmaceutical characteristics of the formulation. Several attempts to establish standard physical and chemical attributes have been attempted by various pharmacopoeias around the world, but without success largely due to technical issues regarding the creation of globule size limits. The United States Pharmacopoeia (USP), which develops drug monographs and chapters relating to drug purity and safety in the United States and whose standards are enforceable by the US Food and Drug Administration (FDA), have made two proposals affecting the stability and safety of commercial lipid injectable emulsions (Driscoll, 2004). Between 1991 and 1998, the USP first attempted to draft Chapter 728 (now 729), entitled "Globule Size Distribution in IV Emulsions," and a monograph entitled "IV Fat Emulsion." USP chapters numbered <1000 must comply with official tests and assays; failure to comply may result in regulatory actions by the FDA. (Those numbered >1000 are informational.) USP monographs are specific to a particular drug (e.g., Phenytoin Sodium Injection) or dosage form (e.g., Lipid Injectable Emulsion) and provide product specifications. Hence, individual drug monographs and chapters numbered <1000 state Pharmacopoeial requirements that must be met by pharmaceutical manufacturers before release to the general public.

Recently, the USP has revised its previous efforts and developed two methods and criteria (under Chapter <729>) to measure the mean droplet size (Method I), and the large-diameter tail >5 μm (Method II) of the globule size distribution to verify the stability of first generation NE. Importantly, it is the latter size limits of Method II that have the greatest implications for infusion safety. Currently, the USP has several relevant issues involving lipid injectable emulsions under consideration. Recently, a new monograph (Lipid injectable emulsions: in-process revision. Pharm Forum, 2006, 32, 350–353) has been proposed that sets specific physicochemical limits on lipid injectable emulsions that must be met by the individual manufacturer in order to meet Pharmacopoeial requirements. In addition, the USP has also recently proposed a new version of Chapter 729 entitled "Globule Size Distribution in Lipid Injectable Emulsion" (Chapter 729. Globule size distribution in lipid injectable emulsions. Pharm Forum, 2005, 31, 1448–1453). This is intended to be a general chapter that outlines the acceptable physical characteristics of the emulsion as the size of globules is critical to the safety of the dispersion. Two physical methods that provide critical information about the globule size distribution (GSD) have been selected. Method I is a qualitative test that reflects the homogenization process of the final product to yield a desirable mean droplet size and therefore is viewed as a manufacturing parameter. It uses light-scattering techniques (either "dynamic" or "static" light scattering) to ascertain the mean droplet size of the formulation. The USP has chosen a universal upper limit (i.e., irrespective of the final lipid concentration: 10%, 20%, or 30% wt/vol) of not >500 nm or 0.5 μm. Method II is a quantitative test that reflects the fineness of the final product whereby the population of large fat globules is minimized and is therefore viewed as a stability parameter. It uses the light obscuration or light extinction method that uses a single-particle (globule) optical sensing technique (LE/SPOS) to ascertain the amount of fat globules found in the large-diameter tail (i.e., >5 μm) of the GSD, indicative of stability. Hence, if the normal population of these large fat globules is known in stable lipid injectable emulsions, higher amounts are associated with instability. The proposed USP limit for this population of fat globules is expressed as the volume-weighted percent of fat >5 μm or $PFAT_5$ of <0.05%. Ultimately, it is expected that the USP limits proposed in Chapter 729 be maintained throughout the shelf life of the native lipid injectable emulsion product.

The major safety issues involving injectable emulsions include impairments in plasma clearance in susceptible patients, and the infusion of an unstable emulsion containing large quantities of potentially embolic fat globules. Recent animal studies investigating the toxicity from the infusion of unstable lipid injectable emulsions have shown evidence of oxidative stress and tissue damage to the liver when recommended globule size limits determined by Method II of the USP are exceeded. Adoption of Chapter <729> of the USP seems appropriate at this time. For further details, Driscoll (2006) elaborated the Pharmacopoeial and safety issues for first generation NE.

2.1.3. Clinical issues of first generation NE

The selection of various vegetable oils, such as those from cottonseed, soybean, or safflower plants, as the lipid sources for the early first generation NEs was principally based on providing a substrate that was high in the essential fatty acid, linoleic acid. According to the work of Holman et al. (1982), it was not until later that a pure safflower oil-based NE was demonstrated to supply insufficient amounts α-linolenic acid, producing a clinical deficiency state involving significant neurologic abnormalities. Interestingly, both cottonseed oil and safflower oil contain very little α-linolenic acid, whereas soybean oil has approximately 10 times more than these per g of oil (Bloch and Shils, 2006). Consequently, the oil composition of certain commercial first generation NEs was modified by the addition of soybean oil, so as to increase the fraction of this essential fatty acid. Even today, with the newer emulsion products containing various mixtures of MCT or other LCT oils, soybean oil is routinely included because of the presence of relatively high amounts of essential fatty acids.

Presently, only omega-6 LCT-based emulsions as either 100% soybean oil or as a 50:50 physical mixture (by weight) of soybean and safflower oils, are available in the United States. Most of the serious adverse effects associated with these emulsions are a consequence of excessively high infusion rates. According to Klein and Miles (1994), LCT-based emulsions infused at rates exceeding 0.11 g/kg/h underlie the major toxicities associated with their use in the clinical setting. Administration of lower amounts of LCTs, either by reducing the infusion rate or the dose of LCTs, or by employing mixtures of oils, such as those composed of MCTs and LCTs, to prepare first generation NE, will decrease the risk of adverse events in susceptible patients.

