Journal of Drug Delivery

Translating Nanotechnology from Bench to Pharmaceutical Market: Barriers, Success, and Promises

Guest Editors: Abhijit A. Date, Rajesh R. Patil, Riccardo Panicucci, Eliana B. Souto, and Robert W. Lee

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Editorial Board

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Editorial

Translating Nanotechnology from Bench to Pharmaceutical Market: Barriers, Success, and Promises

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Nanotechnology is a buzzword of this millennium and it has transformed the face of research in science and technology. The advent of nanotechnology has also influenced the biomedical and pharmaceutical research since last decade. Various nano-architectures have been designed for improving the therapeutic performance of drugs, proteins, peptides, and genes and to achieve their targeting at the site of action. Although nanotechnology has demonstrated dramatic potential in drug delivery research, like any technology, its real success depends on the ability of drug delivery scientists to translate and scale innovations to the commercial pharmaceutical products. It is indeed a very challenging task to successfully overcome manufacturing, clinical, and regulatory hurdles associated with a nanotech product. Nevertheless, the pharmaceutical industry has witnessed commercialization of the nanotechnology-based products for various applications. In the present special issue, we have tried to consolidate various aspects of existing and upcoming nanotechnologies for drug delivery.

Contribution by V. Morigi et al. takes an overview of business potential and market trend of pharmaceutical nanotechnology. The authors have also discussed financial aspects of nanotechnology by citing noteworthy examples of few nanotech products that have already been commercialized. This contribution could be useful to scientists aiming to start up nanotechnological business ventures. Contribution by N. Anton et al. demonstrates how nanotechnology can change the face of conventional drug delivery systems. In this interesting investigation, the authors demonstrate that coating of conventional tablets with lipid nanoemulsion can be used to modulate the release of the drug from tablet matrix. The paper by P. Severino et al. gives an account of potential of solid lipid nanocarriers for the oral delivery of drugs and peptides. The authors have provided information about the lipids that can be used for oral delivery, role of lipids in the oral delivery, toxicological aspects of lipid nanocarriers, and products under clinical development.

S. Banerjee et al. have given a complete overview of polyethylene-glycol- (PEG-) based conjugates for drug delivery. The contribution fosters understanding design aspects of and chemistry behind PEG-based nano-architectures for drug delivery. Furthermore, the paper has a detailed discussion on the various PEG-conjugates available in the pharmaceutical market. Contribution by A. Garcia et al. highlights the potential of particle replication in nonwetting templates (PRINT), a platform technology based on lithographic techniques for drug delivery applications. The contribution clearly demonstrates potential of PRINT technology to generate particles of various, but precise, morphology for a variety of drugs and biotechnology-based therapeutics (proteins and siRNA). The application of PRINT technology for generating aerosols for pulmonary applications has also been described. This contribution is an example of the attributes required from a nanofabrication technique to circumvent manufacturing-related issues in pharmaceutical nanotechnology.

The paper by F. Lallemand et al. delineates various aspects of and challenges in ocular drug delivery and systematically describes development of Novasorb, a cationic nanoemulsion-based platform ocular delivery system. The authors have furnished a detailed description of the formulation development aspects and ocular safety of excipients which is followed by *in vivo* proof-of-concept and clinical development. This paper gives clear insight into various challenges faced for developing nanomedicine for ocular delivery. The paper by J. D. Heidel and T. Schleup throws light on the various applications of self-assembled nanocarriers consisting of cyclodextrin based polymers. The authors describe various developmental aspects of two platforms based on cyclodextrin-based polymers (Cyclosert and RONDEL) which can enable efficient delivery of drugs or nucleic-acid-based therapeutics. The translational aspects of both the nanocarriers and *in vivo* proof-of-concept have also been furnished in this paper. The research paper by J. Rios-Doria et al. gives insight into developmental aspects of a pH sensitive cross-linked polymeric micelle technology (IVECT). The authors describe synthesis of the polymeric micelles, their ability to encapsulate various drugs, and *in vivo* proof-of-concept for anticancer drugs like daunorubicin and BB4007431.

In a nutshell, we believe that this special issue would give readers insight into various aspects involved in translating nanotechnology from bench to pharmaceutical market. Moreover, the special issue also includes some contributions about nanotechnologies that are currently under clinical development.

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Review Article

Poly(ethylene glycol)-Prodrug Conjugates: Concept, Design, and Applications

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Poly(ethylene glycol) (PEG) is the most widely used polymer in delivering anticancer drugs clinically. PEGylation (i.e., the covalent attachment of PEG) of peptides proteins, drugs, and bioactives is known to enhance the aqueous solubility of hydrophobic drugs, prolong circulation time, minimize nonspecific uptake, and achieve specific tumor targetability through the enhanced permeability and retention effect. Numerous PEG-based therapeutics have been developed, and several have received market approval. A vast amount of clinical experience has been gained which has helped to design PEG prodrug conjugates with improved therapeutic efficacy and reduced systemic toxicity. However, more efforts in designing PEG-based prodrug conjugates are anticipated. In light of this, the current paper highlights the synthetic advances in PEG prodrug conjugation methodologies with varied bioactive components of clinical relevance. In addition, this paper discusses FDA-approved PEGylated delivery systems, their intended clinical applications, and formulations under clinical trials.

1. Introduction

The field of drug delivery system (DDS) utilizing polymeric carrier, which covalently conjugates molecule of interest, plays an important role in modern therapeutics [1, 2]. Such polymer-based drug entities are now termed as "polymer therapeutics" and include nanomedicine class that has become immensely critical in recent years [3–5]. The objectives for designing a polymer therapeutics are primarily to improve the potential of the respective drug by (i) enhancing water solubility, particularly relevant for some drugs with low aqueous solubility, (ii) stability against degrading enzymes or reduced uptake by reticulo-endothelial system (RES), and (iii) targeted delivery of drugs to specific sites of action in the body [1, 6].

Poly(ethyleneglycol) (PEG) is the most commonly used nonionic polymer in the field of polymer-based drug delivery [1]. Due to high aqueous solubility, PEG polymer is considered as a versatile candidate for the prodrug conjugation. Ringdorf was the first to propose the rational model for pharmacologically active polymers in 1975 [7]. An ideal prodrug model typically consists of multiple components (Figure 1):

(i) polymer as a carrier;

(ii) drug, peptide, or protein as a biological active component;

(iii) spacer molecule or targeting moiety.

PEGylation, the covalent attachment of PEG to molecules of interest, has become a well-established prodrug delivery system [8, 9]. PEGylation was first reported by Davies and Abuchowski in the 1970s for albumin and catalase modification. Since then the procedure of PEGylation has been broadened and developed thereafter tremendously [10–16]. The remarkable properties of the biologically inert (biocompatible) PEG polymer derive from its hydrophilicity and flexibility. PEG is also considered to be somewhat hydrophobic due to its solubility in many organic solvents. Most used

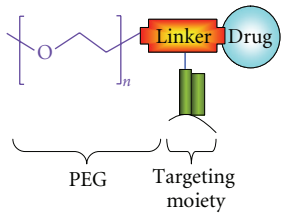

Figure 1: Schematic presentation PEG-based prodrug with targeting agent.

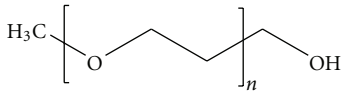

Figure 2: Molecular structure of monomethoxy PEG.

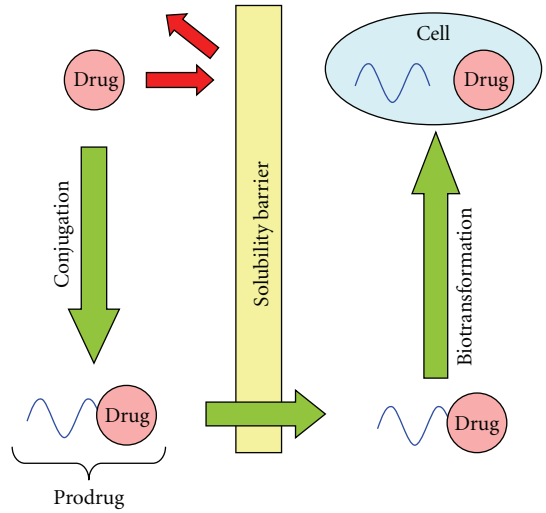

Figure 3: A schematic illustration of prodrug concept.

PEGs for prodrug modification are either monomethoxy PEG or dihydroxyl PEG (Figure 2) [7].

Typically, most of the PEG-based prodrugs have been developed for the delivery of anticancer agents such as paclitaxel, methotrexate, and cisplatin. High-molecular-weight prodrugs containing cytotoxic components have been developed to decrease peripheral side effects and to obtain a more specific administration of the drugs to the cancerous tissues [17]. Favorably, a macromolecular antitumor prodrug is expected to be stable in circulation and should degrade only after reaching the targeted cells or tissues. PEG-drug conjugates can therefore be tailored for activation by extra- or intracellular enzymes releasing the parent drug in situ (Figure 3) [7]. In this paper, we represent an overview on the advances of PEG prodrug conjugates which are being currently used as therapeutics. A short discussion with particular emphasis on the derivatives in clinical practice or still under clinical trials is also provided.

2. Properties of PEG

PEG in its most common form is a linear or branched polyether terminated with hydroxyl groups. PEG is synthesized by anionic polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring. Most useful for polypeptide modification is monomethoxy PEG (mPEG). On the other hand, mPEG is synthesized by anionic ring opening polymerization initiated with methoxide ions. Successful conjugation of PEG with biomolecule depends upon the chemical structure, molecular weight, steric hindrance, and the reactivity of the biomolecule as well as the polymer. In order to synthesize a bioconjugate, both chemical entities (i.e., the bioactive as well as the polymer) need to possess a reactive or functional group such as –COOH, –OH, –SH, or $–NH_2$. Therefore, the synthetic methodology to form a conjugate involves either protection or deprotection of the groups [18].

3. PEG-Based Nanocarrier Architectures and Designs

There is need to design simple and yet appropriate PEG-conjugation methodology. Most commonly used strategies for conjugation involve use of both coupling agents such as dicyclohexyl carbodiimide (DCC) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or use of *N*-hydroxysuccinimide (NHS) esters. Chemical conjugation of drugs or other biomolecules to polymers and its modifications can form stable bonds such as ester, amide, and disulphide. The resulting bond linkage should be relatively stable to prevent drug release during its transport until it reaches the target. Covalent bonds (e.g., ester or amide) are comparatively stable bonds and could deliver the drug at the targeted site. However, in some instances such bonds may not easily release targeting agents and peptides under the influence of acceptable environmental changes [19]. In the past, PEG prodrugs have been designed mostly for the delivery of anticancer agents due to its overall implications in the treatment. However it should be noted that PEG-antitumor prodrug is expected to be stable during circulation and degrade/hydrolyze only on reaching the targeted site. PEG-drug conjugates can therefore be tailored to release the parent drug *in situ* on activation by extra- or intracellular enzymes or pH change.

PEG has limited conjugation capacity since it possesses only one (two in case of modified PEGs) terminal functional group at the end of the polymer chain. To overcome this limitation of PEG, coupling amino acids, such as bicarboxylic amino acid and aspartic acid, to the PEG has been proposed [20, 21]. Such derivatization increases the number of active groups of the original PEG molecule. Using the same method with recursive derivatization, dendrimeric structures have also been achieved at each PEGs extremity. However, in the study the authors encountered low reactivity of the bicarboxylic acids groups towards arabinofuranosylcytosine (Ara-C) binding due to steric hindrance between two Ara-C molecules on conjugation with neighboring carboxylic moieties.

It was suggested that this effect might be overcome by incorporating the dendrimer arms with an amino alcohol (H_2N–$[CH_2$–CH_2–$O]_2$–H).

PEG polymers with hydroxyl terminals can be easily modified by aliphatic chains molecules or small amino acids. For example, antitumor agent 1-β-D-Ara-C was covalently linked to varying molecular weight –OH terminal PEGs through an amino acid spacer in order to improve the *in vivo* stability and blood residence time [22]. Conjugation was carried out with one or two available hydroxyl groups at the polymer's terminals. Furthermore, to increase the drug loading of the polymer, the hydroxyl groups of PEG were functionalized with a bicarboxylic amino acid to form a tetrafunctional derivative. Finally, the conjugates with four or eight Ara-C molecules for each PEG chain were prepared (Figure 4). The authors investigated steric hindrance in PEG-Ara-C conjugates using molecular modeling to investigate the most suitable bicarboxylic amino acid with the least steric hindrance. Typically, hydroxyl groups of PEG are activated by *p*-nitrophenyl chloroformate to form a stable carbamate linkage between PEG and amino acid. The degree of PEG hydroxyl group activation with *p*-nitrophenyl chloroformate was determined by UV analysis of the *p*-nitrophenol released from PEG-*p*-nitrophenyl carbonate after alkaline hydrolysis. Activated PEG was further coupled with amino acid and the intermediate PEG-amino acid was linked to Ara-C by EDC/NHS activation.

3.1. PEG N-Hydroxysuccinimide (NHS) Esters and Coupling Methods. PEG-NHS esters are readily available which are reactive with nucleophiles to release the NHS leaving group and forms an acylated product [23] (Figure 5(a)). NHS is a choice for amine coupling because of its higher reactivity at physiological pH reactions in bioconjugation synthesis. In particular, carboxyl groups activated with NHS esters are highly reactive with amine nucleophiles and are very common entity in peptides and proteins. Polymers containing reactive hydroxyl groups (e.g., PEG) can be modified to obtain anhydride compounds. On the other hand, mPEG can be acetylated with anhydrides to form an ester terminating to free carboxylate groups (Figure 6).

The reactive PEG and its derivatives succinimidyl succinate and succinimidyl glutamate are used for conjugation with drugs or proteins. The coupling reactions involving amine groups are usually of two types: (a) acylation, (b) alkylation. These reactions are comparatively efficient to form a stable amide bond. In addition, carbodiimide coupling reactions or zero lengths crosslinkers are widely used for coupling or condensation reactions. Most of the coupling methodologies involve use of heterobifunctional reagent to couple via modified lysine residues on one protein to sulphydryl groups on the second protein [24], while modification of lysine residues involves the use of a heterobifunctional reagent comprising an NHS functional group, together with a maleimide or protected sulphydryl group. The linkage formed is either a disulphide bridge or as a thioether bond, depending if the introduced group is either a sulphydryl or maleimide, respectively. The thiol group on the second protein may be an endogenous free sulphydryl, or chemically introduced by modification of lysine residues.

4. PEG Prodrug Conjugates as Drug-Delivery Systems

In general, low-molecular-weight compounds diffuse into normal and tumor tissue through endothelia cell layer of blood capillaries [7]. Conjugation of low-molecular-weight drugs with high-molecular-weight polymeric carriers results in high-molecular weight prodrugs (Figure 1). However, such conjugation substantially alters the mechanism of cellular internalization and accumulation. High-molecular-weight drugs are internalized mainly by endocytosis, which is a much slower internalization process over to simple diffusion. Hence in case of endocytosis higher drug concentration outside the cell is required to produce the same cellular effect as corresponding low-molecular-weight drug [7]. Therefore, higher-molecular-weight prodrugs displays lower specific activity compared to its free form of drugs. For example, polymeric anticancer prodrugs are generally less toxic when compared with its free form, yet require substantially higher concentrations inside the tumor to be cytotoxic. Compensation for this decrease in drug efficacy can be achieved by targeting a polymeric drug to the specific organ, tissue, and/or cell [7].

Following two approaches is generally used to target polymeric anticancer drugs to the tumor or cancer cells [25, 26]:

(1) passive targeting,

(2) active targeting.

4.1. Passive Drug Targeting: The EPR Effect. Passive targeting is a drug delivery approach in which drugs are delivered to the targeted site by conjugating with polymer which releases the drug outside the targeted site due to altered environmental conditions (Figure 6(a)). Tumors and many inflamed areas of body have hyperpermeable vasculature and poor lymphatic drainage which passively provides increased retention of macromolecules into tumor and inflamed area of body [27–30]. This phenomenon is called enhanced permeability and retention (EPR) effect [27]. It constitutes one of the practical carrier-based anticancer drug delivery strategies. EPR effect is primarily utilized for passive targeting due to accumulation of prodrug into tumor or inflamed area. Low molecular drugs covalently coupled with high-molecular-weight carriers are inefficiently eliminated due to hampered lymphatic drainage and therefore accumulate in tumors. While EPR effect enhances the passive targeting ability due to higher accumulation rate of drug in tumor and subsequently due to accumulation, prodrug slowly releases drug molecules which provide high bioavailability and low systemic toxicity [30].

Passive accumulation of macromolecules such as PEG and other nanoparticles in solid tumors is a phenomenon which was probably overlooked for several years as a potential biological target for tumor-selective drug delivery.

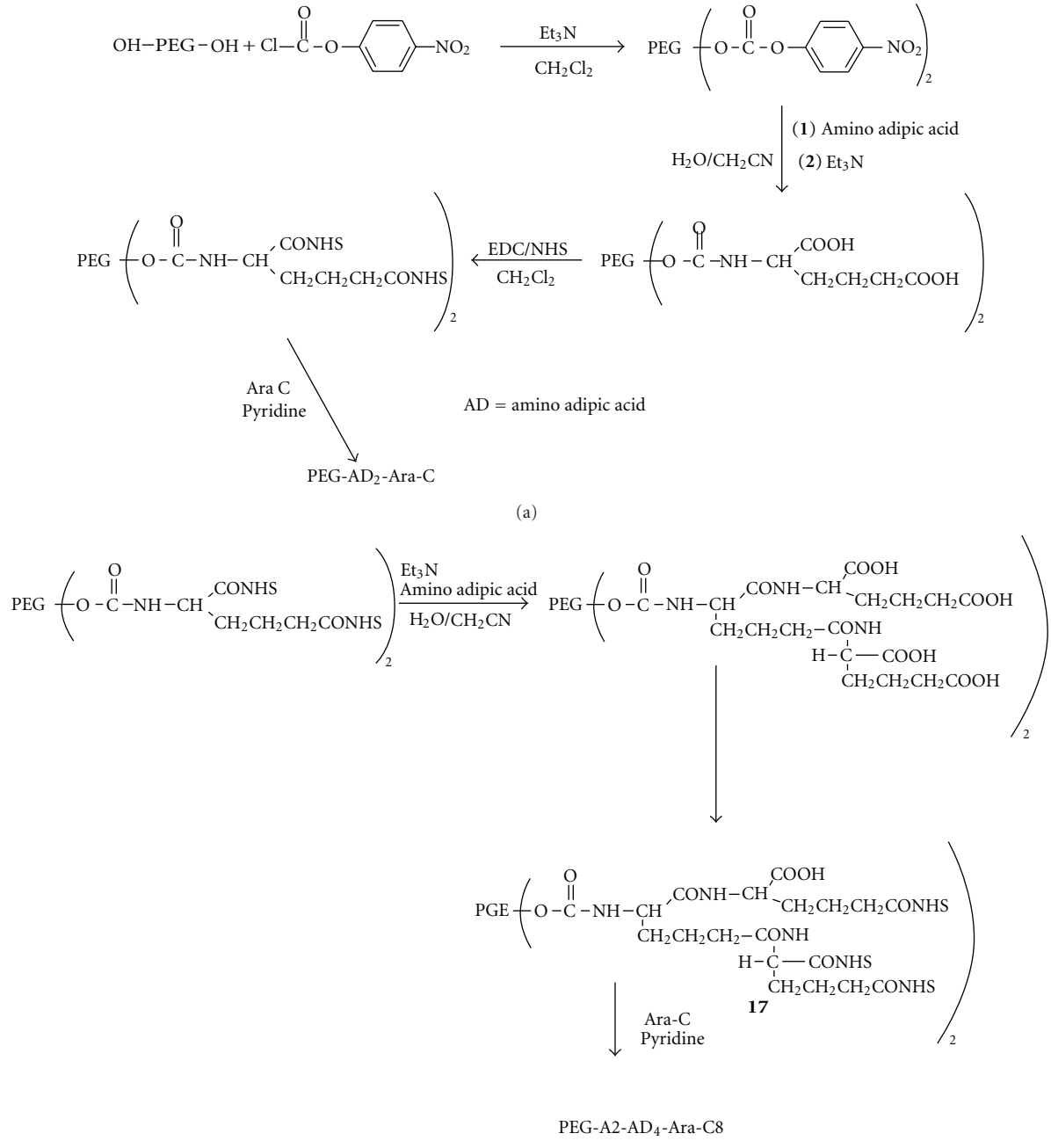

Figure 4: Synthetic schemes for $PEG_{10,000}$-AD_2-Ara-C_4 (7) (a) and $PEG_{10,000}$-AD_2-AD_4-Ara-C8 (8) conjugates (b). The antitumour agent 1-b-D-arabinofuranosylcytosine (Ara-C) was covalently linked to varying molecular weight –OH terminal PEGs through an amino acid spacer in order to improve the *in vivo* stability and blood residence time (reproduced from [22]).

The existence of the EPR effect was experimentally confirmed by David et al., for macromolecular anticancer drug delivery systems [31]. Furthermore, passive targeting increases the concentration of the conjugate in the tumor environment and therefore "passively" forces the polymeric drug to enter the cells by means of the concentration gradient between the intracellular and extracellular spaces and therefore is not very efficient. The more efficient way to provide targeting is by "active targeting" [32].

4.2. Active Targeting. Active targeting approach is based on interaction between specific biological pairs (e.g., ligand receptor, antigen antibody, enzyme substrate) (Figure 6(a)) [33]. Active targeting is achieved by attaching targeting agents that bind to specific receptors on the cell surface—to the prodrug by a variety of conjugation chemistries. Most widely used targeting moieties are peptide ligands, sugar residues, antibodies, and aptamers specific to particular receptors, selectins, antigens, and mRNAs expressed in targeted

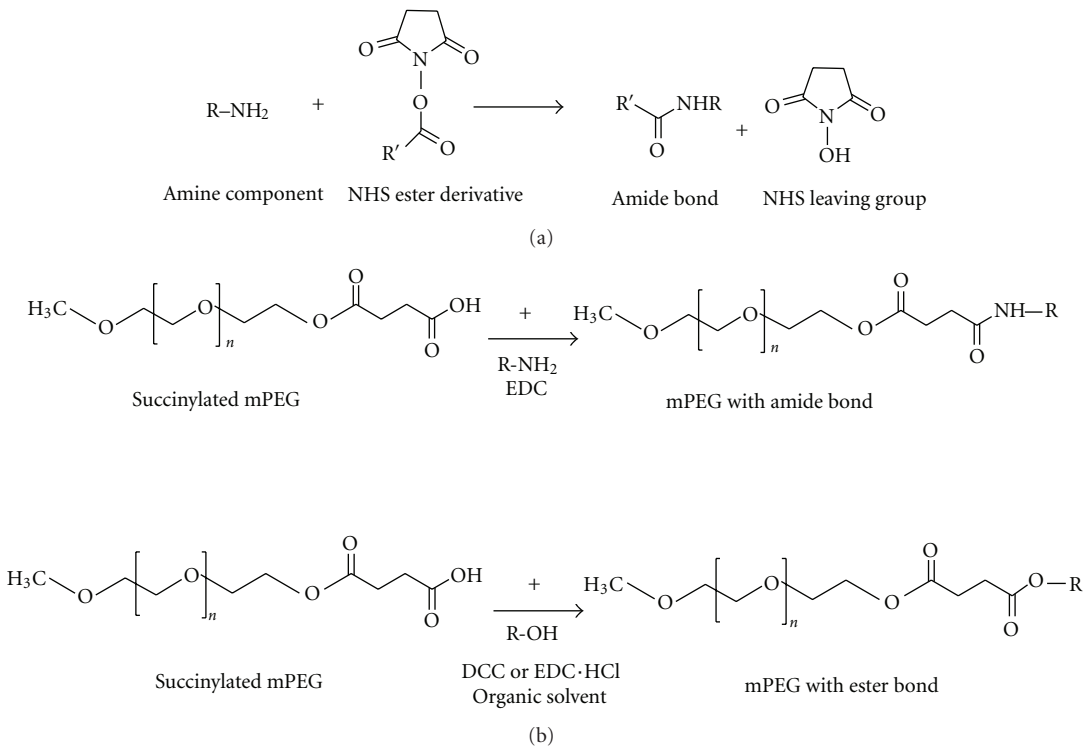

Figure 5: (a) NHS esters compounds react with nucleophiles to release the NHS leaving group and form an acetylated product. (b) PEG can be succinylated to form –COOH group, which can further form amide or ester bond with biomolecules.

cells or organs. The targeted anticancer LHRH-PEG-CPT conjugate is an example of such targeted anticancer drug delivery system [7]. In this system, LHRH peptide is used as a targeting moiety to the corresponding receptors overexpressed in several cancer cells, PEG polymer—as a carrier and CPT—as an anticancer drug. Interaction of these targeting moieties to their target molecule results in uptake of the drug by two main approaches: (i) internalization of the whole prodrug or (ii) internalization of the drug into targeted cells by various endocytosis and phagocytosis pathways [34].