With the development of validated methods of fat globule analysis (Driscoll et al., 2001a,b), potential toxicity of first generation NEs from the infusion of unstable formulations has been an area of heightened research. The historical problems with ascertaining cause and effect of toxicity was largely the result of there being no reliable and quantifiable analytical technique to measure the presence of potentially embolic fat globules found in the large-diameter tail of the GSD (Driscoll, 1997). Without such analytical capabilities, it is not possible to establish a dose–response relationship. Conventional methods of assessing the GSD, such as laser diffraction, microscopy, electrical resistive pore techniques and light scattering, have proved to be wholly inadequate in accurately quantifying the important large-diameter tail of injectable emulsions (Driscoll, 2002). With the formal recognition of the LE/SPOS technique as the proposed stability-indicating assay capable of discerning the details of the large-diameter tail of emulsions by the USP (Chapter 729. Globule size distribution in lipid injectable emulsions. Pharm Forum, 2005, 31, 1448–1453), it is possible to explore this dose–response relationship in animal models.

There have been three studies in animals that have used the LE/SPOS technique in assessing the effects of infusing large fat globule-laden (i.e., unstable) first generation NEs (Driscoll et al., 2005, 2003, 2004). In the guinea pig model (Driscoll et al., 2005), 24-h total nutrient admixtures (TNA) infusions were compared with the stable group ($n = 6$) having a starting $PFAT_5$ of 0.004% versus the unstable group with a starting $PFAT_5$ of 2.4%. The lungs of the animals receiving the unstable emulsion showed evidence of oxidative stress. In the first 24-h TNA infusion study in rats, the starting $PFAT_5$ levels for the stable group ($n = 6$) were 0.007%, whereas the unstable group ($n = 7$) had a $PFAT_5 = 0.682\%$. Infusion of the unstable emulsion was associated with oxidative stress in the liver (Driscoll et al., 2003). The second TNA study in rats was similar, but was conducted over 72 h with a stable group ($PFAT_5 = 0.004\%$) compared against an unstable group at a lower level of instability ($PFAT_5 = 0.117\%$) (Driscoll et al., 2004). In this study, the unstable group exhibited a similar degree of oxidative stress in the liver as the 24-h infusions, but this occurred at lower levels of instability, suggesting a cumulative adverse effect.

The risk of infectious complications with the administration of first generation NEs from exogenous sources can occur in neonates and is likely a consequence of poor aseptic technique (i.e., during the preparation of syringes for lipid delivery) or excessively long infusion times. The Centre for Disease Control (CDC) (O'Grady et al., 2002) and USP Chapter 797 (2006) clearly recommend that as a separate infusion, lipid injectable emulsions not hang for a period exceeding 12 h. Despite this, some advocate 18- to 24-h infusion time in the neonatal ICU setting (Reiter, 2002). A recent review of the risks suggests that adherence to the 12-h hang time is prudent and a safer method of lipid administration in infants (Sacks and Driscoll, 2002).

The clinical utility of first generation NEs goes beyond the provision of essential fatty acids and as a dense source of parenteral calories. The exogenous administration of the essential fatty acids, precursors to important second messengers influencing the metabolic response to injury (i.e., inflammation, vagal tone, pulmonary and renal function, immune regulation) (Bistrian, 2003), will have a profound effect on prostaglandin synthesis in cell membranes. Consequently, the inflammatory response can be greatly exaggerated, so that when essential fatty acids are given in sufficient quantities to critically ill patients, they may produce pathophysiologic effects (Driscoll et al., 2001a,b; Hasselmann and Reimund, 2004; Lekka et al., 2004; McCowen and Bistrian, 2005). These adverse effects are largely mediated by the highly vasoactive prostaglandin-2 series. The omega-3 fatty acids, especially those of marine oil origin, including EPA and DHA, act principally through the less vasoactive prostaglandin-3 series and therefore down-regulate or modulate the extent of the inflammatory response. Thus, beyond the provision of calories or supplements, certain lipids may exhibit favorable or even unfavorable pharmacologic-like effects under some clinical conditions.

The etiology of parenteral nutrition-associated liver diseases (PNALD) may be because of the use of soybean-based emulsions, secondary to proinflammatory metabolites of omega-6 fatty acids (Grimminger et al., 1997), and decreased hepatic clearance of the parenteral lipid (Zaman et al., 1997). Soybean-derived lipids contain phytosterols (e.g., stigmasterol, b-sitosterol, and campesterol) that are linked with impairment of biliary secretion (Clayton et al., 1998). Past and very recent studies have suggested that phytosterols may be the "hepatoxic" or "cholestatic" component of soybean-derived lipid emulsions, with recent molecular mechanisms of phytosterols being suggested (Carter and Shulman, 2007; Carter et al., 2007). It has also been suggested that omega-6 fatty acids may contribute to impaired immunologic function (Wanten and Calder, 2007). This multitude of factors results in a cholestatic, steatotic liver that is especially susceptible to inflammatory insults (e.g., bloodstream infections, surgery, and hepatotoxic medication) (Day and James, 1998). In turn, repeated liver injury results in fibrosis, cirrhosis, and end-stage liver disease. Fish-oil-based emulsions address these problems on several fronts. Omega-fatty acid metabolites are less involved in the inflammatory response (Grimminger et al., 1997), and animal models have shown that parenteral fish oil does not impair biliary secretion and may prevent steatosis (Alwayn et al., 2005; Araya et al., 2004; Van Aerde et al., 1999). Hence, the liver is not predisposed to inflammatory insult, and liver injury can be prevented.