(i) Internalization of the Prodrug. In this system, the drug is cleaved intracellularly after endocytosis. The internalized prodrug exhibits pharmacological activity on reaching the cytosol or the nucleus, which are the sites of action of intracellularly active drugs. This process can be divided into several distinct steps as schematically presented in Figure 6(b). Interaction of a targeted prodrug with a corresponding receptor initiates receptor-mediated endocytosis by formation of an endocytic vesicle and endosomes-membrane-limited transport vesicles with a polymeric delivery system inside [6]. The activity of the drug is preserved during the intracellular transport as the membrane-coated endosome prevents drugs from degradation by cellular detoxification enzymes. Endosomes fuses with lysosomes forming secondary lysosomes. If the drug-polymer conjugate is designed by incorporating an enzymatically cleavable bond then the drug is released from the polymer-drug conjugate by the lysosomal enzymes and might exit a lysosome by diffusion. The advantage of this approach is a high local drug concentration with a potential increase in efficacy [30].

(ii) Internalization of the Drug. In this system, the drug conjugate is cleaved extracellularly.

The microenvironment of tumors has been reported to be slightly acidic in animal models and human patients and the pH value in tumor tissue is often 0.5–1.0 units lower than in normal tissue.

5. Approaches and Applications

5.1. Polymer Conjugates of Therapeutically Relevant Proteins. The potential value of proteins such as antibodies, cytokines, growth factors, and enzymes as therapeutics has been recognized for years. However, successful development and application of therapeutic proteins are often impeded by several difficulties, for example, short circulating $t_{1/2}$, low stability, costly production, poor bioavailability, and immunogenic and allergic potential. An elegant method to overcome most of these difficulties is the attachment of PEG chains onto the surface of the protein. PEGylation of the native protein generally masks the protein's surface, inhibits antibodies or antigen processing cells, and reduces degradation by proteolytic enzymes [6]. In addition, PEGylation of the native protein increases its molecular size and as a result prolongs the half-life *in vivo*, which in turn allows less frequent administration of the therapeutic protein.

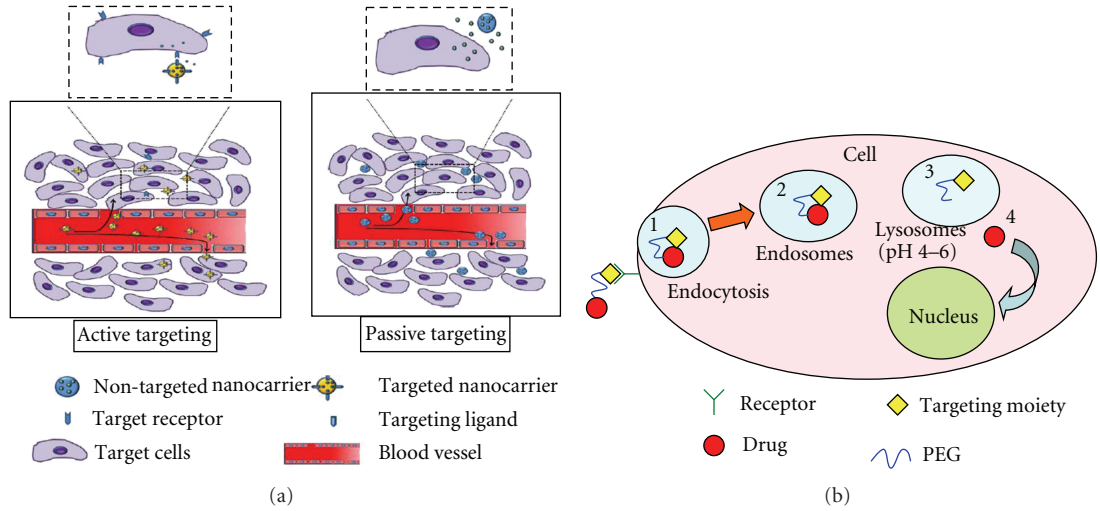

Figure 6: (a) Active and passive targeting by nanocarriers [35]; (b) (1) polymer-conjugated drug is internalized by tumor cells through receptor-mediated endocytosis following ligand-receptor docking, (2) transport of DDS in membrane limited organelles; (3) fusion with lysosomes; (4) the drug will usually be released intracellularly on exposure to lysosomal enzymes or lower pH (pH 6.5–<4.0) [31]. If the drug is bound to the polymer by an acid-sensitive linker then the extracellular release of drug takes place, especially if the drug is trapped by the tumor for longer period of time.

The most common chemical approach for preparing PEG-protein conjugates has been by coupling $-NH_2$ groups of proteins and mPEG with an electrophilic functional group [36]. Such conjugate reactions usually result in formation of polymer chains, covalently linked to a globular protein in the core. Figures 7(a) and 7(b) illustrate the commonly used methods of mPEG-based protein modifying reagents. Derivatives 1 and 2 contain a reactive aryl chloride residue, which is displaced by a nucleophilic amino group by a reaction with peptides or proteins, as shown in Figure 7(b). Derivatives 1 and 2 are acylating reagents, whereas derivatives 3–11 contain reactive acyl groups referenced as acylating agents. Protein modification with all of these agents results in acylated amine-containing linkages: amides derived from active esters 3–6 and 11 or carbamates derived from 7–10. Alkylating reagents 12 and 13 react with proteins forming secondary amine conjugation with amino-containing residues. As represented in Figure 7(a), tresylate 12 alkylates directly, while acetaldehyde 13 is used in reductive alkylation reactions. Numbers 1–13 represent the order in which these activated polymers were introduced [6, 36].

Adagen (pegademase bovine), used for the treatment of severe combined immunodeficiency disease (SCID), is developed using PEG polymer. PEG chemistry may results in side reaction or weak linkages upon conjugation with polypeptides and low-molecular-weight linear PEGs ($\leq$12 kDa). It is prepared by first reacting mPEG (Mw 5000 Da) with succinic anhydride spacer. The resulting carboxylic group of PEG succinic acid is activated with *N*-hydroxysuccinimide (NHS) by using carbodiimide coupling agents. The NHS group is displaced by nonspecific reaction with nucleophilic amino acid side chains [37]. Another PEG prodrug of Enzon (Oncaspar®) is also synthesized by the use of PEG succinimidyl succinate [37]. The PEG ester and thioesters are highly susceptible to hydrolysis and thus modification occurs primarily at the amines forming amides. The PEGylated CERA protein conjugate, a product of Hoffmann-LaRoche (Mircera) is synthesized by attachment of an NHS-activated monomethoxy PEG butanoic acid to lysine 46 and 52 on erythropoietin (EPO) [38, 39]. Also, Hoffman-La Roche, Inc.'s peginterferon α2a (Pegasys) is prepared by conjugating PEG with the side chain and *N*-terminal amine groups of lysine spacer, forming a biscarbamate. Then on activation of the carboxylic acid with NHS, it helps the branched PEG chain linker form stable amide bonds with 11 possible lysine residues. Monosubstituted conjugate can also be synthesized by the same reaction process by limiting the amount of PEG chain linker used in the conjugation step. While, PEG-Intron by Schering-Plough (peginterferon α2b) is a covalent conjugate of interferon alfa-2b linked to a single unit of Mw 12000 PEG [40] is a covalent conjugate of interferon alfa-2b linked to a single unit of Mw 12000 PEG. The interferon conjugates are synthesized by condensing activated PEG, wherein a terminal hydroxy or amino group can be replaced by an activated linker, and reacting with one or more of the free amino groups in the interferon (Figure 8). Condensation with only one amino group to form a monoPEGylated conjugate is a prime feature of this synthesis process.

In other instance, pegvisomant (Somavert) prodrug conjugate is synthesized by covalent attachment of four to six Mw 5000 Da PEG units via NHS displacement to several lysine residues available on hGH antagonist B2036, as well as the *N*-terminal phenylalanine residue is used for acromegaly treatment [41–43]. Similarly, Amgen's pegfilgrastim (Neulasta®) is used to decrease febrile neutropenia manifested infection and this prodrug is a covalent conjugation of Mw 20000 Da monomethoxy PEG aldehyde by reductive amination with the *N*-terminal methionine residue of the filgrastim protein [44]. On the other hand, Krystexxa (pegloticase) by Savient, used for the treatment of chronic gout,

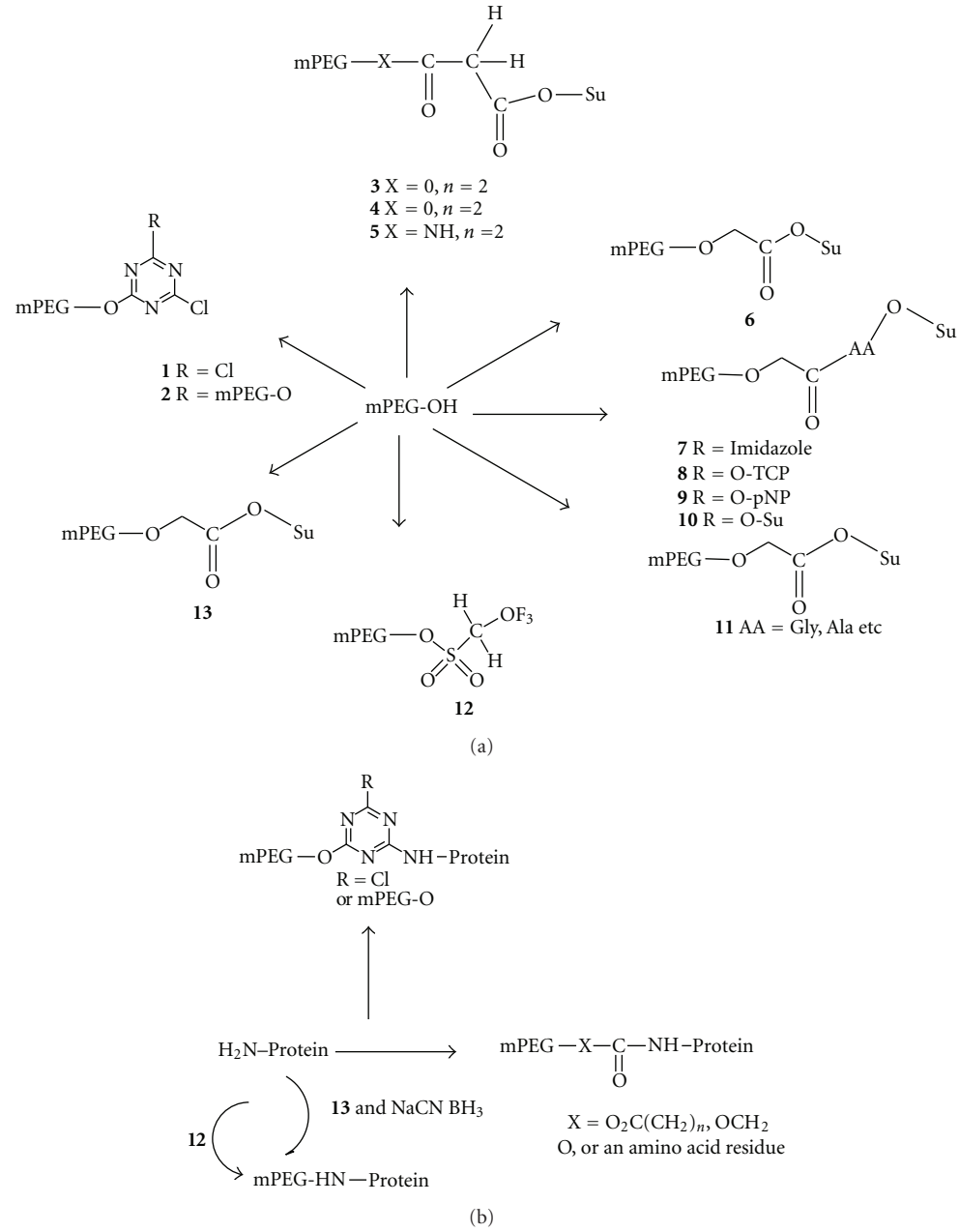

Figure 7: (a) mPEG-based protein-modifying methods. Protein modification with all of these agents results in acylated amine-containing linkages: amides, derived from active esters 3–6 and 11, or carbamates, derived from 7 to 10. Alkylating reagents 12 and 13 react with proteins forming secondary amine conjugation with amino-containing residues. As represented in (b) tresylate 12 alkylates directly, while acetaldehyde (13) is used in reductive alkylation reactions. The numbering (1–13) represent to the order in which these activated polymers were introduced (reproduced from [6, 36]).

is synthesized by using PEG *p*-nitrophenyl carbonate ester [45]. The primary amine lysine side chain is replaced by *p*-nitrophenol to form carbamates, which are further subjected to decrease hydrolysis under mild basic conditions. From the total of 28-29 lysines, approximately 12 lysines on each subunit of urate oxidase are surface accessible in the native tetrameric form of the complete enzyme. In fact, due to the close proximity of some of the lysine residues, PEGylation of one lysine may sterically hinder the addition of another PEG chain [45, 46].

5.2. PEG-Drug Conjugates. PEGylation of drugs does influence the pharmacokinetic properties of drugs and drug carriers and therefore is emerging as an important area in pharmaceutics. PEG has been successful for protein modification but in the case of low-molecular-weight drugs it presents a crucial limit, the low drug payload accompanying the available methoxy or diol forms of this polymer. This intrinsic limitation had for many years prevented the development of a small drug-PEG conjugate, and also because the conjugates extravasation into tumors by EPR effect is directly

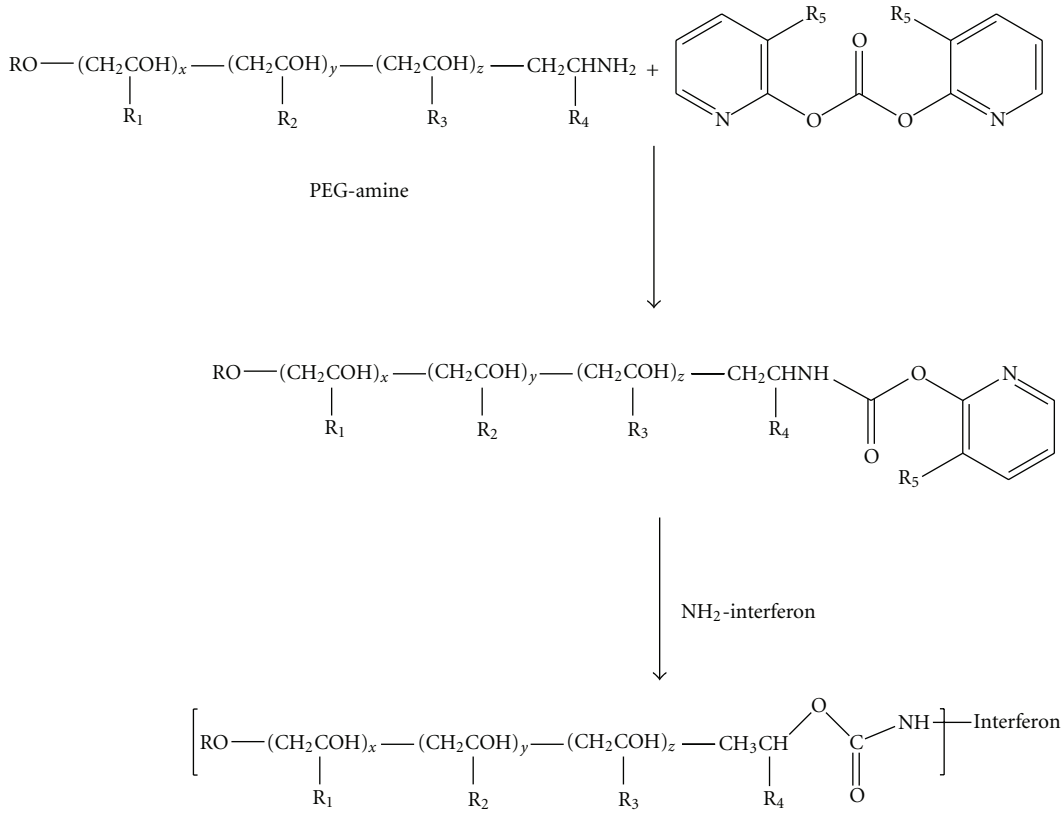

Figure 8: Synthesis of PEG-Intron by conjugating activated PEG with free amino groups in the interferon. R is lower alkyl group, R_1, R_2, R_3, R_4, R'_1, R'_2, R'_3, R'_4, R_5 is H or lower alkyl; and x, y, and z are selected from any combination of numbers such that the polymer when conjugated to a protein allows the protein to retain at least a portion of the activity level of its biological activity when not conjugated; with the proviso that at least one of R_1, R_2, R_3, and R_4 is lower alkyl (reproduced from [40].

proportional to the conjugate's molecular weight. Unfortunately, in case of PEG the use of larger polymer does not correlate well with an increase in the amount of drug selectively delivered into the tumor. In case of PEG, the number of available groups for drug coupling does not change with the length of polymeric chain, as happens instead with other polymers (e.g., polyglutamic acid, and dextran) or copolymers (e.g., HPMA). The latter can have several functional groups along the polymeric backbone: longer polymer chains correspond to an increased number of functional groups [22, 47–49].

A few studies have been conducted recently to overcome the low PEG loading by using multiarm PEGs either branched at the end chain groups or coupling on them small dendron structures (Figure 9) [47, 49–51]. Such multiarm PEG conjugates have recently entered phase I clinical trials [52]. This compound was obtained by coupling a 4-arm PEG of 40 kDa with the camptothecin derivative SN38, through a spacer glycine (Figure 10). The coupling strategy was developed to link selectively the 20-OH group of SN38, thus preserving the E ring of SN38 in the active lactone form while leaving the drug 10-OH-free [53].

Design and synthesis of nontargeted or antibody targeted biodegradable PEG multiblock coupled with N_2,N_5-diglutamyllysine tripeptide with doxorubicin (Dox) attached through acid-sensitive hydrazone bond has also been reported [54–57]. PEG activated with phosgene and NHS was reacted with $-NH_2$ groups of triethyl ester of tripeptide N_2,N_6-diglutamyllysine to obtain a degradable multi-block polymer. The polymer was converted to the corresponding polyhydrazide by hydrazinolysis of the ethyl ester with hydrazine hydrate. On the other hand, the nontargeted conjugate was prepared by direct coupling of Dox with the hydrazide PEG multi-block polymer. Whereas the antibody-targeted conjugates, a part of the polymer-bound hydrazide group, was modified with succinimidyl 3-(2-pyridyldisulfanyl) propanoate to introduce a pyridyldisulfanyl group for subsequent conjugation with a modified antibody. Dox was coupled to the remaining hydrazide groups using acid-labile hydrazone bonds to obtain a polymer precursor. In addition, human immunoglobulin IgG modified with 2-iminothiolane was conjugated to the polymer by substitution of the 2-pyridylsulfanyl groups of the polymer with –SH groups of the antibody. It was demonstrated that Dox was rapidly released from the conjugates when incubated in phosphate buffer at lysosomal pH 5 and 7.4 (blood).

5.3. Incorporation of Spacers in Prodrug Conjugates. To construct a prodrug, various spacers have been incorporated along with the polymers and copolymers to decrease the crowding effect, to increase the reactivity, and reduce steric

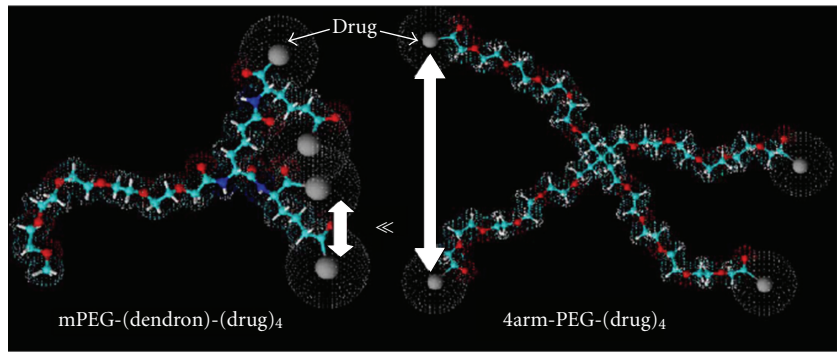

Figure 9: Schematic representation of higher steric entanglement in PEG dendrons with respect to multiarm PEGs (reproduced from [52]).

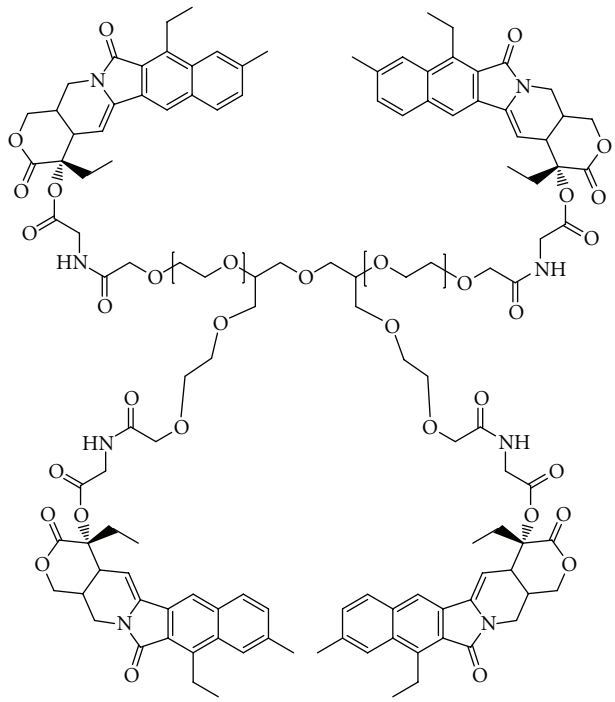

Figure 10: ENZ-2208: $^{4°K}$4 arm-PEG-$(SN38)_4$ (reproduced from [53]).

hindrance [6, 58]. The application of a spacer arm can enhance ligand-protein binding and also provide multiple binding sites. Ideal spacer molecules possess the following characteristics:

(1) stable during conjugate transport,

(2) adequate drug conjugation ability and,

(3) being able to release the bioactive agent at an appropriate site of action.

Amino acid spacers such as alanine, glycine, and small peptides are most commonly used due to their chemical versatility for covalent conjugation and biodegradability. Heterobifunctional coupling agents containing succinimidyl have also been used frequently as spacers.

Polymer spacers are used to enhance the conjugation ratio of an antibody with a drug by introducing them between the targeting antibody and the drug. The use of an intermediate polymer with drug molecules carried in its side chains increases the potential number of drug molecules able to attach to that antibody by modification of only a minimum amount of existing amino acid residues (Figures 7(a) and 7(b)) [59].

6. PEG Therapeutics: Clinical Applications and Challenges for Development

PEG-based therapeutics were initially dismissed as interesting, but impractical to be translated in clinical setups. However, a growing number of products have shown that they can satisfy the stringent requirements of regulatory authority approvals (Table 1). Clinically used PEG conjugates are described below.