Recent work in a murine model of nonalcoholic fatty liver disease showed that administration of omega-3 fatty acids protected the liver against injury, whereas standard omega-6 fatty acids failed to do so (Alwayn et al., 2005). The data suggest that supplementation of omega-3 fatty acids might be beneficial in ameliorating parenteral nutrition (PN)-induced hepatic steatosis. Subsequently, the same group reports two infants with severe PN-associated liver disease where the clinical courses in both cases were reversed by the administration of parenteral omega-3 fatty acids (Gura et al., 2006). Although the data are preliminary and will require further study, the results are very promising as a potential treatment in this potentially life-threatening complication. Furthermore, the same authors (Gura et al., 2008) recently compared safety and efficacy outcomes of a fish-oil-based fat emulsion in 18 infants with short-bowel syndrome who developed cholestasis (serum direct bilirubin level of >2 mg/dl) while receiving soybean emulsions with those from a historical cohort of 21 infants with short-bowel syndrome who also developed cholestasis while receiving soybean emulsions. The primary end point was time to reversal of cholestasis (three consecutive measurements of serum direct bilirubin level of $\leq$2 mg/dl). More importantly, the authors have not observed any deleterious adverse effects of treatment. These benefits may be because of the absence of soybean oil or because of the pharmacologic effects of fish oil. However, this hypothesis is difficult to test because of the need to provide essential fatty acids in parenterally fed patients. Ideally, a prospective randomized, controlled trial comparing fish-oil emulsions with soybean emulsions in the treatment of established parenteral nutrition-associated liver diseases should be conducted. But this type of study would be difficult to conduct, because some may consider it unethical to perform a study where children with preexisting parenteral nutrition liver injury could potentially be randomly assigned to a treatment group in which they would continue to receive a soybean oil-based parenteral lipid emulsion. A prospective, randomized trial is currently being underway at Children's Hospital Boston, Harvard Medical School, Boston, MA, USA to assess the efficacy of fish-oil-based emulsions in the prevention of cholestasis in which in infants who have never been exposed to either type of lipid emulsion are randomly assigned to either conventional soybean oil emulsion or fish-oil emulsion at the start of their parenteral nutrition course.

In summary, from a clinical perspective, newer first generation NEs show great promise in certain patient settings, most notably in the intensive care unit in both adults and infants. The clinical use of alternative oils, such as MCT, fish oil and olive oil show benefits over conventional soybean oil formulations. In adults, for example, the administration of omega-3 fatty acids via soybean oil-based lipids produces a heightened inflammatory response via production of 2-series prostaglandins, whereas substitution of a portion of the lipid with omega-3 fatty acids via fish oil can favorably dampen the inflammatory response. In infants, for example, the substitution of soybean oil with fish oil has recently been shown to reverse parenteral nutrition-associated liver disease. These advances should lead to safer infusion therapy in patients receiving lipid injectable emulsions. Further, the current techniques for analyzing the pharmaceutical integrity of first generation NE are now better defined, allowing standardization of these dosage forms and, ultimately, the

formal establishment of Pharmacopoeial standards, and perhaps even compounding standards during the period of clinical use for these complex formulations.

2.2. Second generation NE

An easy and substantial associability of lipophilic bioactive compound with the MCT or other vegetable oil-based NEs, however, makes the NE to be used as vehicles/carrier for the formulation and delivery of drugs with a broad range of applications. These applications extend right from enhanced solubilization or stabilization of the entrapped drug to sustained release and site-specific delivery. Hence the NE used for these applications are termed as second generation NE. Fittingly, the second generation NE can be administered by almost all available routes including topical [percutaneous (Amselem and Friedman, 1998) and ocular (Tamilvanan and Benita, 2004)], parenteral, oral, nasal (Tirucherai et al., 2002) and even aerosolization to the lungs (Mizushima et al., 1983). Typical o/w NE is basically comprising about 0.5–50% of a first component of an oil or oil mixture, about 0.1–10% of a second component of an emulsifier, about 0.05–5% of a non-ionic surfactant and an aqueous component with the mean droplet size being in the submicron range, i.e., below about 500 nm and preferably between about 100 and 300 nm.

2.2.1. Opsonization of second generation NE

The lipid-induced enhancement in oral bioavailability of many drugs having poor water solubility is a well-known documented fact when the drugs are incorporated into NE. However, direct intravascularly or locally administered conventional first and second generation NEs could be taken up rapidly by the circulating monocytes for clearance by the reticuloendothelial cells (through organs such as the liver, spleen and bone marrow). Regardless of the residence time of NEs within the vascular system, much of an injected dose is taken up via endocytosis by the cells of reticuloendothelial systems (RES) to end up in the lysosomal apparatus. NEs can be considered as being artificial chylomicrons and enter the fat metabolism pathway through the adsorption of apolipoproteins (apos) and the subsequent action of lipoprotein lipase (LPL). It appeared that uptake of conventional NEs by the RES cells is lysosomotropic resulting in the localization of emulsion droplets inside the lysosomes where they are degraded by local enzymes. Furthermore, the extent of clearance is enhanced by the adsorption of opsonic plasma proteins onto NE surfaces. However, hydrophobic particles like NEs are also taken up by macrophages without the necessity of opsonization.

An opsonization process is the adsorption of protein entities capable of interacting with specific plasma membrane receptors on monocytes and various subsets of tissue macrophages, thus promoting particle recognition by these cells. Classical examples of opsonic molecules include various subclasses of immunoglobulins, complement proteins like C1q and generated C3 fragments (C3b, iC3b), apolipoproteins, von Willebrand factor, thrombospondin, fibronectin and mannose-binding protein. Reports that the coemulsification or incubation of NE with gelatin prior to intravenous injection enhanced its rate of clearance from the blood by fixed RES cells of the liver, lung, spleen and bone marrow have led to the development of an artificial NE for RES functionality tests (Illum et al., 1989). The mechanisms employed are interactions with circulating plasma opsonin proteins, thereby exploiting a receptor-mediated process involving fibronectin. It should also be emphasized that the interaction of particles with blood protein may have effects beyond opsonization. These may include interference with the blood-clotting cascade, a process that may lead to fibrin formation, and anaphylaxis because of complement activation.

When given by other parenteral routes, for example, intraperitoneally, subcutaneously, or intramuscularly, the majority of NE droplets enter the lymphatic system and eventually the blood circulation where particles behave as if given intravenously. Liver, spleen and bone marrow uptake is significantly lower. Indeed, relative to the NEs size, lymph nodes take up a much greater (over 100-fold) proportion than any other RES tissue. Lymphatic transport was predominantly associated with chylomicron-based transport (Porter et al., 1996).

2.2.2. Long circulation concept on second generation NE

There is an increasing interest in developing injectable NE that is not cleared quickly from the circulation when NE is designed to reach non-RES tissues in the vascular system, extravascular sites of action, or to act as circulating drug reservoirs. It has been thought that initial clearance rate by the RES can be affected by the presence of large numbers of NE particles occupying available RES receptors or exhausting opsonizing factors (Saba, 1970). Overloading or saturating the RES by single large doses or repeated administration may lead to subsequent remain of injected LE particles in the circulation and could be used to affect distribution patterns. Davis et al. (1992) confirmed that the infusion of NEs into the rabbit can cause slight temporary impairment of the RES as determined by subsequent administration of a radiolabeled colloid probe. An infused NE can cause RES blockade by one or both of two mechanisms. Immediately after infusion some of the NE particle may be recognized as foreign and are then cleared by the RES (largely the Kupffer cells of the liver). In addition, or alternatively, the Kupffer cells of the liver may become overloaded by NE remnants that will result from normal metabolism of the NE by tissue lipases (Davis et al., 1992). The study by Ueda et al. (2001) is supporting in a way the RES overloading or saturation concept. They prepared NEs consisting of soybean oil and egg yolk phosphatides and studied the effect of injection volume on the pharmacokinetics of oil particles and incorporated menatetrenone (vitamin K_2) after intravenous injection as o/w NEs in rats. At 3.0 ml/kg injection volume, which equates to 180 ml for a person weighing 60 kg, these NEs showed a prolonged plasma half-life in rats following intravenous administration. In contrast, at 0.1 ml/kg injection volume, which equates to 6 ml for a 60 kg person, these NEs were shown to disappear from the circulation soon after intravenous administration to rats.

2.2.3. Approaches for making long-circulating second generation NE

To augment substantially the NE half-life in blood circulation, the two different approaches that are being investigated most actively so far on second generation NE involve the use of either structured lipids as oil core in final NE or emulsion droplet surface modification using a coemulsifier with highly hydrophilic chains like polyoxyethylene (POE) and amphipathic polyethylene glycol (PEG) derivatives. Deckelbaum et al. (1990) explored how enzyme affinity and enzyme activity (LPL and hepatic lipase) regulate hydrolysis of phospholipid-stabilized emulsions of MCT versus LCT. It was shown that MCT NE are more readily hydrolyzed by LPL and hepatic lipase than LCT NE because of greater MCT solubility and mobility at the oil–water interface. The major factors involved are the affinity of the lipases for the interface and accessibility of individual substrate molecules to the lipases. In mixtures of LCT and MCT NEs, a higher affinity for the LCT-containing particle results in partitioning of the lipases away from the MCT NE with consequently diminished MCT hydrolysis. Hedeman et al. (1996) prepared NE using structured lipids and explored its potential to prolong the *in vivo* circulation time. Takino et al. (1994) developed NE for lipophilic drugs with the potential for prolonged circulation in the blood or hepatic targeting. A coating with sphingomyelin (SM) in the surface of the oil droplets resulted in avoidance of the

RES (Takino et al., 1994). In an another study of Takino et al. (1993) demonstrated further that ^{14}C cholesteryl oleate administered with oil droplets of a conventional NE was rapidly taken up from the circulation by the RES cells, while those containing SM survived in the circulation for a considerably longer period. Similarly, it has further been confirmed that the presence of SM in NE delays the removal of emulsion particles from rat plasma (Arimoto et al., 1998). The results of Kurihara et al. (1996) indicated that hydrogenated castor oil (HCO60) emulsions, when compared with conventional lecithin-stabilized emulsions, are more stable to LPL and show low uptake by RES organs, long circulation's in the plasma and high distribution in tumors. Lin et al. (1992) confirmed that HCO60 is a good emulsifier for the preparation of NE with better stability and prolonged and selective delivery properties. Thus, these sterically stabilized NEs could show potential as effective carriers for highly lipophilic antitumor agents to enhance the drug delivery in tumors. This was confirmed by Sakaeda et al. (1994) who found that the rate of selective delivery of Sudan II to liver, lungs and spleen could be suppressed by using HCO60-based NE. Conversely, the use of saturated MCT in NE was the most effective way to increase blood concentration of Sudan II, resulting in higher distribution to liver, lungs, spleen and brain (Sakaeda and Hirano, 1995). Furthermore, an o/w type NE containing HCO60 was shown to be superior in the selective distribution of adriamycin–HCl to the liver and in decreasing concentration in heart and kidney (Yamaguchi et al., 1995). Recently, Ueda et al. (2003) reported the effect of using a series of HCOs having different oxyethylene numbers such as HCO10, HCO20, HCO30, HCO60 and HCO100 on the pharmacokinetics of menatetrenone (vitamin K_2) incorporated in soybean oil (SO)-based NE in rats. Plasma half-life of menatetrenone after administration as the NE prepared by HCO with 10 oxyethylene units (SO/HCO10) was similar to that after the administration as SO/egg yolk phosphatides (SO/EYP), but was shorter than that as the NEs prepared by HCOs with >20 oxyethylene units (SO/HCO20, SO/HCO30, SO/HCO60 and SO/HCO100). These findings clearly demonstrate that 20 oxyethylene units in HCOs are minimum requirements for the prolongation of the plasma circulation time of the incorporated drug in SO/HCOs NEs. The above-described studies suggest the involvement of oil or structured lipids in the enhancement of systemic circulation of the NE.