6.1. PEG-Proteins Conjugate

6.1.1. Adagen (mPEG per Adenosine Deaminase). Enzon's Adagen was among the first few PEG-protein conjugates to enter the clinic with FDA approval in 1990 [37]. It is used as a placement therapy to treat severe combined immunodeficiency (SCID) disease. SCID is an autosomal recessive genetic disorder caused by adenosine deaminase deficiency. It is usually fatal in children unless the patient is kept in protective isolation or undergoes a bone marrow transplant. As an alternative, Adagen is administered intramuscularly every 7 days. It is a replacement therapy and is repeated for the rest of the life by the patients following the dosing schedule: 10 U kg^{-1}, 15 U kg^{-1}, and 20 U kg^{-1} for the first three doses, and the weekly maintenance dose of 20 U kg^{-1}. However, immune related problems have been reported for pegademase and its long-term treatment benefits are yet to be elucidated. Also, the high cost of treatment ($200,000–$300,000 per annum per patient) is an obvious disadvantage [60–62].

6.1.2. Oncaspar® (mPEG-L-Asparaginase). Oncaspar (pegaspargase) is an antineoplastic drug from Enzon Pharmaceuticals Ltd. and was approved by FDA in 1994. Oncaspar is

Table 1: PEG therapeutic systems with in the market or clinical development.

Product name	Description	Clinical use	Route of admin.	Stage
		PEG-protein conjugates		
Oncaspar	PEG-asparaginase	Acute lymphocytic leukaemia	iv/im	Market
Adagen	PEG-adenosine deaminase	Severe combined immune deficiency syndrome	im	Market
Somavert	PEG-HGH antagonist	Acromegaly	sc	Market
PEGIntron	PEG-Interferon alpha 2b Hepatitis C	Hepatitis C	sc	Market
NeulastaTM	PEG-rhGCSF Chemotherapy	Chemotherapy-induced neutropenia	sc	Market
Pegasys	PEG-interferon alpha 2a hepatitis C	Hepatitis C	sc	Market
CimziaTM	PEG-anti-TNF Fab	Rheumatoid arthritis, Crohn's disease	sc	Market
Mircera	PEG-EPO	Anaemia associated with chronic kidney disease	iv/sc	Market
Puricase	PEG-uricase	Gout	iv	Market
Macugen	PEG-aptamer	Age-related macular degeneration	Intraviteal	Market
		PEG-drug conjugates		
NKTR-102	PEG-irinotecan	Cancer-metastatic breast	iv	Phase II
PEG-SN38	Multiarm PEG-camptothecan derivative	Cancer-various	iv	Phase II
NKTR-118	PEG-naloxone	Opioid-induced constipation	Oral	Phase II

a PEG-modified entity of the enzyme *L*-asparaginase and is used for the treatment of acute lymphoblastic leukaemia [63]. PEGylation was attempted to overcome several factors limiting the utility of asparaginase as therapeutic agent such as high clearance, immunologic factors such as antibodies to asparaginase owing to bacterial protein and also inactivation due to conversion to asparagine via asparagine synthetase. Also, the immunological side effects such as hypersensitivity reactions (up to 73%) were major factors that limited clinical utility of *L*-asparaginase [64].

Pegaspargase was developed in the 1970–1980 while it was translated in the clinical trials in the 1980. Taking clues from the preclinical studies, a series of systematic clinical studies revealed the effectiveness of the pegaspargase as compared to its non-PEG-grafted parent drug [65, 66]. Clinical trials demonstrated safety in terms of fewer incidence of hypersensitivity reactions and prolonged duration of action. The trials defined different protocols (weekly or every two weeks) and recipes of multidrug regime to treat different malignancies. The clinical observations from clinical studies for pegaspargase conjugate are summarized in Table 2 [67, 68].

6.1.3. Mircera (Continuous Erythropoiesis Receptor Activator or Methoxy Polyethylene Glycol-Epoetin Beta). Mircera is a PEGylated continuous erythropoietin (EPO) receptor activator (CERA) introduced by Hoffmann-La Roche. It got approved by FDA in 2007 and is currently used to treat renal anemia in patients with chronic kidney disease (CKD). PEGylation of erythropoietin helps to prolong the half-life to approximately 130 h [69]. Darbepoetin alfa (Aranesp, Amgen), a second-generation EPO, due to the inclusion of an amino acid mutation has a higher glycosylation rate, and hence requires only weekly or biweekly injections. On the other hand, third-generation EPO (CERA) requires only monthly administration and thus helps in significantly improving the quality of life. However, it has been reported to have negligible effects on morbidity or mortality like other ESAs [70].

6.1.4. Pegasys (Peginterferon Alfa-2a). Pegasys (peginterferon alfa-2a) (Hoffmann-La Roche) drug is used to treat chronic hepatitis C (HCV) either alone or in combination with antimicrobial ribavirin. Pegasys was approved by FDA in 2002. It consists of a PEGylated interferon alfa-2a intended to mediate antiviral immune response. PEGylated interferon demonstrated higher efficacy by increasing the clearance time of the protein, thus maintaining interferon concentration levels in the blood to control HCV. The clinical study of peginterferon revealed that 180 μg of peginterferon alfa-2a, administered once a week in patients with hepatitis C-related cirrhosis or bridging fibrosis was significantly more effective than 3 million units of standard interferon alfa-2a [71–73].

6.1.5. PEG-Intron (Peginterferon Alfa-2b). PEG-Intron [74] marketed by Schering-Plough is used to eradicate hepatic and extrahepatic hepatitis C virus infection. PEG conjugated with α-interferon (IFN) was approved by FDA for use in 2001. Monomethoxy-PEG-linked interferon has a sustained serum for 48–72 h compared to the native protein half-life of 7–9 h. The recommended dosage for standalone PEG-Intron therapy is 1 mg kg^{-1} per week for 52 weeks on the same day of the week subcutaneously [74, 75].

Interestingly, peginterferon α-2a has a higher market share because peginterferon α-2b is dosed on a body weight basis, whereas peginterferon α-2a is not. As a result, peginterferon α-2a is more frequently utilized to treat hepatitis C

Table 2: Clinical trials and their outcome for pegaspargase conjugate.

Stage	Trial details	Observations/results	Reference
Phase I	31 patients with pegaspargase dose ranging from 500 to 8000 U m^{-2}.	Mean half-life—357 h; dose unrelated hypersensitivity in small population of patients.	[67]
	Patients with advanced solid tumors; pegaspargase dose 250–2000 U m^{-2} every 14 days.	*L*-aspargine level were found to be very low which was again a function of dose. 2000 U m^{-2} dose showed adverse effects such as fatigue, nausea/vomiting and weight loss. Hence dose escalation beyond 2000 U m^{-2} was not evaluated.	[76]
	Low-dose (500 units m^{-2}) in children with relapsed acute lymphoblastic leukemia.	*L*-asparaginase activity >100 U L^{-1} was demonstrated for atleast 1 week. Indicating in possibility reduction in dose.	[77]
	Five patients with AIDS related lymphoma treated with 1500 U m^{-2} every 2 weeks.	Three patients showed complete response.	[78]
Phase II	PEG-*L*-asparaginase as a single agent in patients (22) with recurrent and/or refractory multiple myeloma.	Maximal tolerated dose for single agent PEG-*L*-asparaginase in relapse/refractory multiple myeloma patients was found to be 1000 mg m^{-2} every 4 weeks.	[79]
	Patients earlier demonstrated sensitivity to *L*-asparaginase was treated with pegaspargase and other agents.	36% patients demonstrated complete response while 15% partial response.	[80]
	Newly diagnosed adults (14) with acute lymphoblastic leukemia (ALL) treated with 2000 U m^{-2} pegaspargase and multidrug regimen consisted of vincristine, prednisone, and danorubicin.	93% patients revealed complete response.	[81]
	Seven patients with refractory acute leukemias; dose 2000 U m^{-2} on days 1, 14, and 28 with other agents.	Five patients demonstrated complete response while one showed partial response.	[82]
	An open-label, multicenter study involving 21 patients with recurrent lymphoblastic leukemia with pegaspargase, 2000 U m^{-2} single dose. After 14 days patients were treated with multidrug therapy regime consisting of vincristine, prednisone, and some patients with doxorubicin and intrathecal therapy.	On day 14, 17% of patients (from 18) achieved complete response and 1% partial response. On day 35 (after the multidrug regime therapy), 67% patients demonstrated complete response and 11% showed partial response. The overall response rate was 78%.	[83]
	Pediatric oncology group study: patients with acute lymphoblastic leukemia treated with 2500 U m^{-2} with multidrug regime either weekly or every two weeks.	Highly significant 93% complete response was observed in the patients receiving weekly therapy as compared to 82% in patients receiving every two weeks.	[84]
Phase III	Reinduction of relapsed acute lymphoblastic leukemia: 2500 U m^{-2} pegaspargase on day 1 and 15 or 10,000 U m^{-2} *L*-asparaginase three times a week for 12 doses, both with multidrug regime.	Despite difference in dose and dosing rate the complete response and partial response rates were almost similar (63 and 65% for pegaspargase and *L*-asparaginase, resp.).	[85]
	Randomized trial involving Children with newly diagnosed acute lymphoblastic leukemia; 2500 U m^{-2} pegaspargase on day 1 or 6000 U m^{-2}*L*-asparaginase three times a week for three weeks.	Pegaspargase achieved faster rate of remission. Complete response rate was almost similar (98% versus 100% for pegaspargase and *L*-asparaginase, resp.) despite significant difference in dose and dosing rates.	[86]

[68]. Nevertheless, some reports have suggested that peginterferon α-ribavirin combination therapy has higher risks of neutropenia and thrombocytopenia than interferon α-ribavirin combination therapy [87, 88], although both therapies have been reported to have similar side effect profiles.

6.1.6. Somavert® (Pegvisomant). Pegvisomant (Somavert®) conjugate (Pfizer) is used to treat acromegaly by preventing human growth hormone (hGH) binding to its receptor, because this binding activates the signal pathways that lead to IGF-1 generation. It is a genetically engineered analogue of hGH conjugated with PEG which was approved for use in 2003 [89]. Acromegaly is a chronic metabolic disorder caused when the pituitary gland generates excess hGH after epiphyseal plate closure. GH receptor has two binding sites: (i) binds to site 1 and (ii) then to site 2, inducing the functional dimerization of the hGH receptor. Pegvisomant inhibits the dimerization of the hGH receptor due to its increased affinity for site 1 of the hGH receptor [89]. With eight amino acid mutations at the site, and by the substitution of position 120 glycine to arginine, inhibits hGH receptor dimerization. Overall, PEGylation reduces the activity of the GH receptor antagonist. However, the 4–6 PEG-5000 moieties added to pegvisomant prolongs its half-life and allow once-daily administration immunogenicity as the rate of clearance from the body are greatly reduced, making it an effective drug against acromegaly [90]. The recommended dosage for

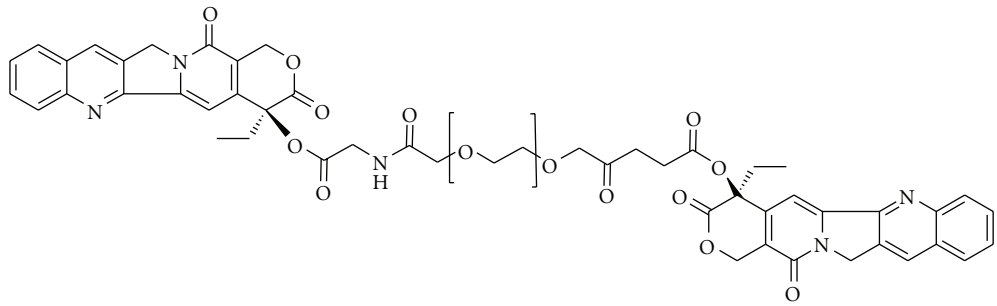

Figure 11: Synthetic structure of pegamotecan, a bisfunctional PEG-CPT conjugate mediated by a glycine spacer.

patients begins with subcutaneous administration of 40 mg dose. The patient can self-administer 10 mg of Somavert daily with adjustments to the dosage of Somavert in 5 mg increments depending on the elevation or decline of insulin growth factor-1 (IGF-I) levels [91, 92]. However, because pegvisomant can increase glucose tolerance, care is embarked for the diabetes mellitus patients [93].

6.1.7. Neulasta (Pegfilgrastim). Amgen's pegfilgrastim (Neulasta) is developed using filgrastim (Neupogen, Amgen) from Nektar (formerly Shearwater) PEGylation technology. The conjugate is formed by conjugating a 20 kDa linear monomethoxy-PEG aldehyde with Granulocyte-Colony Stimulating Factor G-CSF [94]. Neulasta is used to decrease febrile neutropenia manifested infection and was approved for use in 2002. The PEGylation increases the protein serum half-life to 42 h compared to the serum half-life of 3.5–3.8 h for the unmodified G-CSF. Therefore, the overall dose is reduced to a single cycle dose that is as effective as daily doses of native G-CSF [94–96]. The recommended dose of Neulasta is a single administration of 6 mg subcutaneously once-per-chemotherapy cycle and advised of not delivering it within 14 days before and 24 days after administration of chemotherapeutics [97].

6.1.8. Krystexxa (Pegloticase). Krystexxa (pegloticase) by Savient, a PEGylated mammalian urate oxidase (uricase) was FDA approved in 2010 [98]. It is a recombinant tetrameric urate oxidase used for the treatment of chronic gout. Pegloticase acts by preventing inflammation and pain due to urate crystal formation in plasma. The advantage of pegloticase over other standard treatments is the higher effectiveness in reducing gout tophi [99]. However, pegloticase has been reported to be immunogenic. Subcutaneous and intravenous injections of pegloticase in clinical trials showed production of antibodies [100–102]. However, it was found out that the antibodies produced were due to PEG and not because of uricase. Furthermore, as hydrogen peroxide may be produced during the conversion of uric acid to allantoin by uricase, the long-term safety profile of pegloticase needs to be established. Moreover, the transient local pain, slow absorption, and allergic reactions induced by subcutaneous injections of pegloticase were not observed after intravenous injections. However, intravenous injections are administratively inconvenient because self-administration is difficult and may have caused infusion reactions in multidose trials [103–105].

6.2. PEG-Drug Conjugates. PEG low-molecular-weight drug conjugates that entered the clinical trials are mostly from the camptothecin (CPT) family, namely, camptothecin itself, SN38, and irinotecan (Table 1). Although the first PEG based products were anticancer agents, subsequently other PEG therapeutics were developed and introduced for the treatment, for example, infectious diseases (e.g., PEG-interferons), and age-related diseases including macular degeneration and arthritis. Moreover, building of these first generation compounds, the pipeline of polymer therapeutics in clinical development continues to grow.

6.2.1. Prothecan (PEG-Camptothecin). Pegamotecan is a product of Enzon Pharmaceuticals, Inc. which is PEG prodrug of the DNA damaging agent. The prodrug conjugate was conceived by coupling two molecules of CPT to a glycine-bifunctionalised 40 kDa PEG, yielding a drug loading of only approximately 1.7% (w/w) [105] (Figure 11). The CPT prodrug was designed with the aim of doubling the loading capacity to increase the drug half-life in blood by PEGylation and to stabilize CPT by acylation of the active lactone configuration of CPT [105]. The conjugation to PEG considerably enhanced CPT solubility and bioavailability at the tumor site. The maximum tolerated dose of the conjugate in phase I trials was determined at 7000 mg m^{-2} when administered for 1 h *i.v.* every 3 weeks, both for heavily and minimally pretreated patients. Phase I clinical studies underlined partial response in some cases and indicated that the conjugation to PEG notably improved the pharmacokinetics of the compound. Similarly, in phase II studies the same amount and administration schedule was recommended [106].

6.2.2. NKTR-102 (PEG-Irinotecan). The multiarm PEG design was employed for the synthesis of NKTR-102 by Nektar Therapeutics in which the drug was conjugated to a four-arm PEG for the treatment of solid tumors [107]. The plasma half-life evaluated for NKTR-102 in a mouse model taking into consideration the active metabolite SN-38,

released from irinotecan demonstrated prolonged pharmacokinetic profile with a half-life of 15 days compared to 4 h with free irinotecan [53]. While in phase I clinical trial the safety, pharmacokinetic and antitumour activity of NKTR-102 were evaluated on patients with advanced solid tumors, (e.g., breast, ovarian, cervical, and non-small-cell lung cancer). Interestingly, 13 patients showed significant antitumor activity and reduction of tumor size ranging from a 40% to 58%, while 6 patients showed minor response only [22]. The cumulative SN38 exposure in patients treated with NKTR-102 was 1.2- to 6.5-fold higher than that predicted for irinotecan. The maximum tolerated dose (MTD) of the conjugate was to be 115 mg m^{-2} and the toxicity was manageable (diarrhea and not neutropenia is dose limiting). Noteworthy, that the patients enrolled in this study had failed the prior anticancer treatments or have tumors with no standard treatments available. Multiple phase II studies are ongoing with NKTR-102 alone or in combination with cetuximab for the treatment of ovarian, breast, colorectal, and cervical cancer [53].

6.2.3. EZN-2208 (PEG-SN38). The multiarm PEG-SN38 conjugate which recently entered phase I clinical trials (year) showed an increased drug loading of 3.7 wt.% with respect to pegamotecan. SN38 is an active metabolite of irinotecan and has 100- to 1000-fold more cytotoxic activity in tissue cell cultures than irinotecan. However, SN38 is practically insoluble in water and hence cannot be administered intravenously [53]. This PEG conjugation enhanced the solubility of SN38 by about 1000-fold. The conjugate acts as a prodrug system with a half-life of 12.3 min of SN38 release in human plasma. Even though the drug release is quite rapid, the PEG conjugate accumulates in tumor mass by EPR effect. In fact, EZN-2208 showed a 207-fold higher exposure to SN38 compared to irinotecan in treated mice, with a tumor to plasma drug concentration ratio increased over the time during the four-day-long pharmacokinetic and biodistribution studies [108]. Earlier, the derivatives demonstrated promising antitumor activity *in vitro* and *in vivo*. Especially, in mouse xenograft models of MX-1 breast, MiaPaCa-2 pancreatic, or HT-29 colon carcinoma, treatment with the conjugate administered either as a single dose or multiple injections exhibited better results than irinotecan [56]. However, recently Enzon Pharmaceuticals, Inc. announced the discontinuance of its EZN-2208 clinical program, following conclusion of its phase II study. The decision was taken in light of evolving standards of care for the treatment of metastatic colorectal cancer (mCRC). The company planned to continue to enroll studies for the other PEG-SN38 programs, which included a soon-to-be fully enrolled phase II study in metastatic breast cancer, a phase I study in pediatric cancer, and a phase I study in combination with Avastin (bevacizumab injection) in solid tumors [109].

7. Clinical Perspective

Early polymer therapeutics were developed as treatments for life-threatening diseases (cancer and infectious diseases), the emerging products, and clinical development candidates are designed for a much broader range of diseases. NKTR194, an opioid drug, being developed by Nektar using their advanced polymer conjugate technology platform is presently in the preclinical stage [110]. It has been designed to act peripherally without entering the CNS so that the gastrointestinal bleeding, CNS side effects, and cardiovascular risks associate with NSAIDs and COX-2 inhibitors used for treating moderate pains. NKTR-171 is another drug being designed by Nektar to treat neuropathic pain without CNS side effects is in the early research stage. NKTR-125 also in the research stage combines Nektar's PEGylation technology with potent antihistamine to enhance its anti-inflammatory properties and minimize the side effects.

BAX 855, Baxter's most advanced longer-acting candidate, is schedule to move into phase I clinical trial in 2011 [110]. It is a PEGylated FVIII molecule, which utilizes Nektar's PEGylation and Baxter's proprietary plasma and albumin-free platform. Preclinical animal studies have revealed that 1 injection of BAX 855 per week imparted similar FVIII levels as that of 3 injections of Advate given approximately every alternate day. In addition, Nektar and Baxter have collaborated to design long-acting clotting protein for hemophilia using Nektar's innovative PEGylation and releasable linker conjugate technology [110].

Convincingly, there are pioneering new approaches in research, for example, PEG-recombinant human HA-degrading enzyme, (rHuPH20) developed to degrade HA (it often accumulates in the tumor interstitium) with the aim of decreasing interstitial tumor pressure and to enhance penetration of both low-molecular-weight and nanosized anticancer agents [111, 112]. The latter provides an interesting opportunity for combination therapy.

8. Conclusions

PEG is currently the only water soluble polymer, widely accepted in therapeutics with market approval for different drugs. The reason for the wide utility of PEG is because its decreased interaction with blood components (low plasma protein binding) and high biocompatibility. PEGylated drugs such as peginterferon α and pegfilgrastim have proven their cost-effectiveness in the market, and products like pegvisomant and certolizumab pegol demonstrate that PEGylated forms will be marketed regardless of the prior commercialization of their non-PEGylated counterparts. This trend indicates that the long-term prospects for the biopharmaceutical PEGylated protein market are high. Due to significant clinical advantages, PEGylation is an essential proposition in delivering drugs and other bioactives. The therapeutic advantages of G-CSF, IFN, and EPO have been acknowledged, and PEGylation offers an attractive means of replacing the original market, given the assumption that biosimilars will appear soon after patents expire. Moreover, PEGylation allows drugs to be distinguished from simple biosimilars. The critical perspective of PEGylation is now envisioned to achieve cellular targetability and therefore suitable chemistry is being explored. Advanced forms of PEGs and their various architectures are designed and being introduced (e.g., hyper

branched polyglycerols) [113]. Therefore, the importance of conducting comprehensive investigations on recently introduced potent peptides, proteins, oligonucleotides, and antibody fragments for PEGylation cannot be overemphasized.

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Review Article

Nanotechnology in Medicine: From Inception to Market Domination

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Born from the marriage of nanotechnology and medicine, nanomedicine is set to bring advantages in the fight against unmet diseases. The field is recognized as a global challenge, and countless worldwide research and business initiatives are in place to obtain a significant market position. However, nanomedicine belongs to those emerging sectors in which business development methods have not been established yet. Open issues include which type of business model best fits these companies and which strategies would lead them to sustained growth. This paper describes the financial and strategic decisions by nanomedicine start-ups to reach the market successfully, obtain a satisfactory market share, and build and maintain a competitive defendable advantage. Walking nanomedicine-product from the hands of the inventor to those of the doctor, we explored the technological transfer process, which connects laboratories or research institutions to the marketplace. The process involves detailed analysis to evaluate the potentials of end-products, and researches to identify market segment, size, structure, and competitors, to ponder a possible market entry and the market share that managers can realistically achieve at different time horizons. Attracting funds is crucial but challenging. However, investors are starting to visualize the potentials of this field, magnetized by the business of "nano."

1. Introduction

Globally defined as the application of nanotechnology to the clinical arena, nanomedicine has its roots in the same basic concepts and principles of nanotechnology; that is, materials with the nanoscale features present unique characteristics, otherwise absent at a macroscopic level [1]. Just as nanotechnology benefits from mathematics and engineering, nanomedicine too has a multidisciplinary nature involving notions and techniques borrowed from biology, chemistry, and physics [2]. As a result of this successful marriage, nanostructure materials display emerging functions that have exceptional benefits when applied to medical devices.

The success of nanotechnology in the healthcare sector is driven by the possibility to work at the same scale of several biological processes, cellular mechanisms, and organic molecules; for this reason, medicine has looked at nanotechnology as the ideal solution for the detection and treatment of many diseases. One of the many applications of nanotechnology to the medical sector is in the field of drug delivery. The advent of protocols and methods for the synthesis, functionalization, and use of nanoparticles and nanocarriers has flooded the scientific and clinic community with new therapeutic approaches from molecular targeting to radiofrequency ablation and from personalized therapies to minimally invasive techniques.

While most members of the investment community are able to grasp the meaning of nanotechnology and can expertly launch and manage a viable product into the market,

they are limited in their conceptual understanding of this scientific discipline and the intricate inner workings behind the product's functionality [3]. On the contrary, those involved in the scientific research recognize that nanomedicine is an expansion of nanotechnology but have very little understanding of the business expertise required to develop their technologies into a commercial product [3]. Cooperation is therefore needed between the two factions in order to lead nanomedicine-based inventions to a successful market position.