Using the established formulation approaches by which the emulsion droplet surfaces could be altered might, however, be of more realistic and even further useful for a wide array of drug targeting purposes. Steric barrier or enhanced hydrophilicity effect exerted by POE chain having surfactants when added as coemulsifier into the phospholipid-stabilized NE allows, to some extent, the passive/inverse drug targeting to the lung, kidneys and areas of inflammation (Liu and Liu, 1995; Lee et al., 1995). Addition of POE-based surfactants into the otherwise hydrophobic phospholipid-stabilized NE is particularly effective against plasma protein adsorption onto NE surfaces because of the hydrophilicity and unique solution properties of POE-based surfactants, including minimal interfacial free energy with water, high aqueous solubility, high mobility and large exclusion volume (Lee et al., 1995). In addition, colloidal particles presenting hydrophilic surfaces with a low contact angle will be almost ignored by phagocytic cells (Davis and Hansrani, 1985), although NEs are not supposed to be recognized as foreign by the body to some extent. Examples of POE chain containing surfactants employed so far in NE are Tween 80, Span 80, Brij and poloxamer 188. The effectiveness of these polymeric surfactant molecules to intercalate at the oil–water interface with strong bounding to the phospholipid molecules could also be checked/judged through an *in vitro* monolayer experiment (Levy et al., 1991).

In general, the modification of particulate carriers using amphipathic PEG-containing molecules results in the prolongation of their blood circulation time (Harris et al., 2001; Bhadra et al., 2002). Therefore, similar to POE, a phosphatidylethanolamine derivative with polyethylene glycol (PEG-PE) is also incorporated as a coemulsifier into NE (termed as pegylated NE) to augment its circulation half-life time (Wheeler et al., 1994). Liu and Liu (1995) studied the biodistribution of NEs coated with phosphatidylethanolamine derivatives with three different molecular weight PEGs (m.w. 1000, 2000 and 5000). Among them, the PEG-2000 was able to prolong the circulation time of NE probably due to the increased hydrophilicity of the droplet surface and/or the formation of a steric barrier. A dipalmitoyl phosphatidylcholine (DPPC) stabilized NE was prepared by Lundberg et al. (1996) and the effect of addition of PEG-PE, polysorbate 80 or Pluronic F-68 on the metabolism of DPPC-stabilized NE was studied. They have employed two radioactive markers, [^{14}C] triolein (TO), which is susceptible to the action of LPL and [^{3}H] cholesteryl oleate ether (CO ether), which is not, in order to study the fate of NE following injection into tail vein of female BALB/c inbred mice. Hence the removal of ^{14}C-TO represents the triglyceride metabolism, whereas the other one is a core marker to represent whole particle removal by RES organ uptake. The NEs with DPPC as sole emulsifier were rapidly cleared from the blood with only 10–11% of CO or TO left in circulation after 1 h. However, addition of PEG-PE gave a prolonged clearance rate especially during first 3 h. A further addition of cosurfactant polysorbate 80 or Pluronics F-68 resulted in a marked extension of the circulation lifetime during first 6 h. The notable effects of polysorbate 80 and Pluronic F-68 can apparently be attributed mainly to the decrease in droplet size, although an additional influence due to the increased hydrophilicity may not be ruled out.

2.2.4. Antibody conjugation onto long-circulating second generation NE

In order to bring the colloidal carrier more closer to otherwise inaccessible pathological target tissues, homing devices such as antibodies and cell recognizing proteins are usually linked somehow onto the particle surfaces. Antibodies are proteins able to specifically recognize an antigen (for example, a pathogen or a tumor agent). It has been observed over the last two decades that progression of cancer is often accompanied by the overexpression of one or several proteins, called tumor antigens. The use of monoclonal antibodies for the treatment of cancer has been suggested as a means of targeting cancer cells while sparing normal cells (Farah et al., 1998). To avoid interference of PEG chains of PEG-PE-containing NE with antibody localization on the surface of the colloidal carrier, the coupling of antibodies to the terminal ends of the PEG chains has attracted much attention in recent years. The methods used to link the homing devices such as antibodies onto microspheres (another DDS) consist of simple, direct adsorption to covalent bonds. However, the PEG-PE emulsifier-containing NE can usually be linked only through a conjugation process (through a covalent link) with antibodies. Such an anchorage of antibodies achieved by conjugation process at the distal ends of PEG chain emulsifiers orienting from oil–water interface of NE may provide the "active" targeting of biological drug compounds to life-threatening diseases including various forms of malignancy (Song et al., 1996).

Decker et al. (1995) defined two fundamental properties dominating the delivery of drugs from NEs: first, the concentration of the compound in the lipid phase of the emulsion is directly proportional to the concentration of the compound in the cell at equilibrium, and second, the rate of transfer is directly proportional to the concentration of particles in contact with cells. Moreover, the transfer is consistent with direct partitioning from the lipid phase of the emulsion to cells and occurs by the direct collision of emulsion particles with cells. Essential requirements of this "active" targeting

approach include identification of recognition features (receptors) on the surface of the target, and the corresponding molecules (ligands) that can recognize the surface. Indeed, NE with appropriate ligands anchored on their surface must be able to access the target, bind to its receptors and, if needed, enter it. In the case of cells, intracellular entry will possibly be carried out by receptor-mediated endocytosis or through an adsorptive pinocytosis to a minor extent. Access to the target is expected to occur easily in the intravascular space, as for instance is the case of populations of circulating cells, or where cells (e.g. certain malignant cells) are separated from the blood or other biological fluids by leaky capillaries. Obviously, because of the submicron size range (175–400 nm in diameter) of the NE, the more they circulate and therefore the greater their chance of reaching respective targets. Thus, antibody associated NEs are more likely to offer a means to introduce drugs into the desired intracellular sites. Conjugation of an anti-B-cell lymphoma monoclonal antibody, LL2, to long-circulating drug-carrier NEs has been reported by Lundberg et al. (1999).

Benita and colleagues have reported the results of the conjugation of an anti-ferritin mAb(AMB8LK) to oil droplets of cationic nanoemulsions (Goldstein et al., 2005). It was shown that the processes used for the preparation of these immunoemulsions did not affect the physicochemical properties of the emulsions (average droplet size of 120 nm with a zeta potential of +50 mV) and did not alter the immunoreactivity of the mAb. Again from the same research group, this was again confirmed in a separate study with respect to the conjugation of trastuzumab to cationic emulsions because the trastuzumab immunoemulsion was significantly bound to the well-known breast cancer cell line SKBR-3 overexpressing the HER2 receptor (Goldstein et al., 2007). In addition, the stability over storage time at room temperature was followed up during all the period of the animal experimentation. Before any injection, the size, zeta potential, pH, and drug content were determined and found unchanged.

Paclitaxel is a highly promising drug against advanced and refractory cancers, such as breast and ovarian carcinomas. However, there is a need for the development of an i.v. formulation of the drug, which is safer and better tolerated than the present Cremophor EL-based preparation (Taxol). Reformulation could also provide a possibility to improve the efficacy of paclitaxel-based anticancer therapy. Paclitaxel is difficult to formulate for i.v. administration because of its poor aqueous solubility and general hydrophobicity. Indeed, paclitaxel can be incorporated into the internal oil phase of an emulsion (Lundberg et al., 2003). However, upon i.v. administration, the emulsion formulation is diluted infinitely, and the paclitaxel partitioned in favor of the serum owing to its log*P* of 4.7. Thus, the biofate of paclitaxel in the emulsion is similar to the biofate of paclitaxel in the commercial product (Lee et al., 2005). It has already been reported by other authors that for a drug to be retained in an emulsion following i.v. administration, the log*P* should be >7 (Takino et al., 1994); otherwise, the drug will be released rapidly in the serum, loosing the advantage of long blood circulation and possibility of organ-passive targeting. To allow paclitaxel to remain entrapped in the internal oil phase of an emulsion, paclitaxel lipophilicity should be markedly increased by esterification with a fatty acid as recently reported by Lundberg et al. (2003) who entrapped in an anionic emulsion paclitaxel oleate. Therefore, Benita and colleagues have synthesized paclitaxel palmitate (calculated log*P* of 9) and incorporated into the cationic emulsion before antibody conjugation (Goldstein et al., 2007). The objectives of this study were to assess the efficiency of anti-HER2 immunoemulsions loaded with paclitaxel palmitate in a well-established *in vivo* pharmacologic model of prostate cancer that overexpresses the HER2 receptor and to examine whether or not it can activate the complement system. Apparently, they have concluded that a more improved specific drug delivery system to prostate tumor and disseminated prostate metastases is needed in terms of targeting and intracellular uptake. It is intended to conjugate an additional mAb to the actual immunoemulsion that exhibits antiangiogenic activity. Thus, the novel bifunctional immunoemulsions will elicit a more efficient effect than the actual encouraging targeted drug delivery system, allowing for specificity to the prostate cancer cells, whereas the antiangiogenic mAb will diminish the resistance of the cancer cells and should enhance the cell uptake of paclitaxel palmitate.

2.2.5. Second generation NE for RES-related disease treatment

Apart from non-RES-related disease treatment through target specific antibody conjugation, the second generation NEs may also be useful for RES-related disease treatment. Certain lipoprotein or polysaccharide moiety inclusion into the NE would help to achieve this concept. In general, uptake of small colloidal drug carriers by the phagocytotic mononuclear cells of RES in the liver can be exploited to improve the treatment of parasitic, fungal, viral and bacterial diseases such as, for example, leishmaniasis, AIDS and hepatitis B. The approach to use NE as a drug carrier against microbial diseases is superior to free antimicrobial agents both in terms of distribution to the relevant intracellular sites and in treating disseminated disease states effectively. As already discussed conventional NE particles are capable of localizing in liver and spleen, where many pathogenic microorganisms reside.

Rensen et al. (1995) demonstrated the active/selective liver targeting of an antiviral prodrug (nucleoside analogue, iododeoxyuridine) incorporated in a NE complexed with ligands like recombinant apolipoprotein E (apo E) using Wistar rat as animal model because its apo E-receptor system is comparable to that of humans (Mahley, 1988). Whereas the parent drug did not show any affinity for emulsion due to hydrophilic property, derivatization with hydrophobic anchors allowed not only incorporating at least 130 prodrug molecules per emulsion particle but also without imparting any effect on the NE structure and apo E association to NE. Furthermore, without being bound by theory, the apo E component helps to disguise the NE particle so that the body does not immediately recognize it as foreign, but may allow the body to perceive it as native chylomicrons or very low density lipoproteins (VLDL). The small size and the approximately spherical shape allow the NE particle to exhibit similar physicochemical properties to native chylomicrons or VLDL (hydrolyzed by LPL) whereas the incorporated prodrug remained associated with the NE remnant particles following injection into the blood circulation of the rat. Because the carrier particles are not recognized as foreign, the systemic circulation of the drug increases, thus increasing the likelihood of drug delivery to the target tissues (up to 700 nM drug concentration in liver parenchymal cells). Additionally, the clearance rate of the drug decreases, thereby reducing the likelihood of toxic effects of the drug on clearance tissues since accumulation of the drug in other part of the clearance tissues is reduced. Thus, specific organs may be targeted by using NE carrier particles as described above, due to target cells comprising high levels of specific receptors, for example but not limited to apo E receptors.