2. Nanomedicine Market

With 76% [4] of the publications and 59% [4] of the patents, drug delivery is the market segment that dominates the nanomedicine sector. In vitro diagnostics represent the second leading field, contributing with 11% [4] of the publications and 14% [4] of the patent filings. According to the European Commission [4] in a global vision, clustering the publications in the three geographical areas USA, Europe, and Asia (Japan, China, South Korea, Taiwan, Singapore, and India), Europe is leading with 36% [4] of the worldwide publications, followed by the USA with 32% [4] and Asia with 18% [4]. Considering all patent applications in the different fields of nanomedicine, USA hold a share of 53% [4], Europe has 25% [4], and Asia 12% [4]. Biopharmaceutical and medical devices companies are well aware of the potential applications of nanotechnology to the healthcare sector, as demonstrated by the increasingly growing partnerships between these enterprises and nanomedicine startups.

According to a research report from the Business Communications Company (BCC) Research, despite the catastrophic consequences of the 2008-2009 crisis on capital markets, the global nanomedicine sector, which was worth $53 [5] billion in 2009, is projected to grow at a compound annual growth rate (CAGR) of 13.5%, surpassing $100 billion in 2014 (see Figure 1(a)) [5]. One of the largest segments of this market is represented by anticancer products. Valued about $20 billion [5] in 2009, it is expected to reach $33 billion [5] in 2014, growing at a CAGR of 11% [5] (see Figure 1(b)).

3. Financing of Nanomedicine

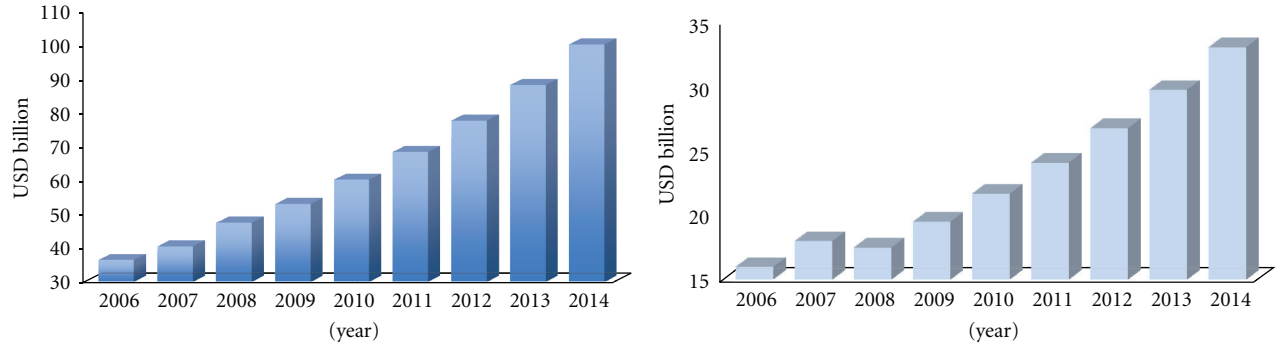
3.1. Common Issues in the Investments on Innovation. The primary output of innovation is obtaining the know-how, which the inventor initially possesses. Unfortunately, the confidentiality of this knowledge can be breached and its use by one company cannot preclude the use of the same by another one. Therefore, investors approaching novel projects are aware of the fact that they will not be able to easily appropriate the total returns of the investment undertaken. As a consequence, there is a lack of attractiveness in financing innovative projects. In fact, from the perspective of economic theory, it is complex to find funding for innovative ideas in a competitive market place [6]. Even in large firms, there is evidence of shortages in resources to spend on the innovative projects that the managers would like to undertake [6]. There are a number of reasons for this phenomenon: low expected returns due to an incapacity to capture the profits from an invention, the exaggerated optimism in undertaking an investment on breakthrough projects, and most notably the uncertainty and risk associated with these projects. Technology-based companies can also consider imitating the inventions developed by competitors. However, Edwin et al. [7], using survey evidence, found that imitating is not costless and could result in expenses equal to 50% [7] to 75% [7] of the cost of the original invention, not eliminating the underinvestment problem. Policymakers are trying to change the funding situation, by facilitating the invention process, rationalizing the interventions through government encouragement of innovative activities, sustaining the intellectual property system, allowing Research and Development tax incentives, and supporting research collaborations. Nonetheless, the path that leads the nanoscale outcome from the laboratory to the marketplace is long and expensive, putting the inventor in a position of disadvantage.

3.2. Asymmetric Information, Credibility, and Commitment. The financing and management of innovative products in nanomedicine—like many young and innovative multisectoral fields—happens in a context of both financial and product markets failures. These make the financing and management of innovation a particularly complex process, which is also reflected in the corporate governance structure of innovative firms.

Asymmetric information, transaction costs, intangible goods, credibility, and commitment issues, jointly with high and unique risks, make it impossible for traditional financial institutions to be part of the picture, paving the way for angel investors, seed and venture capital investors, or other forms of nontraditional financial institutions.

The asymmetric information issue is partly due to the different information set in the hands of the innovator as opposed to that of the possible provider of funds [8], which gives rise to a "two-sided incentive problem" [9]: the best incentive to reconcile the conflicting behavior of entrepreneur (unobservable efforts) and venture capitalist (monitoring costs) is multistage financing. In an alternative approach, staged financing solves the lack of credibility and of an adequate commitment technology on the part of the entrepreneur.

The credibility and commitment issues arise because the entrepreneur possesses a "unique human capital" [10]: once the Venture Capital has provided financing, the entrepreneur can decide to withdraw and, therefore, hold the VC hostage of his/her decisions. In such conditions, the VC would not provide financing, as the entrepreneur cannot make a credible commitment not to withdraw. The solution in this case is the "staged capital commitment" similar to Hellmann [9] with a different rationale: the unique human capital of the entrepreneurs must be blended with the firms in various sequential stages. This leads to a progressive increase in the expected value of the firm (in terms of a future initial public offering), so that the initial investments become the collateral



(a) Global nanomedicine market size (USD billions)

(b) Anticancer products market size (USD billions)

Figure 1: (a) This graph shows the global nanomedicine market size, measured in terms of revenues, such as sales revenues, grants revenues, and milestones. From 2006 to date, a steady growth has occurred, which is expected to continue through 2014, at a CAGR of 13.5% [5]. (b) The graph illustrates the market size for the anticancer applications segment. Except for a slight decrease in 2008, the market has and is predicted to expand by a factor of steady growth [5].

(the firm itself) for the VC, providing the right incentive to continued financing.

The two approaches also require both the entrepreneur and the VC to participate in the ownership of the firm (as financing happens with shares) and therefore an evolving strategic and managerial relationship between the two parties in an evolutionary view of the firm [11]. Often the VC possesses very good managerial skills, due to its experience in dozens of startups, while the innovating entrepreneur has little or none. Against this backdrop, the staged financing with shares (i.e., joint ownership) also helps addressing the key issue of management decisions: at the beginning of the "relationship," the entrepreneur has the most detailed technical knowledge and almost complete managerial powers to set up all the technical work that needs to be embodied into the firm. As this knowledge is transferred to the firm, other managerial aspects take priority (e.g., competition, finance, governance) where the VC has better skills. By increasing VC ownership in stages, management powers can be transferred to VC-appointed managers, with specific skill in running an evolving start-up firm and take it adequately to the market, usually with an IPO.

Due to significant concern and disapproval for fundraising in support of innovation, fledgling nanomedicine companies do not have an endless number of financial options. Therefore, in order to establish start-up companies, cofunders generally commit their own money and expertise into it. This is one aspect that represents the internal capital of the startup, as opposed to the external one, which has to be collected from other sources. At this stage start-up companies turn towards government and foundations' grants (i.e., the National Institutes of Health, and the National Science Foundation programs), in order to finance the research and development of their innovative products. These funds are also intended to protect the intellectual property of these novel discoveries and to attract professional investors.

In order to expand and sustain their business, nanomedicine startups usually begins by turning to angel investors—private financiers who provide seed funding—then to venture capitalists (VCs). The interaction and support of these professional investors is essential to assess whether a market entry is possible and to decide which market share managers can realistically achieve at different time horizons. In fact VCs enter at a specific moment of the life of the company when it is still in an early stage, but has already strongly proved its value and perspective. According to Paul A. Gompers and Yuhai Xuan, the general role of VCs is to alleviate asymmetric information between private venture capital-backed targets and the public acquirers, building a bridge between the two parts [12]. These funds plan investment decisions in order to decrease possible agency costs that afflict young entrepreneurial companies. Venture capitalists usually add value to companies in which they invest beyond pure financing, providing managerial expertise, industrial experience, contacts and—not least—momentum [12]. There is strong evidence of VCs involvement in the management of the financed nanotechnology companies as they often have higher costs and longer development times compared to an equivalent information technology business. Furthermore, Baker and Gompers [13] asserted that venture capital-backed firms have better boards of directors compared to those not financed by VCs. This evidence confirms the crucial role played by VCs in the economic success of nanomedicine-based products.

Corporate finance literature has devoted a meaningful stream of research to the relevance of board composition as a useful tool against different typologies of asymmetric information and agency costs. The literature has clearly underlined the existence of a connection between firms' performances and board composition. However, notwithstanding these important results, there is not a universally accepted evidence about the optimal board composition that allows the minimization of the above-mentioned agency costs. In the VC literature evidence, a board composed by internal, external, and instrumental [14] should achieve

the result of the minimization of agency costs that is a propaedeutic step for a feasible way out for VC investors.

3.3. Landscape. In 2007 investment in nanotechnology by VCs was US $702 million [15], involving 61 deals. 27% [15] went to healthcare and life science, 31% [15] to energy and environment, and 42% [15] to electronics and IT. Two years later, nanotechnology market captured US $792 million from VCs [15]. Of these, the largest share (51%) [15] went to healthcare and life sciences, followed by energy and environment and electronics and IT, with 23% and 17%, respectively [15]. Doubling the funds invested in the healthcare segment in just two years, the VC industry has demonstrated a clear interest in investment opportunities in the nanomedicine field (see Figures 2(a) and 2(b)).

Although venture capital investors want to continue to be involved in the science and technology of the small scale, they are extremely cautious about large investments in nanotechnology and nanomedicine, as positive returns on investments are expected only in the long term, especially for nanomedicine [3]. VCs and private investors are still burned by the subprime crisis of 2008 [16], which took a serious toll on their assets, causing catastrophic losses to the whole financial community and restricted access to funds. However, the decline of fundraising might also be a result of ordinary funding cycles, with several VCs having already raised enough resources for the short term [17]. Experts see the Wall Street's crisis of 2008, as a possible regime change [16], rather than a temporary market malfunctioning. After four decades of fairly straightforward access to relatively inexpensive capital, capital markets are currently undergoing major changes [16]. According to the National Science Foundation, innovation is an essential source of competitiveness for economy [18] and represents an excellent opportunity to sustain the economic recovery after the 2008 crisis. As usually happen after a crisis, investors become risk adverse, adopting more rigid risk-cover policies, but there is evidence that the nanobusiness seems to be too attractive not to invest in.

4. Business Strategies

The main business area characterizing a nanomedicine company, as well as pharmaceutical and biotechnology industries, is the research and development (R&D). Choosing the R&D strategy, managers evaluate two possible options. The first is based on the idea to perform the entire process inside the company, composing a highly experienced team of scientists. The second option is based on universities or research institutes and is founded on the reliance on leading academic laboratories created over time by "scientific stars." This second possibility will certainly reduce company costs as these academics frequently cofound the companies based on their discoveries and become part of the scientific boards. We have gathered strong evidence of this second option for the R&D strategy in the companies we analyzed. The commercialization of the research-based product might represent another business area of the nanomedicine company. However, the typical option considered and adopted by managers is to license out the manufacturing and commercialization of the nanomedicine-based product to larger companies. If this is the case, the business model pursued will not include commercialization, and the company will be technology and research based.

The commercialization of the nanomedicine products/technologies is currently driven by startups and small-medium enterprises (SMEs) [4], and it is performed through three types of business models.

(1) *The development of a nanotechnology platform that can be used to add value to second-party products*: this business model seems to be particularly attractive for drug delivery companies, which typically license their particular technologies out to pharmaceutical industries. Otherwise the drug delivery system is tailored and applied to a specific drug complying the particular instructions of the larger company [4].

(2) *The development and manufacturing of high-value materials for the medical device and pharmaceutical industry*: several startups and SMEs merely provide nanomaterials for the manufacture of medical devices or nanotechnology-enhanced drugs [4].

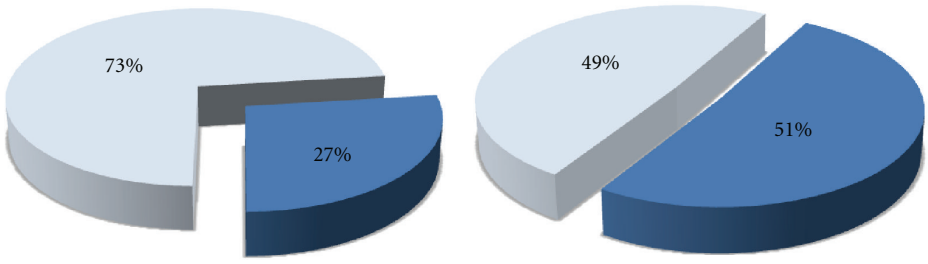
(3) *The development of nanotechnology improved medical devices or pharmaceuticals*: companies adopting this business model intend to develop a proprietary product pipeline as well as trying to bring to the market place new or standard drugs delivered with a drug delivery system or else to develop, for example, a new diagnostic platform based on nanotechnologies [4].

5. Regulatory Risk

The US Food and Drug Administration's long approval procedure and regulations make nanomedicine products different from those of other industries using nanotechnologies with no limitations due to regulatory bodies. As a consequence, the expenditure to bring a nanomedical product to the marketplace is so huge that pharmaceutical and biotechnology industries have no alternative but focus on the blockbusters that can please the stockholders [3]. Nanoparticles are not inevitably hazardous, but they have unique properties that question their safety. It is reasonable to presume that nanomaterials are "new for safety evaluations purposes" [3], and therefore they merit careful regulatory oversight by FDA both before and after entering the marketplace. In this arena, federal agencies like the FDA and the US Patent and Trade Mark Office (PTO), impose a sort of order, for the protection of the population safety, while encouraging the development of these products.

The advent of nanomedicine, beside causing changes in the biopharmaceutical industries' business model and value chain, brought two crucial regulatory issues: difficulties in product classification and a lack of scientific expertise on the part of the FDA [19].

On the basis of the product's principal method of action, the FDA classifies nanoproducts as drugs, devices, or combination thereof. For regulatory purposes, the FDA applies the same requirements to each part of the combination product



(a) Venture capital funding of nanotechnology in 2007. Total investment amount: USD 702 millions. Fundings spread across 61 deals. Investment average size: $11.51 million

(b) Venture capital funding of nanotechnology in 2009. Total investment amount: USD 792 millions. Fundings spread across 91 deals. Investment average size: $8.6 million

Figure 2: Venture capital investors. Captivated by the great potential of future development, in only two years VCs have shifted their focus on the "science of the tiny things", nearly doubling investments in this sector.

and verifies whether the manufacturer gave the correct definition to the product. The definition becomes extremely ambiguous novel for nano-based drug delivery devices as they can be considered either devices (carriers) or drugs (effectors) [19, 20]. The FDA will face exceptional challenges in efficiently regulating such products. In order to successfully do so, a strong scientific knowledge of the field is essential together with a better understanding of the potential risk associated to the exposure of patients to nanomedical products [19].

6. Best Practices in the Clinic

Bringing new products to the market has always represented a great challenge, especially when it comes to highly innovative products with high risk/high return. Despite the numerous entry barriers of the nanomedicine market, there are some noteworthy examples of nano-based FDA-approved products that successfully reached the market, impacting medicine and anticipating a change in the healthcare arena.

Within the anticancer products segment, Doxil and Abraxane are two main examples of success in the clinic. Sequus Pharmaceuticals was the first company to sell doxil, the liposomal formulation of Doxorubicin, a powerful but toxic chemotherapeutic, initially approved for treatment of Kaposi's sarcoma in the USA in 1995 [21]. Sequus was then acquired in 1998 by ALZA Pharmaceutical for US $580 millions [22], which subsequently merged with Johnson and Johnson in 2001 in a US $12.3 billion deal [22]. The other approved nanotherapeutic agent, Abraxane, instead, was originally sold by Abraxis Biosciences, which was acquired in June 2010 by Celgene Corporation for US $2.9 billions [23]. Granted by the orphan drug designation in January 2005 by the FDA, this product consists of albumin nanoparticles containing paclitaxel, and is indicated for the treatment of breast cancer [21]. Conventional chemotherapies consist of injections of cytotoxic drug intravenously, which indiscriminately kill both healthy and tumor cells. The clinic success of Doxil and Abraxane was driven by their ability to concentrate preferentially in tumors, because of the gaps (otherwise called endothelial fenestrations) characterizing the blood vessels that supply the cancerous mass. Nanoparticles of the right size can penetrate these "gates" and passively diffuse into the tumors [24]. Thanks to this generation of chemotherapies, patients are now benefiting from new treatment strategies for delivering drugs through nanotechnology carriers with lower systemic toxicity and improved therapeutic efficacy [21].

The economic success of these nanomedical products is driven by an urgent demand of new anticancer therapies able to better fight this highly aggressive and increasingly frequent disease. In fact, the FDA problematic regulatory process, the unsteady funding situation, and the expensive and lengthy R&D process did not thwart the development and success of Doxil and Abraxane.

Despite being the most profitable, anticancer delivery systems are not the only clinically approved nanomedical products. In fact, advances in nanomedicine are bringing breakthroughs in other problematic areas of medicine. Following are some examples of successful nano-enabled biomedical products currently on the market.

The first successful application of nanoparticles in the clinic was Omniscan, the leading injectable paramagnetic resonance product of Amersham. This contrast agent was approved for magnetic resonance imaging (MRI), launched in 1993, and utilized ever since both in neurology, to detect strokes and brain tumors, as well as in cardiology. This contrast agent—originally developed by Salutar—has prolonged half-life in patients with renal insufficiency. After the conduction of preclinical testing, Salutar was acquired by Nycomed, which in turn purchased Amersham International, in 1997. Currently, Amersham and its rights on

Omniscan are propriety of General Electric Healthcare. The deal was closed in 2003 for US $9.5 billion on an all-stock transaction. According to Yan et al. [25] and as confirmed by Spiess [26], there are 12 different MRI contrast agents currently on the market [27]. Magnevist was marketed by Bayer Schering Pharma as their first intravenous contrast agent employed in the clinic. In 2004, the company demonstrated that the product safely and effectively eases the visualization of cranial and vertebral anatomy among cancers and wounds, and since then it is diffused worldwide with that specification of use [28]. Another competitor is OptiMARK, a gadolinium-based contrast agent (the only FDA-approved for administration by power injection) for MRI of brain, liver, and spine [29] produced by Mallinckrodt; it allows the visualization of lesions with atypical vascularity. Finally, MultiHance is the first extracellular fluid contrast agent to pose interaction with plasma proteins. Bracco Group produces this contrast agent—an Italian company specialized in diagnostic imaging, drugs and devices—and is utilized in diagnostic MRI of the liver and central nervous system (CNS). It was launched in Europe in 1998 and received the FDA approval for market the product in the United States in 2004 [30].

Returning to the segment of the pharmaceutical applications of nanomedicine, it is important to remember the two FDA-approved nanoparticles-based drugs applied for the treatment of severe fungal infections: AmBisome (liposome for injection), sold by Gilead Sciences and Fujisawa Healthcare and Abelcet (lipid complex), marketed by Elan Corporation. Liposomal formulation of amphotericin B (AmBisome, in its trade name) was originally one of the income-making drugs of NeXstar Pharmaceuticals. The company, along with its products portfolio, was then acquired by Gilead in March 1999. For what concerns Abelcet (the conventional amphotericin B), its North America rights were acquired by Enzon Pharmaceuticals in 2002, in an operational and profitable deal of $360 million (including facilities and operating assets related to the development, production, and sale of the drug). The drug was employed in the treatment of patients with aggressive fungal infection associated to cancer, organs' transplantation, and other postsurgical complications [31]. We wanted to emphasize these two specific products also because they have been subject of a "pharmacoeconomic study." As a result of the analysis, that involved the two drugs in the empirical treatment of persistently febrile neutropenic patients with presumed fungal infection, AmBisome was found to be more cost-effective compared to Abelcet [32].

RenaZorb sold by Spectrum Pharmaceuticals represents another case of a nano-enabled product, which fruitfully reached the marketplace for the treatment of hyperphosphatemia in end-stage renal disease (ESRD) and potentially chronic kidney disease (CKD). RenaZorb is a lanthanum-based phosphate-binding agent currently in clinical trial, utilizing Spectrum's proprietary nanoparticle technology [33]. The economic and clinical success of this nanoparticle is mainly driven by the clinical scenario. According to the National Kidney Foundation, only in the US are estimated to be more than 20 million people with CKD with numbers expected to double over the next decade. These patients live on kidney dialysis and are potential candidates for phosphate binder therapy [34].

In the light of all this overview of the best practices in the clinic, anticancer remains the biggest share of the nanomedicine market, besides for number of publications and patents, also for number of commercialized products. Increasing acceptance with the general public of the employment of nanotechnologies in the clinic, along with popular widespread sensitivity for the aggressiveness of cancer, can be considered strong drivers for the commercial success of this segment. Furthermore, the first tangible considerable returns due to commercial triumphs represent an undoubted source of attraction for investors. On their part, financiers must realize the importance of providing the substantive funds, necessary to gain the solid results and successful drugs as well as devices and therapies the market requires. The effective investments on Doxil and Abraxane, as well as on the other mentioned successful products, are prime examples of this practice.

7. Conclusions and Future Promises

Despite the issues nanomedicine still has to face, investments in this market are predicted to increase. New applications of nanomedicine have been demonstrated, and the resulting expansion of the potential market makes the risk more appealing. Ferocious financial collapse elevated sunk costs of the essential R&D process, tricky access to funds, uncertainty of expected returns, and the extremely meticulous, and lengthy FDA regulatory process has not deterred the investors' community. On the other hand, the promises of great future potential developments in the different market segments and high returns connected to the high risk of the innovation investments make this market still considerably attractive. Compared to the 2007 benchmark, VCs in 2009 decided to double their investments in this sector, at the expenses of the information technology market. The fact that nanomedicine dominates the VC funding in the healthcare market is surely a good predictor of the bright future landscape of expansion of this promising area of research.

Moreover, good returns could even be the result of more accurate assessments of the investments' risks. A pharmacoeconomic analysis would allow the efficient allocation of the monetary resources and the maximization of the highest health return at the lowest costs. A cost-effectiveness analysis (CEA) is structured with a comparison of the costs and effects of two or more treatments, which are under examination. Whereas in the very early stage of the drug development cycle the high failure rate for novel drug molecules is largely due to a not adequate therapeutic index, in the clinical development phase, this rate originates from economic reasons. Therefore, the development of unsuccessful drugs has to be abandoned very fast, in order to save resources for more promising compounds. This saving is obtained through an accurate economic evaluation performed in the early stages of the development process. The benchmark is represented by life-years saved by the investigated nanotherapeutic; if a nano-enabled therapy does not

save sufficient life-years to break-even, it should not be developed further [35].

The major limit to the success of this kind of analysis is given by the scarcity of clinical data concerning nanomedicine. The best solution to this issue is collaboration. According to Bosetti and Vereeck [35], economists and investors specialized in health market should work closely with healthcare providers, researchers, patients associations, doctors, and technologists of all kinds, to create a shared platform able to facilitate communication between parties with the ultimate aim to reduce the high risks associated to investments in nanomedicine. As a result, also patients will benefit from these investments, in terms of innovative techniques, therapies, devices, and drugs designed to extend and improve their lives.

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Review Article

Successfully Improving Ocular Drug Delivery Using the Cationic Nanoemulsion, Novasorb

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Topical ophthalmic delivery of active ingredients can be achieved using cationic nanoemulsions. In the last decade, Novagali Pharma has successfully developed and marketed Novasorb, an advanced pharmaceutical technology for the treatment of ophthalmic diseases. This paper describes the main steps in the development of cationic nanoemulsions from formulation to evaluation in clinical trials. A major challenge of the formulation work was the selection of a cationic agent with an acceptable safety profile that would ensure a sufficient ocular surface retention time. Then, toxicity and pharmacokinetic studies were performed showing that the cationic emulsions were safe and well tolerated. Even in the absence of an active ingredient, cationic emulsions were observed in preclinical studies to have an inherent benefit on the ocular surface. Moreover, clinical trials demonstrated the efficacy and safety of cationic emulsions loaded with cyclosporine A in patients with dry eye disease. Ongoing studies evaluating latanoprost emulsion in patients with ocular surface disease and glaucoma suggest that the beneficial effects on reducing ocular surface damage may also extend to this patient population. The culmination of these efforts has been the marketing of Cationorm, a preservative-free cationic emulsion indicated for the symptomatic treatment of dry eye.

1. Introduction

Ophthalmic diseases are most commonly treated by topical eye-drop instillation of aqueous products. These formulations, however, raise technical problems (e.g., solubility, stability, and preservation) and clinical issues (efficacy, local toxicity and compliance). Conventional aqueous solutions are limited to water-soluble molecules and by the fact that within two minutes after instillation over 80% of the product is eliminated via the nasolacrimal drainage system limiting ocular penetration of the drug to less than 1% of the administered dose [1]. Consequently, pharmaceutical companies have been faced with the challenge of developing a formulation for topical administration which would expand the range of potential active ingredients, remain longer on the ocular surface, and provide sustained therapeutic concentrations in addition to meeting the regulatory criteria for approval. The main challenges in ocular drug delivery and key considerations to develop an ophthalmic preparation are listed in Table 1.

Nanotechnologies are currently considered the best solution to improving the ocular delivery of ophthalmic drugs even though products reaching the market using nanotechnologies are still rare [2]. Some reasons for this are that most of the nanosystems, even the pharmaceutically efficient ones, have encountered technical issues such as stability of colloidal systems [3], requirement for new excipients or use of organic solvents noncompliant to regulatory standards, unknown or unacceptable toxicity profiles [4], or unique scale-up and manufacturing requirements.

Notwithstanding, nanotechnology remains a promising approach for ophthalmic drug delivery. Compared to currently available approaches for administering eye drops, nanosystems with bioadhesive properties (e.g., cationic nanoemulsions) are more efficient at delivering the appropriate concentrations of bioactive molecules to the eye. The

Table 1: The main challenges in ocular drug delivery and key considerations.

Challenges
Absorption: only 3 to 4% ocular bioavailability after topical administration with traditional eye drops
Poorly-soluble drugs: conventional aqueous eye drops not suitable for lipophilic drugs (40–60% of new chemical entities)
Patient compliance: multiple instillations are often needed with eye drops to reach therapeutic levels
High tolerability/comfort requirements limit the formulation options
Excipient choice: few excipients listed in ophthalmology (oils, surfactants, polymers…)
Posterior segment drug delivery: no topical system for the posterior segment; invasive treatments are used due to lack of alternatives
Considerations
Anatomy & physiology of the eye: mucus layer, eyelids, metabolism, blink wash-out…
Tear composition: lipid outer layer, stability of the tear film, enzymes…
Disease state: impact of keratitis or inflammation on absorption and clearance…
Ocular comfort: tolerability of the formulation, pH, osmolality, viscosity, drop size…
Patient expectations: type of packaging and squeeze ability impacting compliance…
Drug loading: impact on absorption, efficacy, dosing regimen, compliance…

mechanism underlying the bioadhesiveness of nanosystems is an electrostatic interaction which prolongs the residence time on the ocular surface [5]. To create an electrostatic interaction with the negatively charged cells of the ocular surface, the vector should be positively charged. This is the advantage of the Novasorb cationic nanoemulsion technology.

The aim of this article is to describe the development of the cationic nanoemulsion technology from bench to patients. The first stage of development after an initial proof-of-concept carried out at the University of Jerusalem was to formulate the nanoemulsion with a cationic agent, an oily phase and surfactants compliant with international pharmacopeias (i.e., US and EU pharmacopeias). The objective was to provide a stable and sterile cationic nanoemulsion loaded with an active ingredient approvable by the regulatory agencies. The completion of a full preclinical package and clinical trials in patients with ocular surface disease has led to the successful launch of the first product based on the cationic nanoemulsion technology.

2. Cationic Nanoemulsion for Ocular Delivery

As the neuroretina is an extension of the central nervous system, the external eye and its adnexa are designed to protect the internal ocular structures, particularly from harmful chemicals [6]. The first ocular barrier is the eyelid which acts as a shutter preventing foreign substances from contact with the ocular surface. The second barrier is the tears which are continuously secreted to wash the ocular surface of exogenous substances. Hence, the tears are mainly responsible for the short residence time and low absorption of drugs applied topically to the eye. The last protective ocular barrier is the cornea. The neuronal system of the cornea is able to detect changes in pH and osmolality which can induce reflex blinking and tearing. Also, the cornea forms a tight structural barrier made of three different tissue layers with alternating hydrophilic and lipophilic properties to prevent the intraocular absorption of unwanted substances [7].

Many attempts have been made to prolong the exposure time of topically applied ocular treatments and to improve their bioavailability, therapeutic efficacy, or patient compliance by reducing the number of required administrations [8–10]. Hydrogels, now widely used in the ophthalmic pharmaceutical industry, have enabled, for example, a decrease in the frequency of timolol administrations from two instillations daily to only one. Several excipients with either viscosifying or bioadhesive properties are commonly used (carbopol gels, cellulose derivatives, dextran, gelatin glycerin, polyethylene glycol, poloxamer 407, polysorbate 80, propylene glycol, polyvinyl alcohol, polyvinyl pyrrolidone) to prolong the ocular residence time. The use of such excipients, however, remains applicable to only hydrophilic drugs and the advantage of increasing the viscosity must be balanced against the potential disadvantage of inducing ocular disturbances due to the blurring of vision as a result of a change in the refractive index on the ocular surface. Furthermore, other disadvantages of higher viscosity are that more viscous solutions do not easily exit from the bottle tip and may impose limits to the sterilization options during manufacturing. Most recently, sophisticated approaches like punctal plugs with active ingredient [11], contact lens-releasing glaucoma medications, and injectable biodegradable micro- and nanoparticles were proposed but are today at too early a stage to be available to patients [8].

In addition to the challenges of increasing exposure, numerous lipophilic and poorly water-soluble drugs have become available in recent years that could be applicable to the treatment of a variety of ocular conditions. These drugs represent a formulation challenge for pharmaceutical scientists because of aqueous solubility limitations. Dosage forms for topical ocular application of lipophilic drugs include oily solutions, micellar solutions, lotions, ointments, and suspensions. The ocular administration of such dosage forms is not only uncomfortable for the patient but also of limited efficacy. Despite a large variety of submicron-sized colloidal carriers in the ophthalmic drug delivery field, nanoparticles and liposomes attract most of the attention since they appear to have the potential to yield greater efficacy over existing formulations [12, 13].

In the last decade, oil-in-water-type lipid emulsions, primarily intended for parenteral applications, have been investigated and are now being exploited as a vehicle to improve the ocular bioavailability of lipophilic drugs [14, 15]. Among these, nanoemulsions are considered excellent alternative formulations to deliver lipophilic drug substances

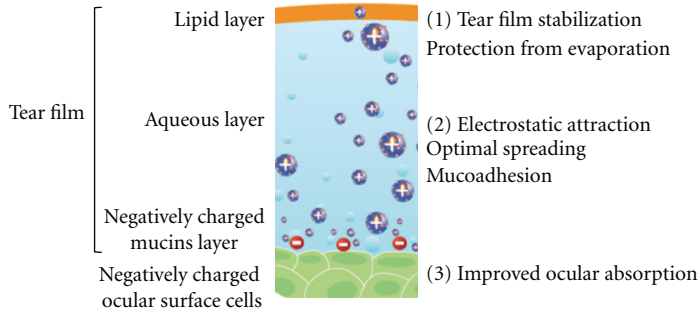


Figure 1: Cationic nanoemulsion interacting with negatively charged corneal cells. The effects of the cationic emulsion are (1) to bring lipids to stabilize the tear film, (2) to interact electrostatically with mucins, and (3) to improve ocular absorption.

to the eye. Emulsions provide a high encapsulation rate, an enhanced stability of the active ingredient, and enhanced ocular penetration. The first marketed ophthalmic emulsion drug product was Restasis (Allergan), a preservative-free anionic emulsion of cyclosporine A (CsA) at 0.05% indicated to increase tear production in patients whose tear production is presumed to be suppressed due to ocular inflammation. Although approved by FDA in 2002, Restasis was never accepted by European authorities. Other emulsion-based eye drops available on the US market are artificial tears (Soothe (Bausch & Lomb) and Refresh Endura (Allergan)). Other ophthalmic nanoemulsions are under development and among them are the products resulting from the Novasorb technology, originated from work at the Hebrew University of Jerusalem by Professor Simon Benita and developed by the French pharmaceutical company Novagali Pharma.

The Novasorb technology platform is based on the cationic nanoemulsion approach. The overall Novasorb strategy exploits the fact that the corneal and conjunctival cells and the mucus layer of glycosyl amino glycans lining the ocular surface are negatively charged at a physiological pH [16]. When applying a positively charged formulation to the eye it is likely that an electrostatic attraction will occur prolonging the residence time of the formulation on the ocular surface (Figure 1). In addition, the nanosize of the oil droplets creates a huge contact surface with the ocular surface cells enabling enhanced absorption. This approach was primarily conceived for oral administration [17] and it was adapted a few years later to ocular delivery by Klang et al. [18] to deliver indomethacin and Abdulrazik and coworkers [19] who intended to deliver cyclosporine A.

The potential of cationic emulsions for ophthalmic drug delivery was rapidly seen to offer advantages over the existing topical drug delivery vehicles [20–22]. However, this drug delivery approach was not exempt of hurdles and technology challenges particularly in the formulation phase as we will see further. During the development (from nonclinical to clinical), the products had to go back to the formulation stage to optimize their physicochemical properties due to stability, toxicity, or pharmacokinetic issues. Up to three generations of cationic nanoemulsions were then tested and patented over the 10 years of development [23–25].

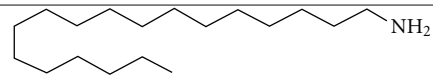
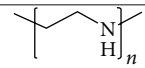
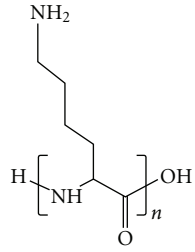
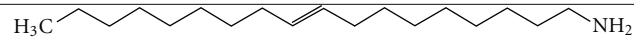
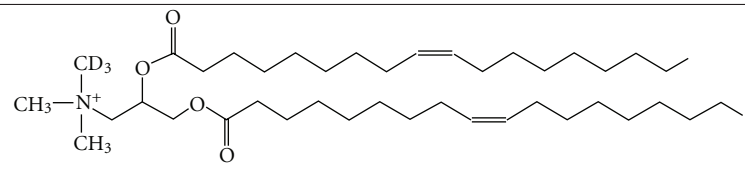
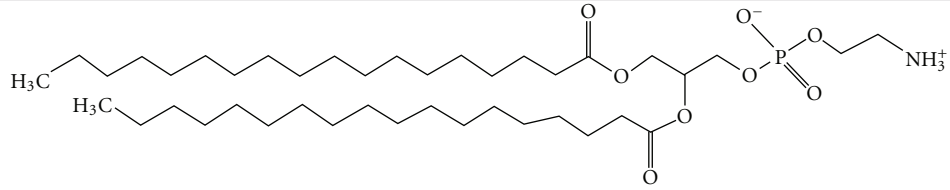
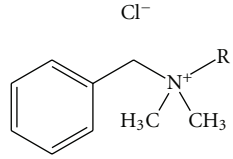
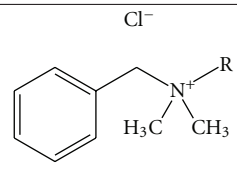
3. Formulation Development

3.1. Cationic Agent. The surface charge of the nanoemulsion is defined by the zeta potential. It corresponds to the electric potential surrounding the oil nanodroplet at the plane of hydrodynamic shear. It is measured by electrophoretic mobility. The latter depends on the nature of the cationic agent, its concentration and the electrolyte environment of the oil nanodroplets. In addition to increasing the residence time on the negatively charged ocular surface, the positive charge of the cationic agent contributes to the stabilization of the emulsion by creating an electrostatic repulsion between the oil droplets of the nanoemulsion [26]. Evidence that the specific nature of the cationic molecule may be responsible for improved uptake properties was supplied by Calvo et al. who showed that two different types of cationic indomethacin loaded nanocapsules (coated with poly-L-lysine or chitosan) resulted in completely different drug kinetics profiles [27]. Therefore, the cationic agent selected needs to be carefully considered prior to starting pharmaceutical development as the success of the formulation is highly dependent upon the choice of the cationic agent as will be discussed further.

Novagali showed that below a zeta potential of +10 mV, nanoemulsions could not be autoclaved without destabilizing the oil droplets. Therefore, the first challenge of the Novasorb technology was to make a cationic emulsion with a zeta potential sufficiently high to stabilize the nanoemulsion, yet with a cationic surfactant concentration as low as possible to avoid compromising the safety of the nanoemulsion. The optimal range for the zeta potential was demonstrated to be between +20 mV and +40 mV. Review of the literature revealed that of the numerous cationic agents described (Table 2) most of them are surfactants, indeed the positively charged region of the molecule does not enter the oil core of the droplet but instead remains at the surface, rendering them very useful for emulsions. Unfortunately, very few are listed in pharmacopeias or accepted for ophthalmic products due to stability or toxicity issues.

Compared to anionic and nonionic surfactants, cationic surfactants are known to be the most toxic surfactants [28]. Therefore, in order to develop the Novasorb technology it was necessary to find an appropriate cationic surfactant

Table 2: Chemical structures of common molecules used as cationic agent in drug delivery.

Stearylamine	
PEI	
PLL	
Oleylamine	
DOTAP	
DOPE	
Benzalkonium chloride	
Cetalkonium chloride	

which would provide a sufficiently high cationic charge, have a low toxicity, and conform to regulatory standards.

Stearylamine is one of the most widely used cationic lipids in the academic world especially for the manufacture of cationic liposomes [29] or cationic emulsions [19]. However, since this primary amine is very reactive towards other excipients and active ingredients and not described in any pharmacopeias, it was not a reasonable choice for pharmaceutical development. Oleylamine is another cationic lipid that has been used to manufacture ophthalmic emulsions [30], but this lipid also has stability concerns due to its primary amine function and the presence of an unsaturated site in the aliphatic chain.

Other cationic molecules usually used for DNA transfection are also frequently used for the formulation of cationic drug delivery systems: poly(ethylenimine) (PEI) and poly-L-lysine (PLL). PEI is an organic polymer that has a high density of amino groups that can be protonated. At physiological pH, the polycation is very effective in binding DNA and can mediate the transfection of eukaryotic cells [31]. It has been used as a cationic agent in micelles [32], nanoparticles [33], albumin nanoparticles [34], liposomes [35], and nanosized cationic hydrogels [36]. However, while some authors claim this polymer to be safe some others such as Hunter [37] have reported PEI to be extremely cytotoxic. PLL is a polymer made of several lysines (amino acid). Lysine

possesses a NH_2 function which is ionized at a physiological pH conferring several cationic charges to that polymer. It is sometimes used as cationic agent in drug delivery systems such as microparticles [38]. However, toxicity has been reported [39], and this polymer is not authorized for use in ophthalmic formulations.

Cationic lipids, DOTAP (N-(1-(2,3-dioleoyloxy) propyl)-N,N,N trimethylammonium) chloride and DOPE (dioleoyl phosphatidylethanolamine), represent another potential class of cationic agents. These are amphiphilic molecules with a fatty acid chain and a polar group bearing a cationic charge. Their main advantage is that they are biodegradable and well tolerated. DOPE, which also harbors a negative charge, is a neutral "helper" lipid often included in cationic lipid formulations like cationic nanoemulsions [40]. Cationic solid lipid nanoparticles were successfully made with DOTAP to transport DNA vaccines [41]. Hagigit and colleagues [42, 43] showed that using DOTAP was better than the seminatural lipid oleylamine to make stable cationic emulsions. Moreover, DOTAP cationic emulsion enhanced the penetration of antisense oligonucleotides after either topical ocular instillations or intravitreous injection. But like most of the seminatural lipids, these agents are chemically unstable and need to be stored at −20°C, thus drastically limiting their industrial use.

The primary limiting factors against the use of the previously cited cationic agents in the Novasorb technology, even though they showed potential in the formulation of cationic drug delivery systems, is that (1) they are not listed in US and EU pharmacopeias or (2) their toxicity on the ocular surface has not been well documented, and (3) none of these cationic agents has been successfully commercialized in a pharmaceutical product. Consequently, Novagali chose to limit its search for the appropriate cationic agent among those already registered, used in ophthalmic products, or compliant to pharmacopeias.

Other excipients previously accepted by health authorities were then considered. Quaternary ammoniums usually used as preservatives have surfactant properties and the potential to give a cationic charge to the nanoemulsions. These agents include cetrimide, benzalkonium chloride, benzethonium chloride, benzododecinium bromide, and cetylpyridinium. As preservatives these products protect against infectious contaminants by electrostatically binding to the negatively charged surface of bacteria and mycoplasma and disrupting their cell membranes. The disadvantage of quaternary ammoniums is that their effect on cell membranes is not limited only to microorganisms but they are also capable of injuring epithelial cells lining the ocular surface by the same mechanism of action. It was consequently not obvious to foresee these molecules as cationic agents, therefore, quaternary ammoniums were not initially considered for use in emulsions. In 2002, Sznitowska revealed findings that the preservative efficacy of this class of surfactants was diminished or neutralized in the presence of emulsions [44]. Part of the quaternary ammonium is bound to the emulsion, resulting in the presence of less free surfactant molecules in the aqueous phase to exert their antimicrobial action, and, consequently, their toxic effect on the ocular surface epithelia. Novagali Pharma exploited this physicochemical property to make a new type of cationic nanovector using benzalkonium chloride (BAK) and cetalkonium chloride (CKC) as cationic agents. CKC is a highly lipophilic ($\log P = 9.5$) component of BAK. It is hence mostly included in the oily phase providing a higher zeta potential on surface of the oil droplets while leaving relatively no free molecules to induce ocular surface toxicity. BAK (and CKC as a component of BAK) has been routinely used as a preservative in other marketed eye drop solutions (e.g., BAK is used in Xalatan) and is accepted as compliant with regulatory requirements for ophthalmic products. These excipients used in lower concentrations as cationic agents in emulsions have been demonstrated to be safe for the eye as we will see in the toxicology chapter of this article. More importantly, the use of BAK and CKC as cationic surfactants only in emulsions are now protected by several granted and pending European and US patents (e.g., EP1655021 [25], EP1809237 [45], EP1809238 [46], and EP1827373 [47] which are granted).

Table 3: Excipients which can be used in an ophthalmic emulsion.

Function	Excipients
Osmotic agents	Mannitol, glycerol, sorbitol, propylene glycol, dextrose
Oils	Medium chain triglycerides, mineral oil, vegetal oil such a castor oil
Cationic agents	Benzalkonium chloride, cetylpyridinium chloride, cetrimide, benzethonium chloride
Surfactants	Polysorbates, cremophors, poloxamers, tyloxapol, vitamin E-TPGS
Buffers, salts, and anions	To be avoided if possible
Water	Water for injections
Others	Viscosifying agents: preferably neutral Preservatives: preferably nonionic and hydrophilic

3.2. Other Formulation Issues. Following the choice of the cationic agent, other excipients, that is, nonionic surfactants, osmotic agents, and oils, need to be selected and their appropriate concentration decided (Table 3). The excipients authorized for ophthalmic use are quite numerous and this step of screening was mainly time dependent. An emulsion is a system which is by essence unstable. The stability is further ensured by the combination of excipients with the surfactants; this combination also defines the size of the emulsion. The concentration of surfactants should be a compromise between stability and toxicity. The most commonly used surfactants are poloxamers, polysorbates, cremophors, tyloxapol, and vitamin E TPGS.

To choose the appropriate excipients and their concentration, parameters like the final osmolality and pH of the nanoemulsion need to be considered. The product to be applied on the eye surface should have these parameters close

to physiological values. This introduces another difficulty as the buffers and osmotic agents may also hide the surface charge of the cationic nanodroplets and potentially destabilize the emulsion. Normal tears have a pH between 6.9 and 7.5 [48]. The literature indicates that the ocular instillation of 20 μL of a buffered solution at pH 5.5, 0.067 M is quickly brought to pH 6–6.5 in the tears [49]. Furthermore, it is usually known that a low pH is well tolerated if it is rapidly brought back to normal tear pH [50], therefore it can be assumed that buffering is not so important. In the case of Novasorb, the emulsion can be slightly buffered with a tris buffer (Cationorm) or not buffered at all, leaving the natural pH of the mixture. In that case, the tears rapidly restore the physiological pH of the lacrimal film.

Neutral osmotic agents, such as polyols (glycerol, mannitol, or sorbitol) were used. The lipid emulsions more or less physically resemble a simple aqueous-based eye drop dosage forms since more than 90% of the external phase is aqueous irrespective of the formulation composition. The main difference is its visual aspect: a milky white appearance. The final specifications are summarized in Table 4. It should be noted that even though BAK or CKC is present in the product as the cationic agent, the formulations are not preserved [51]. Thus, emulsions are packaged in single use vials filled by the Blow-Fill-Seal technology. Finally, the vehicle typically has a formula as presented in Table 5. Active ingredient is added in the oily phase but some hydrophilic molecules could be added in the aqueous phase to create a combination product.

The size of the oil nanodroplets is of utmost importance as it contributes to the stability of the emulsion and to the ocular absorption. To our knowledge, it has not yet been demonstrated that ocular absorption is correlated to the size of the nanovectors even if it is logical that the smaller the object, the higher the expected uptake. As discussed by Rabinovich-Guilatt et al. [21], there are several mechanisms of absorption of nanoparticles in the cornea. In the case of cationic nanoemulsions, positively charged nanodroplets of oil are not likely to penetrate the cornea as the drops are bound to the negatively charged mucus. Therefore, the delivery of the active ingredient is probably related to a passive diffusion linked to the enhanced retention time.

An additional factor favoring drug absorption is linked to the small size of the nanodroplets, that is, the interfacial area available for drug exchange. If the mean diameter of an oil droplet is 150 nm, and the volume of emulsion administered on the ocular surface is about 30 μL, the number of oil nanodroplets administered is close to 10^{10}. Consequently, with such an extraordinarily elevated specific surface of exchange (almost 1,000 mm^2) the diffusion of the active ingredients to the targeted tissues is greatly improved. Thus, a small droplet size of the nanoemulsion should consequently be associated with an improved clinical efficacy of the drug.

The manufacturing process is a three-step process as described in Figure 2. The first step is a phase mixing under magnetic stirring at 100 rpm for a few minutes followed by a high shear mixing at 16,000 rpm during 10 min at that stage the oil droplets of the emulsion have a size of approximately 1 μm. To reach a submicronic size (150–200 nm) the emulsion is submitted to a high pressure homogenization at 1,000 bars under cooling.

Table 4: Final specifications of the cationic nanoemulsions.

Specifications	Values
Aspect	Milky white to translucid
pH	5.5–7
Osmolality	180 to 300 mOsm/kg
Zeta potential	+20 to +40 mV
Mean oil droplet size	150 to 300 nm
Sterility	Sterile
Viscosity	1.1 m^2/s
Surface tension	Similar to tears: 41 mN/m

Stable cationic nanoemulsions were selected over hundreds of prototypes after being submitted to screening stress tests (freeze/thaw cycles, centrifugation, and heat test at 80°C). In addition, a deep physicochemical characterization including measurement of pH, osmolality, zeta potential, droplets size, interfacial and surface tension, aspect, and viscosity was systemically performed on prototypes. All these tests are able to discriminate a potential destabilization of the emulsions like creaming, coalescence Ostwald ripening, and phase separation and to set final specifications of the drug product as described in Table 4.

Finally, the product should be sterile. Since the sterilization process can have a major impact on the physical integrity of the emulsion, it should be taken into account at an early stage during the development of the formulation. A sterilizing filtration is not possible for emulsions as it uses a filter with 0.22 μm size pores that can clog during filtration. Aseptic processes are too expensive. The remaining option was heat sterilization; however, this can be performed only on very stable emulsions, and hence the need of a careful choice of the above-mentioned excipients.