To address this issue, the saccharide moieties of glycolipids and glycoproteins on the cell surface are considered to play an important role in various intercellular recognition processes. For instance, Iwamoto et al. (1991) investigated the influence of coating of the oil droplets in NE with cell-specific cholesterol bearing polysaccharide, such as mannan, amylopectin or pullulan on the target ability of those formulations. They have observed a higher accumulation of mannan-coated NE in the lung in guinea pigs. Thus selective drug targeting through NE bearing ligands would not only lead to an improved drug effectiveness and a reduction in adverse reactions but also offer possibility of applying highly potent drugs. Hence, the

composition of NE plays an important role concerning intercellular cell recognition processes and indeed, cell recognizability is also being improved by the incorporation of apoproteins or galactoproteins onto the NE particles to enhance their specificity (Grolier et al., 1992).

Overall, although second generation NE is usually used as a means of administering aqueous insoluble drugs by dissolution of the drugs within the oil phase of the NE, employing surface modification/pegylation by the attachment of targeting ligands (apo E, polysaccharide and antibody) onto the droplet surface of NE may be of useful in both passive and active drug targeting purposes. Thus receptor-mediated drug targeting using ligands attached NE seems to hold a promising future to the achievement of cell-specific delivery of multiple classes of therapeutic cargoes and this approach will certainly make a major contribution in treating many life-threatening diseases with a minimum of systemic side effects.

2.3. Third generation NE

To increase cellular uptake, cationization strategy is one of the change progressively occurred in last decades particularly on the surfaces of non-viral, colloidal carrier systems such as liposomes, nano- and micro-particulates and nanocapsules. For making the surface of these lipidic and polymeric carrier systems a cationic property, some cationic lipids/polymers are usually added into these systems during/after preparation. But, adding alone the cationic substances in phospholipid-stabilized first generation NE does not help to obtain a physically stabilized emulsion on a prolonged storage period. However, using different cationic lipids as emulsifier and additional helper lipids as coemulsifier, for example, 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1-palmitoyl-2oleoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)$_{2000}$] ($PEG_{2000}PE$), reports are available to prepare NEs with positive charges on their droplets surface (Kim et al., 2001a,b). Alternatively or on the other hand, after the inclusion of cation forming substances like lipids (stearyl or oleyl chain having primary amines), polymers (chitosan) and surfactants (cetyltrimethylammonium bromide) during the preparation of second generation NE allows the formation of a stabilized system with positive charges on it. Further, the positive charge caused by stearylamine was also confirmed by a selective adsorption of thiocyanate. Its adsorption was correlated with increasing stearylamine concentration (Elbaz et al., 1993). So, NE consisting of complex emulsifiers, i.e., phospholipid-polyoxyethylene surfactant-cationized primary amine or polymer combination can conveniently be termed as third generation NE.

2.3.1. Gene therapy using third generation NE

The extemporaneous addition of the solid drug or drug previously solubilized in another solvent or oil to the preformed first and second generation NE is not a favored approach as it might compromise the integrity of the emulsion. However, since therapeutic DNA or single stranded oligos are water soluble due to their polyanionic character, the aqueous solution of these compounds need to be added directly to the preformed third generation NE in order to interact electrostatically with the cationic emulsion droplets and thus associate/link superficially at the oil–water interface of the emulsion (Teixeira et al., 1999; Choi et al., 2002). During *in vivo* condition when administered via parenteral and ocular routes, the release of the DNA and oligos from the associated emulsion droplet surfaces should therefore initially be dependent solely on the affinity between the physiological anions of the biological fluid and cationic surface of the emulsion droplets. The biological fluid that contains mono- and di-valent anions in parenteral route is plasma. Similarly the ocular fluids that contain mono- and di-valent anions in ocular topical route are tear fluid, aqueous humor and vitreous. Moreover, these biofluids contain multitude of macromolecules and nucleases. There is a possibility that endogenous negatively charged biofluid's components could dissociate the DNA and oligos from cationic NE. It is noteworthy to conduct during the preformulation development stages an *in vitro* release study for therapeutic DNA and oligos-containing NE in these biological fluids and this type of study could be considered as an indicator for the strength of the interaction occurred between DNA or oligo and the NE. However, it is interesting to see what could happen when the third generation NE is applied to *in vitro* cell culture models in the presence of serum. The serum stability of LE/DNA complex was reported (Yi et al., 2000). Further studies are, however, necessary to be carried out to understand clearly the origin of the serum stability of this NE. In addition the transfection efficiency of this NE was not affected by time up to 2 h post-LE/DNA complex formation. This means thus that the third generation NE allows the experimenter to have a wider time window to work within during transfection study. A comparison between squalene, soybean oil and linseed oil-cored NE having different concentrations (1–30 mg/ml) of cationic lipid DOTAP was investigated on transfection levels of DNA in COS-1 cell and intravenous administration into Balb/c mice (Kim et al., 2003). Among the oil cores tested, the squalene-cored LE formed a stable complex with DNA and yielded high transfection levels even in the medium containing 80% serum. Further, the squalene also showed the most potent luciferase activity in tissue lysates, especially lung lysates, suggesting that *in vitro* cell culture system containing 80% serum is well mimicking the *in vivo* situation.