3.3. Drug Loading. The Novasorb technology platform was ultimately designed to be loaded with active molecules. Emulsions are clearly adequate for lipophilic drugs with a log P of 2-3 (P: octanol/buffer pH 7.4 partition coefficient) preferentially nonionizable, and such candidates are numerous. Even so, the cationic emulsion with no active ingredient itself possesses beneficial properties. Its composition comprising oil, water, surfactants, and glycerol reduces evaporation of tears from the ocular surface while lubricating and moisturizing the eye. Altogether the components confer a protective effect by augmenting each layer of the tear film. Based on the inherent properties of the Novasorb technology, restoring the deficient layers of the natural tear film, Cationorm, a preservative-free cationic emulsion containing no active ingredient, has been commercialized globally for the relief of dry eye symptoms (Table 6).

Nearly 40% of new chemical entities have a low aqueous solubility, therefore potential candidates to be loaded into Novasorb [52]. Novagali Pharma incorporated about 40 lipophilic active ingredients of various therapeutic classes

Table 5: Composition of a typical vehicle from Novasorb technology.

	Excipients	Function	Concentration % w/w
Oily phase	Medium chain triglyceride	Internal phase	1 to 2
	Cetalkonium chloride	Cationic agent	0.005
	Tylopaxol	Surfactant	0.2
Aqueous phase	Poloxamer 188	Surfactant	0.01
	Glycerol	Osmotic agent	1.5 to 2.5
	NaOH	pH adjuster	Ad pH 6-7
	Water for injections	External phase	Ad 100

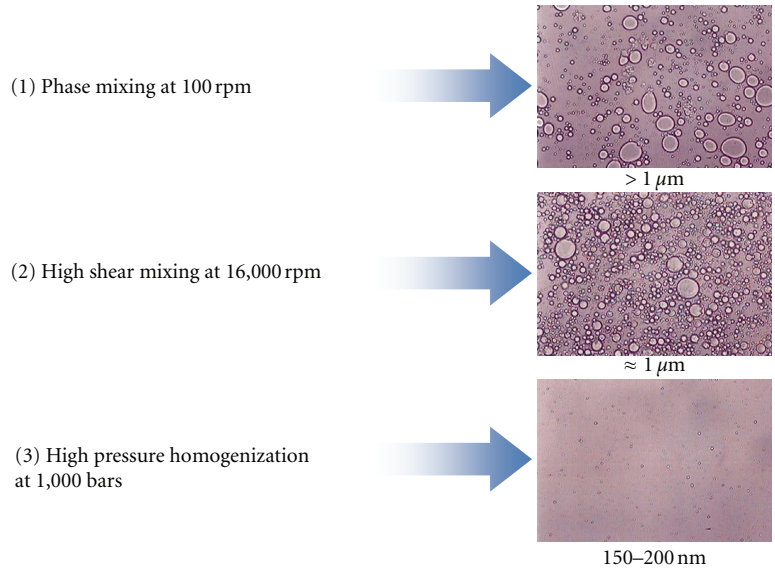


Figure 2: Three manufacturing steps of the process necessary to decrease the oil droplet size of the emulsion. Optical microscopy pictures of the emulsions are presented.

(NSAID, SAID, antibiotics, antifungals, etc.) proving the versatility of this emulsion. Herein, we will only focus on the most advanced products. Despite topical administration in solvents yielding poor bioavailability, CsA, a very lipophilic immunomodulatory drug, is widely used by ophthalmologists due to its recognized therapeutic potential for the treatment of ocular diseases (dry eye, allergy, and inflammation) [53]. CsA was considered an excellent initial candidate to evaluate the potential of the Novasorb cationic emulsion to improve the efficacy of an established drug. Therefore, the primary challenge in the development of a cationic emulsion containing CsA was to design the optimal formulation [53] for topical delivery. Today, Novagali Pharma has developed two products based on the Novasorb technology loaded with CsA: Cyclokat for the treatment of dry eye and Vekacia for the treatment of vernal keratoconjunctivitis.

Latanoprost, a lipophilic prostaglandin analogue, is a potent intraocular pressure lowering agent currently marketed as Xalatan (Pfizer,) for the treatment of glaucoma and ocular hypertension. In Xalatan, the active ingredient, latanoprost 0.005%, is solubilized in water by 0.02% of BAK. Despite being the leading antiglaucoma medication, there are two drawbacks of Xalatan that may have impacted its huge commercial success: (1) the formulation was not stable at room temperature necessitating storage at 5°C and (2) BAK in the formulation as a preservative and solubilizing agent causes ocular surface toxicity which probably resulted in decreased compliance. As the patent protecting this molecule is expiring in 2011, there was an opportunity to improve upon the disadvantages of Xalatan. Hence, Novagali launched the development of Catioprost, a preservative-free cationic emulsion loaded with latanoprost for the treatment of elevated intraocular pressure (IOP) while protecting and improving the ocular surface.

4. Nonclinical Development

The nonclinical development is divided into the safety evaluation and the pharmacokinetic studies.

4.1. Safety. Establishing the safety of the new nanotechnology was an important goal of the nonclinical development program. Toxicity is a major concern in nanotechnology as the behavior of the nano-object is difficult to predict [4].

Table 6: Main product based on Novasorb technology marketed or to be marketed.

Product	Active ingredient	Indication	Status
Cationorm	Medical device	Dry eye	Marketed
Cyclokat	0.1% cyclosporine A	Severe dry eye	Phase III
Vekacia	0.1% cyclosporine A	Vernal keratoconjunctivitis	Phase III
Catioprost	0.005% latanoprost	Glaucoma associated with ocular surface disease	Phase II

Therefore, numerous studies were conducted to ensure the ocular safety of the cationic emulsion.

As the active ingredients used in Novagali's emulsions (CsA and latanoprost) are already used in other drug products only the toxicity of the vehicle and the final product was evaluated.

Before the development of Novasorb, preliminary data regarding the ocular safety of some cationic emulsions on the eye were already available [54]. A subchronic toxicity study performed in rabbits demonstrated that a cationic emulsion containing 3 mg/mL stearylamine was found to be safe and well tolerated after repeated topical ocular administrations [54]. In addition, a local tolerance study in rabbit eyes demonstrated that a 1 mg/mL oleylamine ophthalmic emulsion instilled eight times per day for 28 days was relatively well tolerated [21]. These data, even though promising, were not sufficient to support further development as Novasorb utilizes cationic agents (CKC and BAK) that are usually used at higher concentrations as preservatives. The safety profile of Novasorb cationic emulsions using BAK as a cationic agent was thus evaluated in both *in vitro* and *in vivo* models as listed in Table 7.

Table 7: Listing of safety screening and regulatory toxicity studies performed in order to test Novasorb technology in humans.

Nonclinical studies type	Safety studies for Novasorb alone and loaded Novasorb
Safety screening	(i) Draize test
	(ii) Demonstration in a repeated acute rabbit toxicity model that BAK and CKC containing emulsion are well tolerated
	(iii) Ocular safety evaluation of newly developed *in vitro* corneal wound healing model and in an acute *in vivo* rabbit model
	(iv) *In vivo* toxicity evaluation of latanoprost cationic emulsion in the rabbit
Regulatory toxicity studies	(i) *In vitro* evaluation of the cytotoxic potential by indirect contact
	(ii) Delayed-type hypersensitivity evaluation in the Guinea pig
	(iii) Ocular irritation test in the rabbit (short term: 72 h) following a single application
	(iv) Determination of the physical compatibility of Novasorb with contact lenses
	(v) 28-day ocular tolerance in the rabbit
	(vi) Evaluation of the potential to induce delayed contact hypersensitivity (local lymph node assay)
	(vii) Evaluation of the corneal sensitivity following repeated applications in albino rabbits
	(viii) Phototoxicity and photoallergic potential evaluation following topical applications in the Guinea pig
	(ix) 6-month ocular toxicity in the dog and rabbit

4.1.1. Safety of Novasorb as Vehicle. During the formulation work, emulsion prototypes were quickly evaluated by the Draize test which, despite a few limitations, allowed the identification of the least irritating nanoemulsion. This test consists of instilling 30 to 50 μL of the product into one eye of 6 New Zealand white rabbits and monitoring to observe any abnormal clinical signs such as redness of conjunctiva, swelling, or increased blinking which may indicate irritation. The test does not give objective values as it is operator dependent but gives a good idea of how the product will be tolerated.

Other *in vitro* and *in vivo* tools were used. In an *in vitro* scrapping assay using human corneal epithelial (HCE) cell monolayers, a cationic emulsion containing 0.02% BAK as a cationic agent was as well tolerated as a phosphate buffered saline (PBS) solution while an aqueous solution of 0.02% BAK revealed toxicity.

An acute toxicity rabbit model was used which allows for the characterization of the mechanism underlying the toxicity observed during the conventional Draize tests [55]. In the experiment, 15 instillations of test eye drops are administered at 5 min intervals, with observations performed over 96 hours. Clinical signs, *in vivo* confocal microscopy, and conjunctival impression cytology were performed to assess the safety profile of the different cationic emulsions with BAK or CKC as the cationic agent. This study demonstrated that cationic emulsions using BAK or CKC as the cationic agent were very well tolerated while the tested 0.02% BAK solution was responsible for corneal epithelial cell death related to the proinflammatory and proapoptotic activity of BAK.

4.1.2. Safety of Novasorb Loaded with Active Ingredients. The safety profile of the Novasorb used as a vehicle for lipophilic drugs such as cyclosporine (Vekacia/Cyclokat) and

latanoprost (Catioprost) was evaluated in animal models [56]. These studies demonstrated that neither of the two active ingredients (CsA or latanoprost) has an impact on the safety profile of the cationic emulsions as both drug-loaded cationic emulsions were as well tolerated as the cationic emulsion vehicle (Figure 3). For example, in the acute toxicity rabbit model, repeated instillations of Cyclokat/Vekacia (CsA-containing 0.05 and 0.1% CsA cationic emulsions) were as well tolerated as Restasis (0.05% CsA anionic emulsion), and Catioprost (preservative-free latanoprost 0.005% cationic emulsion) was better tolerated than the 0.02% BAK-preserved Xalatan. Local tolerance studies in the rabbit confirmed that chronic instillations (4–6 times daily over 28 days) with Cyclokat/Vekacia and twice daily for Catioprost were well tolerated by the rabbit eyes.

All the previous *in vivo* data were obtained in rabbits with a healthy ocular surface. However, it was of interest to also assess the effect of Catioprost on damaged corneas to more closely mimic the clinical situation experienced when elderly patients are started on glaucoma therapy. For that purpose, a rat model of debrided cornea was used to assess the effect of Catioprost, its emulsion vehicle, and Xalatan (the commercially available product of latanoprost) on the ocular surface healing process. The *in vivo* data demonstrated that Xalatan delayed corneal healing, while both Catioprost and its cationic emulsion vehicle (without latanoprost) promoted healing of the ocular surface and restored the function of the injured epithelium, thus confirming the better safety profile of the Novasorb cationic emulsions and confirming that Novasorb could hasten the repair of ocular surface damage. Novasorb was hence shown to be safe, but prior to human testing several other studies were necessary to fulfill the various European and American guidelines. These studies cited in Table 7 included *in vitro* evaluation of the cytotoxic potential by indirect contact, a delayed-type hypersensitivity evaluation in the guinea pig, an ocular irritation test in the rabbit (short term: 72 h) following a single application, a determination of the physical compatibility of Novasorb with contact lenses, a 28-day ocular tolerance in the rabbit, an evaluation of the potential to induce delayed contact hypersensitivity (local lymph node assay), an evaluation of the corneal sensitivity following repeated applications in albino rabbits, an evaluation of potential phototoxicity and photoallergy following topical applications in the guinea pig and finally a 6-month ocular toxicity in the dog and rabbit. The description of these entire assays can be found in the various regulatory guidelines.

4.2. Proof-of-Concept Studies and Pharmacokinetics. In parallel to ensuring the safety, proof-of-concept studies were performed in order to validate the cationic nanoemulsion technology in the ocular delivery of active molecules.

To assess the effect of the cationic charge on the ocular surface, Novagali Pharma has performed static and dynamic contact angle and surface tension studies on harvested rabbit eyes according to a method adapted from Tiffany [57]. This experiment showed that Novasorb cationic emulsions have a better spreading coefficient on the cornea and conjunctiva

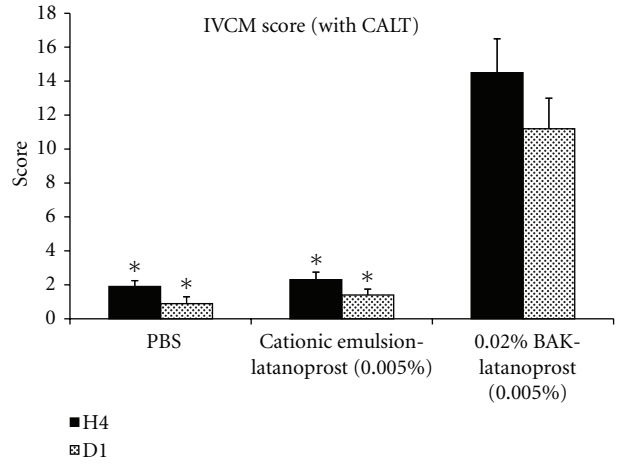


Figure 3: *In vivo* confocal microscopy score of rabbit ocular surface following repeated instillations with Novasorb cationic emulsion of latanoprost. IVCM images of rabbit ocular surface and conjunctiva associated lymphoid tissue (CALT) were used to assess the safety of the cationic emulsion of latanoprost by scoring the alterations observed following repeated instillations. Note that the lower the score the better the tolerance. PBS was used as a negative control. (*) $P < 0.0001$ compared with 0.02% BAK-latanoprost (0.005%). Adapted from Liang et al. [56].

than conventional eye drops and anionic emulsions. This improved spreading coefficient leads to better ocular surface wettability. Optimal spreading of the cationic emulsion confers protective filmogenic properties and reduces tear washout. Figure 4 illustrates the behaviour of the cationic emulsion which spread over the eye very rapidly compared to other formulations. It has been well described that oil-in-water emulsions enhance drug absorption by facilitating corneal or conjunctival absorption or prolonging the contact with the eye, thus improving drug delivery [58].

Early pharmacokinetic studies were performed to evaluate CsA absorption following the application of experimental 0.2% CsA cationic and anionic emulsions [19]. The data demonstrated that the cationic emulsion was almost two-times better at delivering CsA to ocular tissues than an anionic emulsion, even though the latter contained 0.01% BAK and 0.2% deoxycholic acid as a mild detergent that can disrupt cell membranes and serve as a permeation enhancer.

Restasis (Allergan) is an anionic emulsion of CsA (0.05%) that has been shown to readily penetrate ocular tissues without significant systemic passage [59, 60]. Pharmacokinetic (PK) studies designed to evaluate the ocular and systemic CsA distribution following single and multiple dosing with cationic emulsions NOVA22007 (cationic emulsion at 0.05%) or Cyclokat (cationic emulsion at 0.1%), compared to Restasis as a reference, confirmed the beneficial role of the cationic charge in enhancing the ocular penetration of CsA [61] in Novasorb cationic emulsions.

Single-dose PK data demonstrated that the 0.05% CsA cationic emulsion was more effective than Restasis at delivering CsA to the cornea (Cmax: 1372 versus 748 ng/g; AUC: 26477 versus 14210 ng/g.h, resp.). Furthermore, multiple-dose PK confirmed that there was no systemic absorption,

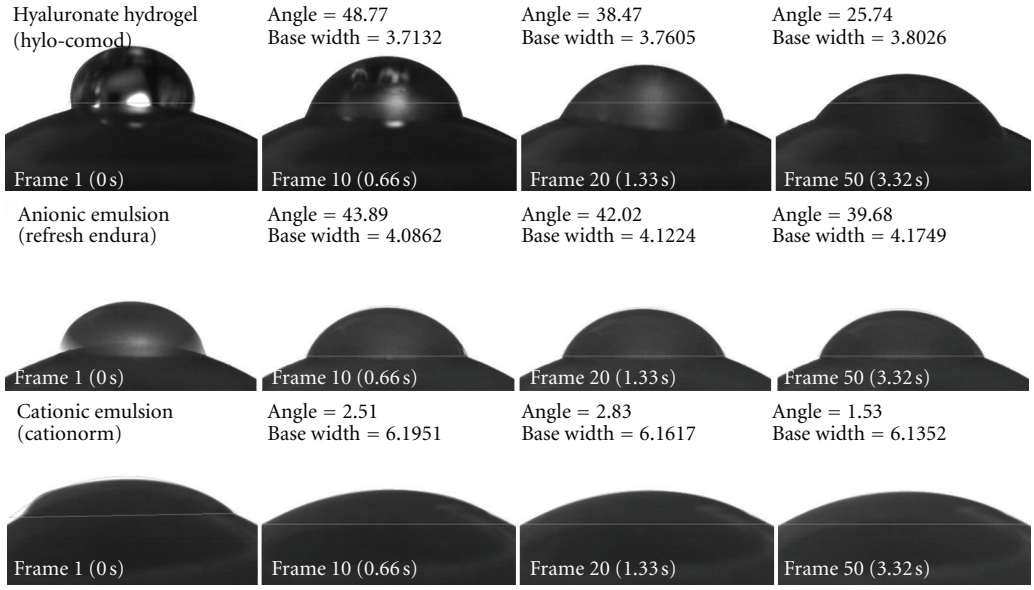


Figure 4: Dynamic contact angle measurement and base width of an eye drop instilled on rabbit eyes. Photos taken at 0, 0.66, 1.33, 3.32 seconds after instillation of hyaluronate hydrogel (Hylo-COMOD), anionic emulsion (Refresh Endura), and cationic emulsion (Cationorm). Contact angle and base width values confirm the optimal and fasted spreading of cationic emulsions compared to anionic emulsions and hyaluronic acid based product.

with values below the limit of detection (LOD, 0.1 ng/mL) for the CsA-cationic emulsion (see Figure 5). The use of 3H-CsA also demonstrated that the systemic distribution following repeated instillations was indeed low and comparable for both the CsA-cationic emulsion and Restasis and confirmed that the improved local absorption with the CsA-containing cationic emulsion did not translate into increased systemic CsA levels.

In addition, the electroattractive interactions between the positively charged oil droplets of the cationic emulsion and the negatively charged ocular surface cell epithelia might also explain the 50% lower contact angle observed with cationic emulsions versus anionic (negatively charged) emulsions, and the higher spreading coefficient [18]. A low contact angle, better spreading coefficient, and an increased residence time of the cationic emulsions may all contribute to the better drug absorption of lipophilic drugs solubilized in cationic emulsions.

The cationic emulsions designed for the treatment of dry eye disease (Cyclokat) and vernal keratoconjunctivitis (Vekacia) were not tested in pharmacodynamic models as there are no reliable experimental models for these pathologies. However, pharmacokinetic studies with CsA cationic emulsions in animal models demonstrated (see previous paragraph) that the tissue concentrations of CsA were above the therapeutic concentration (50–300 ng/g of tissue according to Kaswan [62]) in both the cornea and conjunctiva. Therefore, the safety and efficacy of these CsA-containing cationic emulsions were first demonstrated in phase II and III clinical trials (see the following section).

In contrast, the safety and efficacy of Catioprost (preservative-free latanoprost 0.005% cationic emulsion) was initially evaluated in an established cynomolgus monkey model of ocular hypertension [63], and compared to Xalatan. Both latanoprost formulations shared the same efficacy profile, and the intraocular pressure (IOP) reduction lasted 24 h. Additionally, a comparison of the local tolerance of Catioprost and Xalatan following twice-daily repeated instillations in rabbits over a 28-day period revealed, although both products were well tolerated, there was a 42% lower incidence of conjunctival redness in rabbits treated with Catioprost. Overall, the results of the preclinical models suggested that Catioprost appears to be as potent as Xalatan for the reduction of IOP with an improved safety profile.

As listed in Table 8, some pharmacokinetic studies are compulsory prior to human testing. They include the single- and multiple-dose pharmacokinetic studies, the determination of systemic exposure, plus the toxicokinetic studies following repeated instillations. The full nonclinical package gave a high confidence that Novasorb technology alone or loaded with active ingredients was fully safe and could provide high concentration of active ingredient in ocular tissues. The next step of the development was then the clinical evaluation in human.

5. Clinical Development

An IND-enabling dossier was prepared allowing for conduct of a first-in-man clinical trial. This dossier was prepared according to guidance received through regulatory interactions with health agencies (FDA, EMA). Indeed, early exchanges with health agencies about technologies are possible to discuss technology specific requirements (efficacy, safety) and anticipated clinical and regulatory development programs.

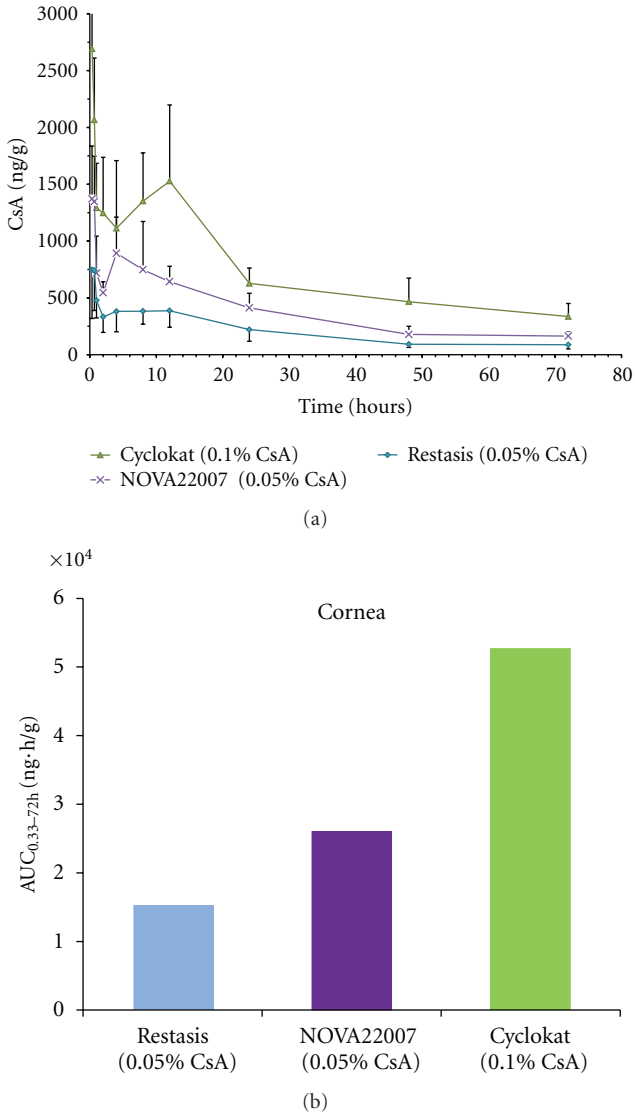


Figure 5: (a) Changes in corneal CsA concentration with time after a single unilateral topical administration in pigmented rabbits. The error bars represent standard errors. (b) Cornea absorption (AUC) following a single instillation in pigmented rabbits.

Table 9 describes the different clinical trials carried out to the evaluate Novasorb technology with or without an active ingredient. The clinical development was first performed with a drug-free cationic emulsion formulation (vehicle). The first clinical trial was carried out with the first generation of the cationic emulsion in 16 healthy volunteers. The safety and tolerance of four-times daily instillations was evaluated over 7 days of treatment. The product was shown to be safe and well tolerated. Since the vehicle harbors intrinsic properties of ocular surface protection, it was then tested in two phase II clinical trials aiming at evaluating the efficacy, tolerance, and safety of Cationorm in patients with mild to moderate dry eye (results are detailed in the next section).

A cationic emulsion containing CsA was subsequently evaluated in patients with either dry eye disease (DED) or vernal keratoconjunctivitis (VKC). Highlights of some

Table 8: Listing of proof-of-concept and regulatory pharmacokinetics studies performed in order to test Novasorb technology in humans.

Nonclinical studies type	Studies for Novasorb alone and Novasorb loaded
Proof-of-concept	(i) *Ex vivo* measurement of contact angle and surface tension of cationic emulsions on rabbit eyes
	(ii) Evaluation and comparison of the wound healing potential of the cationic emulsion versus artificial tears in a rabbit model of corneal abrasion
	(iii) Evaluation of the efficacy of a 0.1% cyclosporine A cationic emulsion in the management of keratoconjunctivitis sicca in the dog
	(iv) Evaluation of the efficacy of a cationic emulsion of 0.005% latanoprost at reducing elevated intraocular pressure in glaucomatous monkeys
	(v) *In vitro* and *in vivo* evaluation of a preservative-free cationic emulsion of latanoprost in corneal wound healing models
Regulatory pharmacokinetics studies	(i) Single and multiple doses pharmacokinetic
	(ii) Systemic exposure determination and toxicokinetics following repeated instillations of BAK and CKC-containing cyclosporine A cationic emulsion

clinical results are detailed below in light of challenges faced including efficacy of the "placebo" comparator which was the cationic emulsion vehicle, variability of endpoints, and disconnection between sign and symptoms of ocular surface diseases.

Finally, a phase II program was initiated with Catioprost, the cationic emulsion containing latanoprost. Since the phase II trial is ongoing, no data are available.

5.1. Clinical Evaluation of Cationorm. In the 2007 Dry Eye Workshop (DEWS) report, dry eye disease (DED) is defined as a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. Currently, symptomatic treatment with artificial lubricants is the first line of treatment for patients with DED; however, the disadvantage of most conventional artificial tear solutions is that most of the instilled drug is lost within the first 15–30 seconds after installation, due to reflux tearing and the drainage via the nasolacrimal duct. The prolonged residence time of the cationic emulsion on the ocular surface due to the electrostatic attraction between the positively charged lipid nanodroplets and the negatively charged ocular surface and the augmentation of the tear film layers by the oily and aqueous phase of the emulsion suggested that the Novasorb technology could be inherently beneficial for the ocular surface even in the absence of an active ingredient.

Table 9: Clinical trials performed with Novasorb.

Year	Phase type	Product	Objectives	Indication	No. of patients
2003	Phase I	Vehicle no.1	Tolerance and safety	None	16
2004	Phase II		Tolerance and safety, Exploratory efficacy	Dry eye	50
2005	Phase II	Cationorm (Vehicle no.2)	Efficacy, tolerance, and safety	Dry eye	79
2010	Phase II		Efficacy, tolerance, and safety	Dry eye	71
2005	Phase IIa	Cyclokat	Tolerance and safety Exploratory efficacy	Dry eye disease	48
2008	Phase IIb		Exploratory efficacy, tolerance, and safety	Dry eye disease	132
2009	Phase III "Siccanove"		Efficacy, tolerance, and safety	Dry eye disease	496
2011	Phase III "Sansika"		Efficacy, tolerance, and safety	Dry eye disease	252
2006	Phase IIb/III	Vekacia	Efficacy, tolerance, and safety	Active VKC	118
2009	Phase IIb		Efficacy, tolerance, and safety	Nonactive VKC	34
2011	Phase II	Catioprost	Exploratory efficacy, open-label study	Glaucoma	NA
2011	Phase IIb		Exploratory efficacy, tolerance, and safety	Glaucoma	100

VKC: Vernal keratoconjunctivitis.

Consequently, the ocular tolerance and efficacy of Cationorm, a preservative-free cationic emulsion, were evaluated and compared to Refresh Tears (Allergan) in a one-month, phase II, multicenter, open-label, randomized, parallel-group study enrolling patients with signs and symptoms of mild to moderate DED. Adults with a history of bilateral DED were subjected to a washout period of prior DED treatments during which only artificial tears were allowed. At the inclusion visit patients were randomized to treatment with either Cationorm ($n = 44$) or Refresh Tears ($n = 35$) in both eyes 4 times daily and evaluated at follow-up visits on Day 7 and Day 28. Ocular tolerance and efficacy were assessed at one month. Seventy-nine patients, 86% female with a mean age of 61.6 years, were enrolled in the study. At 1 week and 1 month the mean reduction in individual dry eye symptoms scores and total dry eye symptoms scores were greater in the Cationorm than Refresh Tears treated patients (36% versus 21% at Day 7, and 49% versus 30% at Day 28, resp.) demonstrating that DED symptoms improved better with Cationorm. While the global local tolerance was perceived similarly with both treatments, the study investigators rated the overall efficacy of Cationorm statistically significantly better than Refresh Tears ($P < 0.001$). Additionally, Cationorm-treated patients experienced greater improvements from baseline compared to Refresh Tears-treated patients for the Schirmer test (1.88 versus 1.27 mm) and corneal fluorescein staining (-0.61 versus -0.59) with statistically significant improvements in the tear film break-up time (2.00 versus 1.16, $P = 0.015$) and lissamine green staining (-1.42 versus -0.91, $P = 0.046$). The overall results showed that Cationorm was as safe as, but more effective than, Refresh Tears in patient with mild to moderate DED symptoms.

In a subsequent 3-month, controlled, randomized, single-masked study conducted in Italy, the efficacy of Cationorm was evaluated in adults with moderate dry eye [64]. Seventy-one patients were randomized to treatment with Cationorm, Optive (Allergan), or Emustil (SIFI) 4 times daily, and efficacy assessments were conducted at 1 and 3 months. At 1 month patients treated with Optive and Cationorm experienced a statistically significant improvement from baseline in their dry eye symptoms which was also evident for each of the 3 treatment groups at 3 months. At 3 months, improvements from baseline in the tear break-up time and fluorescein staining were statistically significant for Cationorm and Optive but not for Emustil, and while both Cationorm and Optive significantly reduced tear film osmolarity, only Cationorm showed a statistically significant change compared to Emustil. In this study Cationorm was clearly more effective than Emustil in patients with moderate DED and although not statistically better, the overall improvement in DED symptoms and signs were greater in patients treated with Cationorm than Optive.

The results of the preclinical studies (corneal healing in alkali burn and de-epithelization rabbit models) and clinical trials evaluating Cationorm in patient with DED support its safety and efficacy for the treatment of dry eye symptoms and showed the benefit of the Novasorb cationic emulsion on the ocular surface independent of an active ingredient. However, as we will see, the inherent efficacy of the preservative-free

cationic emulsion on improving symptoms of ocular surface disease presented an unanticipated challenge when used as a vehicle in the evaluation of the efficacy of the preservative-free cationic emulsion loaded with CsA in patients with DED.

5.2. Clinical Evaluation of Cyclokat. In the DEWS definition of DED it is stated that DED is accompanied by an increased osmolarity of the tear film and inflammation of the ocular surface. As such DED can be considered a chronic, bilateral inflammatory condition for which appropriate treatment, particularly for patients unresponsive to symptomatic treatment with artificial tears would include an anti-inflammatory agent. While Restasis, an anionic emulsion of 0.05% CsA, is available for the treatment of DED in the US, despite the widespread use of hospital compounded CsA and even corticosteroids in the EU there has been no approved pharmaceutical drug indicated for patients with DED. Based on the preclinical data showing the potential advantages of a cationic emulsion over anionic emulsions and unmet medical need for an approved topical CsA formulation in the EU, Novagali undertook the development of Cyclokat for the treatment of dry eye disease.

The initial clinical trial of Cyclokat was a phase II, 3-month, randomized, double-masked, placebo-controlled, dose-ranging study enrolling 53 Gougerot-Sjögren patients with moderate to severe DED. The primary objective of the study was to assess ocular tolerance and systemic safety of the cationic emulsion containing CsA at concentrations of 0.025%, 0.05%, and 0.1% compared to the cationic emulsion vehicle containing no active ingredient. An exploratory evaluation of efficacy was a secondary objective. At baseline, 62% of the enrolled patients had a Schirmer test score of $\leq$1 mm at 5 minutes and 49% had a corneal fluorescein staining score of $\geq$3. Over the 3-month treatment period there were no safety concerns and no evidence of systemic absorption of CsA following topical administration of either Cyclokat dose. Patients treated with the 0.1% Cyclokat formulation showed greatest improvements in corneal and conjunctival staining at 3 months and a dose response effect was observed for the reduction of conjunctival HLA-DR staining (a biomarker for ocular surface inflammation) at month 3 compared to baseline (vehicle: −10%; 0.025% CsA: −8%; 0.05% CsA −23%, and 0.01% CsA: −50%).

A second phase II, 3-month, double-masked placebo controlled study comparing Cyclokat 0.05% and 0.1% versus its cationic emulsion vehicle was conducted in 132 patients with mild to moderate DED utilizing the controlled adverse environment chamber. In this study the efficacy and safety of Cyclokat was assessed by the evaluation of coprimary efficacy endpoints (corneal fluorescein staining as the sign and ocular discomfort as the symptom) at month 3 after and during exposure to controlled adverse environment chamber, respectively. Although superiority was not achieved for the coprimary endpoints, there was an overall favorable safety profile and efficacy was demonstrated for the improvement of several secondary endpoints addressing DED signs and symptoms with the results favoring the use of the 0.1% dose for subsequent clinical development.

The Siccanove study was a 6-month phase III, multicenter, randomized, controlled, double-masked trial of Cyclokat 0.1% administered once daily versus its emulsion vehicle in 492 patients with moderate to severe DED. The primary study objective was to demonstrate superiority of Cyclokat on both a DED sign (mean changes in CFS using the modified Oxford scale) and DED symptoms (mean change in global score of ocular discomfort using a VAS). Following a washout period during which only artificial tears were allowed, patients were randomized at baseline to treatment with either Cyclokat ($n = 242$) or its cationic emulsion vehicle ($n = 250$) and evaluated at study visits at months 1, 3, and 6. As early as month 1 ($P = 0.002$), patients treated with Cyclokat showed a statistically significant improvement in the mean change in CFS grade compared to the cationic emulsion vehicle from baseline which continued to improve from month 3 ($P = 0.030$) to month 6, the DED sign coprimary efficacy endpoint. The statistically significant improvements in CFS over 6 months ($P = 0.009$) were complemented by a statistically significant improvement in lissamine green staining ($P = 0.048$) and a reduction in HLA-DR expression ($P = 0.022$) [65]. Additional, post hoc analysis of the Siccanove study data showed that the benefit of treatment with Cyclokat was greatest in patients with the most severe keratitis (as defined by CFS) at baseline (delta in the mean change in CFS from baseline in CFS grade 2–4 = 0.22, $P = 0.009$; 3-4 = 0.32, $P = 0.005$; grade 4 = 0.77, $P = 0.001$) [66]. Although there was a clinically relevant improvement in DED symptoms from baseline the Cyclokat and cationic emulsion vehicle treatment arms, no statistically significant differences were observed at month 6 for the mean change in the global score of ocular discomfort, the DED symptom coprimary efficacy endpoint. However, there was a statistically significant improvement in symptoms for patients achieving a $\geq$25% improvement in the VAS score (50.21% versus 41.94%, $P = 0.048$). The difficulty in demonstrating the benefit of Cyclokat over its cationic emulsion vehicle was in part attributed to the efficacy of the vehicle itself in improving the symptoms of DED as demonstrated in clinical trials for Cationorm. Additionally, the symptoms coprimary endpoint result can be related to poor correlation between dry eye disease signs and symptoms. At baseline in the Siccanove study, while the mean VAS scores increased with the severity of the CFS, the correlation between the VAS score, as an expression of DED symptoms, and the CFS grade, as an expression of a DED sign, at baseline was low (Spearman's correlation coefficient = 0.23) due to the wide variability in the severity of patient reported symptoms. Similarly at month 6 the statistical correlation between mean change in CFS grade and VAS score was low (Spearman's correlation coefficient = 0.094) with only approximately 68% of patients showing concordance in the direction of change in CFS grade and DED symptoms [65]. Although a poor concordance between dry eye disease signs and symptoms has been recognized in the literature, improvement in both signs and symptoms is an expected outcome in randomized clinical trials investigating new DED treatments. Hence several drugs having shown promise for improving DED have failed due to the inability to demonstrate a statistically

Figure 6: Cationorm is the first product marketed based on the cationic emulsion technology.

significant improvement in signs and symptoms of dry eye disease using coprimary efficacy endpoints.

Fortunately, sign and symptom composite responder endpoints, used in registration trial supporting the approval of new treatments for other chronic inflammatory diseases, provide an alternate method to satisfy the requirement of regulatory authorities. The methodological approach of composite responder analysis avoids issues related to high variability when following mean change of signs and symptoms as discontinuous variables. By focusing only on within-patient's improvements, the composite responder approach could resolve the concern related to the poor correlation between signs and symptoms in evaluating the efficacy of new treatment for DED. As such a pivotal phase III trial, the Sansika study, utilizing a composite responder analysis at month 6, has been initiated to evaluate the efficacy of Cyclokat in patients with severe dry eye disease.

6. Conclusion

Novasorb technology is a typical example of a breakthrough formulation technology primarily developed by an academic team and successfully translated to the patient. Eight years were necessary for the first product to reach the market. With three products in the late stages of clinical development and one product on the market, Novasorb has now proven the concept that cationic nanoemulsions can effectively treat ophthalmic diseases with no toxicity (tested successfully in over 1,000 patients) and several other advantages (Table 10). Cationorm (Figure 6) was launched on the French market April 2008 and at the time this article is written more than 550,000 units of treatment were sold in about 10 countries without any pharmacovigilance concerns. Cyclokat, Vekacia, and Catioprost could reach the market within a few years following the successful completion of pivotal registration studies. The reasons for the success of the Novasorb technology are multiple. Since the beginning of the formulation work, the company prioritized the search for only compoundial and ophthalmology accepted excipients, a manufacturing process which is scalable, and finally the animal models and experimental protocols were designed to carefully screen and select the formulation with the highest probability of demonstrate clinical safety and efficacy.

Table 10: Key drivers of cationic emulsion technology Novasorb.

(i) Solubilization of large doses of lipophilic drugs and/or large molecules
(ii) Better penetration through membranes resulting in enhanced bioavailability
(iii) Potential for drug controlled release
(iv) Stable and can be sterilized
(v) Addition of effective novel routes of administration to existing marketed drugs
(vi) Expanding markets and indications
(vii) Extending product life cycles
(viii) Generating new opportunities
(ix) Inexpensive to manufacture

The Novasorb success story also proves that authorities, particularly European authorities, are relatively open to new delivery approaches and new technologies as long as efficacy and safety can be conclusively demonstrated according to well-constructed protocols and studies. Novagali Pharma is now pursuing the next generation of cationic nanoemulsions, which will have enhanced pharmacokinetics properties and new original drug products to expand the reach of ophthalmic indications. Some other improvements such as development of new cationic agents will provide continued support for this promising and effective means of delivering active molecules.

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Research Article

Microfabricated Engineered Particle Systems for Respiratory Drug Delivery and Other Pharmaceutical Applications

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Particle Replication in Non-Wetting Templates (PRINT®) is a platform particle drug delivery technology that coopts the precision and nanoscale spatial resolution inherently afforded by lithographic techniques derived from the microelectronics industry to produce precisely engineered particles. We describe the utility of PRINT technology as a strategy for formulation and delivery of small molecule and biologic therapeutics, highlighting previous studies where particle size, shape, and chemistry have been used to enhance systemic particle distribution properties. In addition, we introduce the application of PRINT technology towards respiratory drug delivery, a particular interest due to the pharmaceutical need for increased control over dry powder characteristics to improve drug delivery and therapeutic indices. To this end, we have produced dry powder particles with micro- and nanoscale geometric features and composed of small molecule and protein therapeutics. Aerosols generated from these particles show attractive properties for efficient pulmonary delivery and differential respiratory deposition characteristics based on particle geometry. This work highlights the advantages of adopting proven microfabrication techniques in achieving unprecedented control over particle geometric design for drug delivery.

1. Introduction

Particulate drug delivery systems play an important role in the treatment of human disease. Particles such as liposomes, protein nanoparticles, and PLGA microparticles are currently used in marketed drug products using a variety of dosage forms [1, 2]. In particular, particle aerosol inhalation therapy is commonplace for the treatment of respiratory disease. Inhaled therapy using pressurized metered dose inhalers (pMDI), dry powder inhalers (DPI), and nebulizers is an attractive route for treatment of respiratory disease, allowing for local delivery of high concentrations of therapeutics in the lung and avoidance of systemic toxicities associated with oral or injectable therapies [3–6]. Despite the prevalence of aerosol therapy, direct drug delivery to the site of disease remains surprisingly inefficient in part due to the lack of control of particle properties, including particle size, in the drug formulation. Although a wide array of devices are available in the market [7], dose delivery efficiencies for dry powder asthma inhalers range from 3 to 15% for children and 10 to 30% for adults, indicating that less than one third of the contained drug actually reaches the lungs; the most advanced pMDIs deliver only 60% of the inhaled material to central and intermediate bronchial airways [4].

The preparation of respirable particles with reproducible and tunable aerodynamic properties remains a challenge [4, 5]. Conventional fabrication of these pharmaceutical aerosols for DPIs is accomplished by techniques such as micronization (milling) or spray drying [8]. These formulation techniques result in polydisperse aerosol populations, with

large particle size distributions and limited control over particle shape. Additional formulation challenges arise with forming dry, nonagglomerating powders comprised of pure active ingredients, especially biologicals like siRNA, proteins, and monoclonal antibodies (mAbs). Indeed, there are currently no marketed dry powder inhaled mAbs or siRNA therapies. The unmet need for improved aerosol drug delivery technologies is large; respiratory diseases including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and influenza are a significant cause of morbidity and mortality worldwide, with an estimated 10 million lung-disease-related deaths in 2004 globally and with health care costs in the US alone of a projected $173 billion in 2010 [9, 10].

In this work, we demonstrate the use of a top-down, roll-to-roll particle nanomolding technology, (PRINT, Particle Replication in Non-wetting Templates) to fabricate monodisperse, nonspherical particles with unprecedented control over size and shape [11–13] and highlight the benefits that this approach can have for drug delivery and particularly respiratory drug delivery. In addition to new results presented in this paper, we highlight other published studies that demonstrate the breadth and applicability of PRINT drug delivery technology for applications beyond respiratory delivery, including systemic delivery.

In previous efforts, PRINT nanoparticles and microparticles have been used to study the effects of particle size on cellular internalization and particle biodistribution *in vivo*. Gratton et al. studied the effects of particle size and shape on cellular internalization and intracellular trafficking and demonstrated significant dependence on particle size and shape in both the internalization rate and internalization pathways of HeLa cells [14]. Interestingly, the authors demonstrated that rod-like particles show a higher internalization rate than equivalent diameter cylindrical particles. Merkel et al. have examined the role that particle modulus plays in particle circulation *in vivo*, finding that low-modulus hydrogel microparticles have elimination half-lives of greater than 90 hours [15]. Increasing the stiffness of these particles by increasing hydrogel crosslink density can reduce the elimination half-life 30-fold and change the accumulation of these particles from the spleen to the lungs and liver. These two studies highlight the importance that flexible control of particle size, shape, and chemistry affords drug delivery vehicles. Additionally, the PRINT manufacturing process has been demonstrated at scales relevant to support preclinical and clinical studies. Liquidia Technologies has initiated a Phase I clinical study of a PRINT vaccine candidate, demonstrating the production of GMP pharmaceutical materials using this novel nanofabrication process, at a scale relevant to clinical development [16].

The outcome of implementing this particle engineering approach for dry powder fabrication is improved aerosol performance applicable to respiratory drug delivery, demonstrated by incorporation of a variety of pharmaceutically relevant compounds. *In vitro* results demonstrate that PRINT particle aerosols possess high respirable dose, high fine particle fraction, and tunable particle aerodynamic diameter. *In vivo* canine deposition studies demonstrate the ability to influence dry powder delivery as a function of particle geometry. These results suggest that this tunable particle engineering approach is a versatile platform for enabling next-generation respiratory drug delivery. We also highlight some of the utility of PRINT for the production of particles for small molecule, protein, and oligonucleotide drug delivery, which demonstrates that PRINT is a versatile formulation approach and should find applicability in oral, parenteral, and topical dosage forms for multiple disease indications.

2. Methods

2.1. Fabrication of Particles for Drug Delivery Using PRINT Technology. PRINT is an adaptation of micro- and nanomolding technologies, rooted in the microelectronics industry, that is used to fabricate monodisperse particles of controlled sizes and shapes using roll-to-roll manufacturing processes. It allows for the fabrication of monodisperse particles with precise control over size, shape, composition, and surface functionalization. Unlike many other particle fabrication techniques, the PRINT method is versatile and gentle enough to be compatible with the multitude of next-generation therapeutic and diagnostic agents, including small molecules, protein biologics, siRNA, and bioabsorbable and hydrophilic polymer matrix materials with embedded pharmaceutical cargo.

An overview of the PRINT process is outlined in Figure 1. As mentioned previously, the particles produced using the PRINT process are templated using polymeric micromolds. The molds themselves arise from replication of a silicon master template (Figure 1(a)), which is fabricated using advanced lithographic techniques. The replication of the master template results in a precise mold having micro- or nanoscale cavities. Molding of pharmaceutical materials and/or excipients occurs through spontaneous filling of the cavities through capillary forces, with no formation of an interconnecting "flash" layer of material between the cavities (Figures 1(b) and 1(c)). The particles are solidified (Figure 1(d)) and removed from the mold by bringing the mold in contact with an adhesive layer that enables the particles to be easily removed from the mold cavities (Figure 1(e)). At this point free flowing powders or stable dispersions can be obtained by dissolving away the adhesive layer from the particles, with the option to then be further purified, chemically modified, or analyzed (Figure 1(f)). Particles can be used as suspensions or dried using evaporation or lyophilization to produce dry powders.

2.2. Fabrication of Particles for Respiratory Drug Delivery. PRINT particles were fabricated and isolated as dry powders as described in previous reports [12, 13, 15, 17, 18]. To highlight the chemical versatility of PRINT particle technology for aerosol delivery of both small molecule and biologic drugs, particles comprised of proteins such as bovine serum albumin (BSA, Sigma-Aldrich) and immunoglobulin G (IgG, Calbiochem), polymers such as poly-lactic-*co*-glycolic acid (PLGA, Mw 30 K, Polysciences), and pharmaceutically relevant compounds such as itraconazole (Spectrum Chemical), zanamivir (Haorui USA), DNase (Worthington

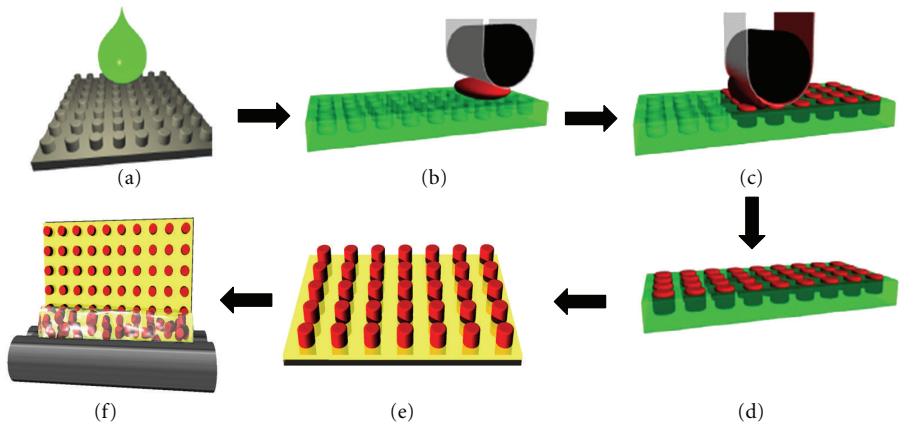


Figure 1: Schematic illustration of the PRINT process. (a) Features on a hard silicon master template are replicated with high fidelity (b) to obtain a soft, polymeric mold with micro- and nanocavities that can then be (c) filled with relevant particle matrix and (d) extracted out of the mold and onto a harvest array for (e) particle collection and purification.