2.3.2. Unique property of the third generation NE

To enhance the drug targeting efficacy of colloidal carriers like nanospheres and liposomes, pegylation/cationization strategy is traditionally made over the surface of these carriers. While surface pegylated colloidal carriers exhibit a prolonged plasma residence time through an escaping tendency from RES uptake following parenteral administration, surface cationized colloidal carriers can facilitate the penetration of therapeutic agents into cell surface possibly via an endocytotic mechanism. These two facts are proved in both liposomes and nanospheres when they possess separately the cationic and pegylatic surface modifications on them. However, cationic emulsion colloidal carrier system, developed in Prof. Simon Benita's Laboratory at Hebrew University of Jerusalem, Israel, differs significantly in a way that it holds the combination of cationic and pegylatic surface properties on it (Floyd, 1999). It has been reported in ocular pharmacokinetic study of cyclosporin A incorporated in deoxycholic acid-based anionic and stearylamine-based cationic NEs in rabbit that when compared to anionic NE the cationic NE showed a significant drug reservoir effect of more than 8 h in corneal and conjunctival tissues of the rabbit eye following topical application (Abdulrazik et al., 2001). Since cornea and conjunctiva are anionic nature at physiological pH (Rojanasakul and Robinson, 1989), the cationic NE would interact with these tissues electrostatically to implicate the observed cyclosporin A reservoir effect. This hypothesis is supported, in principle, by an *ex vivo* study which showed that cationic NE carrier exhibited better wettability properties on rabbit cornea than either saline or anionic NE carrier (Klang et al., 2000).

Studies (Wretlind, 1981; Davis, 1982) have shown that small changes in physical properties of NEs can influence the elimination rate of these formulations from the blood. Indeed, an organ distribution study of stearylamine-based cationic or deoxycholic acid-based anionic submicron NE formulations and Intralipid®, a well-known commercial anionic NE, containing [^{14}C] cholesteryl oleate was carried out following injection into the tail vein of

male BALB/c mice (20–26 g) at a volume of 5 ml/kg (Klang et al., 1998; Yang and Benita, 2000). Since cholesteryl oleate is one of the most lipophilic compounds used in biopharmacy (calculated log *P* value 18.3) and is not prone to degradation in the body, its *in vivo* behaviour can be regarded as reflecting that of the injected NEs in the early phase (Takino et al., 1994, 1998). Following intravenous administration of the various NEs having [^{14}C] cholesteryl oleate to BALB/c mice, the [^{14}C] cholesteryl oleate was found to accumulate in organs such as lung and liver. Furthermore, it was observed that the concentration of [^{14}C] cholesteryl oleate in the lung decreased, but was again elevated over time for both the developed cationic and anionic NE formulations, with a concomitant decrease in the concentration of the radiolabeled compound in the liver. However, within the various LEs distribution patterns observed in liver, a lower [^{14}C] cholesteryl oleate concentration was observed for stearylamine-based cationic NE when compared to Intralipid® while for deoxycholic acid-based anionic NE the observed concentration of [^{14}C] cholesteryl oleate was relatively very low when compared to cationic NE and Intralipid®. In addition, in comparison to both of the anionic NEs, the stearylamine-based cationic NE elucidated a much longer retention time of [^{14}C] cholesteryl oleate in the plasma, indicating clearly a long-circulating half-life for cationic NE in the blood. Thus, the cationic NE can be considered a stealth® long-circulating NE.

The above two studies described clearly the unique characteristics of third generation NE in enhancing ocular drug bioavailability and on the other hand the same NE has the property to circulate longer time in blood following parenteral administration. Excess positive charge at the oil–water interface in conjunction with the projection of highly hydrophilic POE chain (due to the presence of poloxamer 188) towards aqueous phase of the o/w type emulsion is the main reason behind the NE to attain its unique property, which is absent in first and second generation NEs. Still, however a better understanding of the structure of the third generation NE in terms of forces involved in its formation and stabilization must ultimately be obtained in an effort to provide a clearly understood physical basis for uniqueness in its biological efficacy following parenteral and ocular administration.

3. Future perspective

The imaging sciences have also developed enormously and nowadays make use of principles and systems similar to those used in drug delivery and drug targeting. Targeted nanometric contrast agents have been developed based on polymers, lipids and/or proteins that carry radionuclides, paramagnetic elements and/or fluorescent probes suitable for imaging (Koning and Krijger, 2007).

The development of targeted nanocarriers in which therapeutic and imaging agents are merged into a single carrier will certainly be of importance in the near future. Indeed, scientists active in the field of imaging (e.g. nuclear and magnetic resonance imaging) have already started to exploit nanocarriers for molecular imaging. Image-guided drug delivery using these multifunctional nanocarriers, containing therapeutic and imaging agents, will ultimately allow for online monitoring of tumor location, tumor targeting levels, intratumoral localization and drug release kinetics prior and during radio- and/or chemotherapeutic treatment. This system will contribute to a more personalized approach in cancer therapy, as processes in individual patients can be monitored, and physicians can decide on the best suited therapy based on the individual patient's status and response. However, as already said in Section 1, an extremely interesting result of this parallel and potential development using the o/w type NE within image-guided drug delivery is not yet explored fully.

4. Conclusion

First generation emulsions are considered primarily as nutrient carriers to be administered via intravenous routes to bed-ridden patients. Second generation emulsions start initially as drug-carrier systems by solubilizing considerable amounts of lipophilic drugs at the oil phase or at the oil–water interface of the emulsion. This particular merit of emulsions is specifically exploited even commercially for both ocular and parenteral active drugs. Modifications made either in the oil phase or at the o/w interfacial film forming emulsifier molecules allow the emulsions to be able to escape from lipolysis by lipoprotein lipase, apo adsorption, and liver uptake. Such a surface-modified emulsion would prolong the circulation time in plasma and thereby an alteration in *in vivo* disposition of incorporated drugs following parenteral administration. Attachment of homing devices such as antibody and apo E make emulsions that deliver drugs selectively/actively to target sites such as a tumorized organ or hepatic system. Active targeting increases the affinity of the carrier system for the target site, while passive targeting minimizes the nonspecific interaction with nontargeted sites by the RES. Having together a positive charge and a steric stabilizing effect led to the development of third generation emulsions that contain a unique property: plasma half-life prolongation and electrostatic adhesion to ocular surface tissues after topical instillation into eye.

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