Biochemical), and siRNA (Dharmacon) were fabricated. Monodisperse particles from these molds were collected in various aqueous and organic suspensions: for particles consisting of non-water-soluble matrices, such as polymeric and the small molecule itraconazole, distilled water was used to collect the particles from the array; for particles consisting of water-soluble matrices such as zanamivir, DNase, and siRNA, isopropyl alcohol was used to collect the particles from the array. To make porous particles, sacrificial poly(vinylpyrrolidone) porogen are comolded with the drug or drug/excipient blend and selectively removed during the harvesting step. Finally, particles were lyophilized from water or *tert*-butanol in order to obtain dry powder PRINT particles. Itraconazole powder (Spectrum Chemical) was micronized for aerodynamic particle size comparison testing with PRINT particles. Micronization was performed using one pass through the Glen Mills Laboratory Jet Mill.

2.3. Chemical and Bioactivity Analyses of Pharmaceutical Compounds in PRINT Particles. PRINT particles composed of small molecules and biologic materials were analyzed to confirm retention of chemical structure and biological activity during the PRINT process. All chromatographic analyses were performed using the Agilent 1100 liquid chromatography system and analyzed in Empower. A gradient reverse-phase high-performance liquid chromatography (RP-HPLC) method for itraconazole analysis was based off of the European Pharmacopoeia (EP) 5.0 method for the compound [19]. Briefly, the chromatographic procedure is a stability-indicating EP method for itraconazole in which the detection has been modified for use with a diode array. This gradient elution method used a Phenomenex Prodigy ODS (3) 100 angstrom, 4.0×100 mm, 3 μm analytical column with mobile phase A containing 27.2 g/L tetrabutylammonium hydrogen sulphate in HPLC grade water and mobile phase B containing acetonitrile and used a flow rate of 1.5 mL/min with the following gradient conditions: 0 to 20 min, 20 to 50% mobile phase B; 20 to 25 min, 50% mobile phase B; 25 to 30 min, 20% mobile phase B. Itraconazole was detected with a diode array ultraviolet (UV) measurement at 257 +/− 5 nm with reference background correction at 375 +/− 25 nm and at a retention time of 14.42 minutes.

A gradient hydrophilic interaction (HILIC)-HPLC method was used for analysis of zanamivir. Briefly, a Waters Atlantic HILIC Silica 5 μm, 4.6 × 100 mm analytical column was used with mobile phase A containing 10 mM ammonium acetate in 1% methanol and 0.05% phosphoric acid in order to maintain a pH of 3 to 4 and mobile phase B containing 0.1% phosphoric acid in acetonitrile. The method used a flow rate of 1.0 mL/min with the following gradient conditions: 0 to 2 min, 80% mobile phase B; 2 to 7 min, 80 to 60% mobile phase B; 7 to 12 min, 60% mobile phase B; 12 to 17 min, 80% mobile phase B. Zanamivir was detected by UV measurement at 230 nm and at a retention time of 5.52 minutes.

A gradient super-anionic-exchange-(SAX-) HPLC method was used for analysis of siRNA. Briefly, a Dionex BioLC DNAPac PA 200 4 × 250 mm analytical column was used with mobile phase A containing 25 mM $NaClO_4$ and 10 mM Tris, 20% ethanol and mobile phase B containing 250 mM $NaClO_4$ and 10 mM Tris, 20% ethanol, but at a pH of approximately 7.0. The method used a flow rate of 1.0 mL/min with a column temperature of 40 degrees C and the following gradient conditions: 0 to 8 min, 0–100% mobile phase B; 8 to 10 min, 0% mobile phase B. siRNA was detected by UV measurement at 260 nm and had a retention time of 6.37 minutes.

An isocratic size exclusion chromatography (SEC) method was used for analysis of DNase. Briefly, GE Superdex 75 5/150 GL column was used with PBS. The method used a flow rate of 0.3 mL/min, and the protein was detected by UV measurement at 280 nm and at a retention time of 5.14 minutes. In addition to SEC analysis, a DNA-Methyl Green assay was also used to characterize the bioactivity of DNase, as previously performed by others [20]. Briefly, DNA-Methyl Green (Sigma-Aldrich) was solubilized in 0.05 M Tris buffer to a concentration of 0.2 mg/mL. DNase activity, both unprocessed standards (Sigma Aldrich and Worthington) and

DNase from PRINT particles, was obtained by adding DNase samples individually to DNA-Methyl Green and measuring the Methyl Green light absorbance at 640 nm at 2 minute intervals. These measurements were used to obtain an initial linear rate of DNA-Methyl Green degradation, which correlates directly to DNase activity.

2.4. In Vitro Characterization of Particle Size. Aerodynamic particle sizing of all PRINT aerosols was performed using the aerodynamic particle sizer (APS) spectrometer (Model no. 3321, TSI Inc. Shoreview, MN, USA). Dry powder aerosols were dispensed into an aerosol generator using an insufflator device and a volume-calibrated hand pump (Penn Century Inc., PA, USA).

Next-generation impactor (NGI) experiments were used to compare the aerodynamic size distribution of PRINT zanamivir formulations to Relenza. Before testing, NGI stages were coated with silicone oil. To test PRINT formulations, 5 mg of PRINT-zanamivir particles were loaded into a size 3 HPMC capsule, which was loaded into a Monodose device (Plastiape SpA). The loaded Monodose device was attached to an NGI (MSP Model 170) and tested using a 60 L/min flow rate for 4 seconds. Deposited drug was rinsed from the capsule, the device, device adapter, induction port, filter, and each stage of the NGI using 5 to 25 mL HPLC grade water, and the zanamivir content in each rinsate was measured using HPLC and compared to standard curves to determine the absolute weight of zanamivir in the capsule, device, and impactor. Similar methodology was used to measure the aerodynamic particle size distribution of Relenza, with the exception that preseparator stages were used to determine the deposited dose of large (>10 μm) zanamivir/lactose agglomerates.

Laser diffraction was used to determine the geometric size of micronized itraconazole crystals. Specifically, measurements were performed using a Sympatec HELOS instrument, operated at 5 bar primary pressure and 105 mbar secondary pressure.

2.5. Gamma Scintigraphy In Vivo Canine Lung Deposition Imaging. Torus aerosols (1.5 μm and 6 μm) for the *in vivo* canine deposition study were fabricated out of a lactose-albumin-leucine blend (64/32/4 mass ratio) and were further labeled with technitium-99 (Tc99m) by isopropyl alcohol coevaporation. Naïve (unlabeled) PRINT particles were mixed with Tc99m in isopropyl alcohol. Ratios of Tc99m : PRINT particle : IPA were held at 50 mCi : 50 mg : 0.75 mL. The mixture was the gently shaken to mix without coating the material on sides of the vials. The mixture was then evaporated under a gentle stream of N_2. The labeled particles were then immediately loaded into insufflators and used for either validation studies or canine exposures.

In order to confirm the radiolabeling process, the mass median aerodynamic diameter (MMAD) of the materials before and after labeling and the activity median aerodynamic diameter (AMAD) were determined with a next-generation impactor (NGI). The NGI was operated at 30 L/min for all testing. The MMADs of both labeled and naïve aerosols were determined via differential weight analysis of the NGI cups. Following differential weight analysis, the cups were rinsed with 3 mL of water and the water was transferred into a 20 mL scintillation vial. The activity in each cup was quantified with a radio isotope counter. All data were processed to determine the MMAD/AMAD and the geometric standard deviation (GSD) for each aerosol. Based on initial results, it was decided to place a cyclone (URG Corp, model URG-2000-30EC) inline with the aerosol delivery system to remove large agglomerates and achieve an acceptable correlation between the naïve aerosols and Tc99m activity.

In order to estimate the amount of material dosed using the canine endotracheal exposure system, the delivery system efficiency was first determined for each particle group. This was performed by loading the dry powder reservoir with known amounts of each material (1.5 and/or 6.0 μm torus particles) and collecting aerosolized powder on a filter placed at the exit of the endotracheal tube. The amount of material on the filter and the amount of material delivered from the devices were determined via differential weight analysis. The delivery efficiency was calculated as the percentage of material delivered from the dry powder reservoir device that exits the endotracheal tube and is ultimately available to the lower respiratory tract.

At the time of exposure, multiple dry powder reservoirs were loaded to target an aerosol delivery of 10 mCi and ensure sufficient Tc99m deposition in the canine lungs for image analysis. Prior to being exposed, animals were placed on isofluorane anesthesia and apnea was induced by hyperventilation. Immediately following the aerosol exposures, the endotracheal tube was removed and the dogs were transferred to the Siemens E.Cam clinical SPECT gamma camera and a 10 minute planar gamma image was collected. The time lapsed from the start of aerosol exposures until the start of imaging was ~1.5 to 2 minutes, and the time from the start of aerosol exposures until the completion of the imaging was typically ~12 minutes. During image acquisition, the dry powder reservoirs were quantified for radioactivity to determine the amount of activity aerosolized. This value was then multiplied by the predetermined delivery efficiency in order to estimate the lower respiratory tract dose, or dose presented at the exit of the endotracheal tube, for each experiment.

2.6. Canine Lung Deposition Image Analysis. Image analysis was performed with the Siemens ICON software to determine the activity in two canine regions of interest (ROI) for each animal: the lungs and the trachea. In order to correlate the counts in each ROI to activity, a standard curve was prepared for the gamma camera to define the relationship between activity (measured with a radioisotope counter) and counts (from the image analysis). After converting measured counts to radioactivity, the quantified amount of activity in the lung ROI was then divided by the quantified amount of activity in the lung ROI in order to determine whole lung deposition counts normalized to trachea counts for each animal. Statistical differences in this measurement were evaluated by paired, two-tailed t-test across the four animals used for lung deposition imaging.

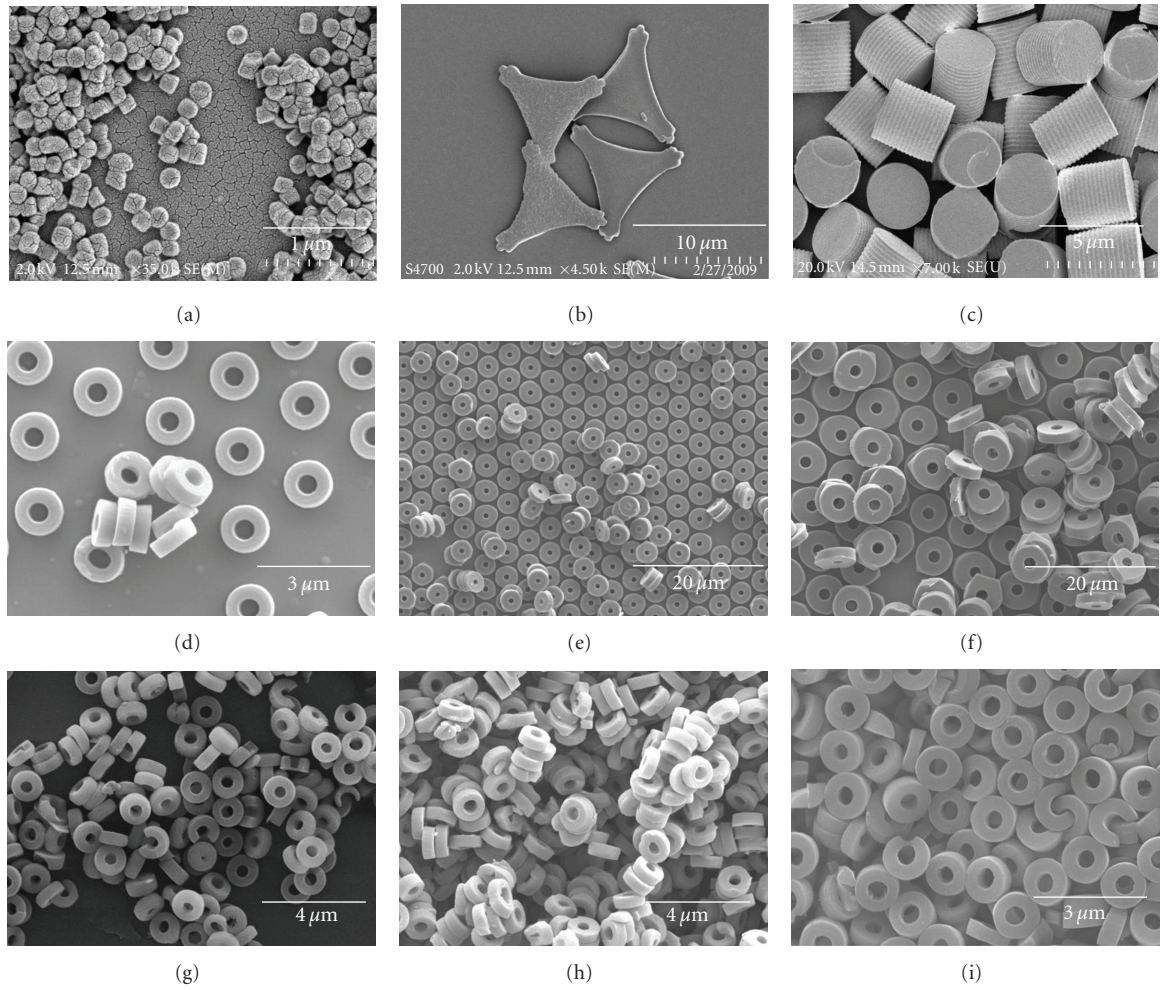


Figure 2: SEM micrographs of diverse PRINT aerosols. (a) BSA/Lactose 200 × 200 nm cylinders; (b) IgG/Lactose10 μm pollen; (c) 30 K PLGA 3 μm cylinders; (d) itraconazole 1.5 μm torus; (e) itraconazole 3 μm torus; (f) itraconazole 6 μm torus; (g) zanamivir 1.5 μm torus; (h) DNAse 1.5 μm torus; (i) siRNA 1.5 μm torus.

3. Results

3.1. Precisely Engineered Particles Containing Pharmaceutically Relevant Components. To illustrate the delivery of relevant therapeutic compounds to the respiratory tract, we fabricated particles with independent control of particle size, shape, and composition. An array of SEM micrographs is shown in Figure 2 highlighting PRINT's versatility: BSA/lactose blend 200 × 200 nm cylinders (Figure 2(a)); IgG/lactose blend 10 μm "pollen" (Figure 2(b)); poly-lactic-*co*-glycolic acid (PLGA, Mw 30 K) 3 μm cylinders (Figure 2(c)); itraconazole (marketed as Sporanox for treatment of fungal infection) molded into 1.5 μm, 3 μm, and 6 μm torus particles (Figures 2(d)–2(f)); 1.5 μm torus particles comprised of pharmaceutically relevant compounds including zanamivir (marketed as Relenza for treatment of influenza) (Figure 2(g)); bovine DNase (recombinant human DNase is marketed as Pulmozyme for treatment of cystic fibrosis) (Figure 2(h)); siRNA (Dharmacon) (Figure 2(i)). The "pollen" shape in Figure 2(b) is a biomimetic design, based on the shape of the pollen *Eperua schomburgkiana*.

In order to confirm that the PRINT particle fabrication process used to generate engineered aerosols did not alter the chemical structure of pharmaceutical compounds, analytical tests were performed to determine the compound integrity following fabrication as compared to the unprocessed or reference compound. Purity of compounds in PRINT particles relative to unprocessed or reference compound was measured to be 99.6% for itraconazole (RP-HPLC), 100% for zanamivir (HILIC-HPLC), 99.2% for siRNA (SAX-HPLC), and 99.0% for DNase (SEC). Additionally, IC_{50} in DNA-Methyl Green assay yielded DNase IC_{50} values for reference DNase (Worthington) and PRINT-DNase of 26.5 and 18.8 Kunitz units/mL, respectively, indicating that PRINT particle fabrication does not alter DNase bioactivity.

3.2. Aerodynamic Characteristics of PRINT Aerosols. Physical characterization of PRINT aerosols confirmed the ability to produce highly dispersible aerosols with controllable and narrow aerodynamic size distributions. Figure 3(a) demonstrates the capability to tune particle aerodynamic size on the basis of particle design. We fabricated torus particles

with geometric sizes 1.5 μm, 3 μm, and 6 μm torus and measured their aerodynamic characteristics using a time-of-flight aerodynamic particle sizer (APS). For these particles, porogen was added to the formulation, then subsequently removed to produce porous particles. The mass median average aerodynamic diameters (MMAD) of these particles were measured to be of 0.83 μm, 1.27 μm, and 2.57 μm with geometric standard deviations (GSD) of 1.68 μm, 1.47 μm, and 1.91, respectively.

To compare the size distributions of PRINT aerosols to conventional fabrication techniques (Figure 3(b)), we compared the mass-weighted aerodynamic particle size distribution (mass median aerodynamic diameter, MMAD) of 1.5 μm PRINT cylinders composed of itraconazole to the particle size distribution of jet-milled itraconazole (geometric size $\times 10$ = 0.77 μm; $\times 50$ = 2.79 μm; $\times 90$ = 7.42 μm). Jet milling is the most commonly utilized technique for preparation of respirable aerosol particles. The PRINT aerosol had a narrower distribution and a higher fraction of drug in the respirable range (less than 5 μm), indicating that the aerodynamic properties of these particles are better suited for inhalation therapies. Moreover, according to well-accepted correlations of aerodynamic particle size and lung deposition, it can be expected that the 1 μm cylinder particles will have enhanced deposition in peripheral airways (alveoli and respiratory bronchioles) compared to the larger particles. The precise control over aerodynamic size of PRINT aerosols may be clinically useful for local drug delivery to the lungs by enhancing deposition efficiency at the site of disease and limiting unintended off-target effects [21].

3.3. Engineered PRINT Aerosols Exhibit Increased Aerosol Delivery In Vitro. We compared the *in vitro* performance of pharmaceutically relevant PRINT particle aerosols to a dry powder marketed product. This was carried out using Relenza (GlaxoSmithKline), a small molecule DPI indicated for treatment of influenza, which contains the active pharmaceutical ingredient, zanamivir (5 mg), blended with micronized lactose (20 mg). 1.5 μm torus PRINT-zanamivir formulations were prepared, directly packaged into capsules, and aerosolized from a low-resistance DPI device (Monodose, Plastiape SpA). Both PRINT-zanamivir and Relenza formulations were characterized with a next-generation impactor (NGI). As shown in Figures 4(a) and 4(b), the PRINT-zanamivir formulation resulted in significantly improved delivery compared to Relenza. For the same fill weight (5 mg), the PRINT zanamivir dosage form showed a smaller MMAD, a similar GSD, 3 to 4 times higher fine particle fraction (FPF) and respirable dose, and 4 to 5 times more deposition of material in the size range of less than 1.6 μm. It is expected that the device retention of the PRINT-zanamivir formulation could be significantly decreased with tuning of the fill weight or device characteristics, which is beyond the scope of the work presented here. These results indicate that finer engineered PRINT particles should correlate to superior drug delivery to the lower respiratory tract (Figure 4(b)). Based on literature studies of the deposition patterns of Relenza in healthy human volunteers, it is known that 77% of the emitted drug from the commercial product is deposited in the oropharynx rather than the lung [22]. Thus, the *in vitro* results presented here suggest that the PRINT-zanamivir aerosol would translate to significantly more efficient lung delivery compared to Relenza.

3.4. PRINT Aerosols with Narrow Size Distributions Exhibit Distinct In Vivo Lung Deposition Patterns. Finally, we demonstrated the ability of PRINT particle aerosols to control *in vivo* pulmonary delivery using a canine deposition model. PRINT aerosols composed of lactose, albumin, and leucine (64/32/4 mass ratio) were prepared, radiolabeled with technetium-99, and aerosolized into the respiratory tract of beagle dogs using an endotracheal dosing apparatus. As shown in the gamma scintigraphic images (Figure 4(c)), significantly more whole-lung deposition was achieved with 1.5 μm versus 6 μm torus particles (1.3 μm and 4.6 μm MMAD, resp.), as would be expected from the relative aerodynamic sizes of these particles. Image analysis and quantification of the radioactivity counts confirmed this observation. In addition, the torus 1.5 μm particles showed a greater than twofold enhancement of whole-lung deposition counts normalized to trachea deposition. This ability to tailor particle lung deposition could have broad applicability for respiratory drug delivery, particularly in scenarios where peripheral lung deposition should be enhanced or avoided depending on clinical application.

4. Discussion

The PRINT fabrication approach predictably controls particle geometric and aerodynamic features, a differentiating attribute as compared to traditional particle generation approaches. In particular, micromolding strategies such as PRINT represent one of the only methods to precisely control particle shape and size. For PRINT, the particle geometry is directly derived from the semiconductor wafer, bringing inherent nanoscale precision to the particle geometry and offering the capability to generate unique, nonspherical shapes. It is possible to control geometric features such as length, aspect ratio, and edge curvature, as well as adding unique features such as fenestrations and biomimetic designs, as shown in Figure 2. The capability of PRINT to prepare micro- and nanoparticles of a diverse set of materials is due to the ability to mold materials in a variety of physical forms. In addition to the detailed studies presented here, particles have been prepared by polymerization [11] or solvent evaporation [23]. This flexibility lends itself to the preparation of pharmaceutically relevant particles such as hydrogels [15], PLGA controlled-release systems [13], stimuli-responsive particles [17], suspension formulations [14], or dry powder aerosols as presented here (Figures 2 and 3). This ability to control particle size, shape, and uniformity should also find advantageous use in many dosage forms, including oral, topical, and parenteral products.

Microfabrication techniques such as PRINT offer the advantage of deterministic control of particle geometry that is inherent from the use of semiconductor manufacturing techniques. In the case of PRINT technology, the same master template can be used to create each batch of micromolds and

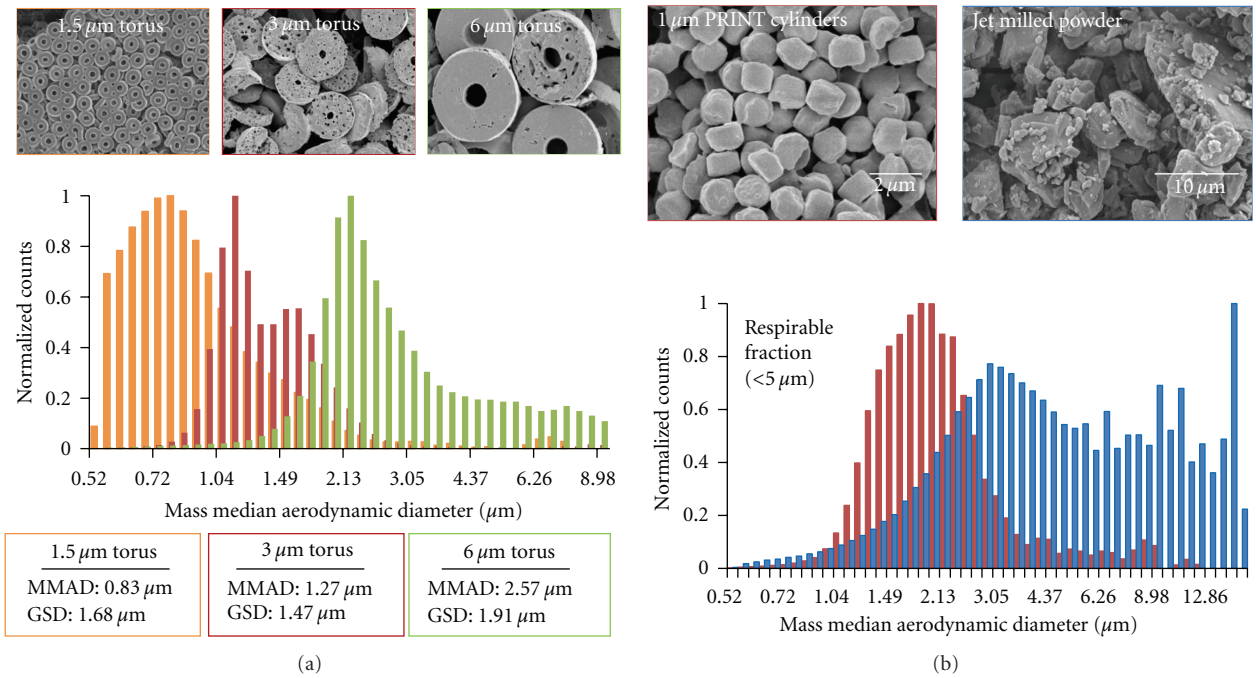


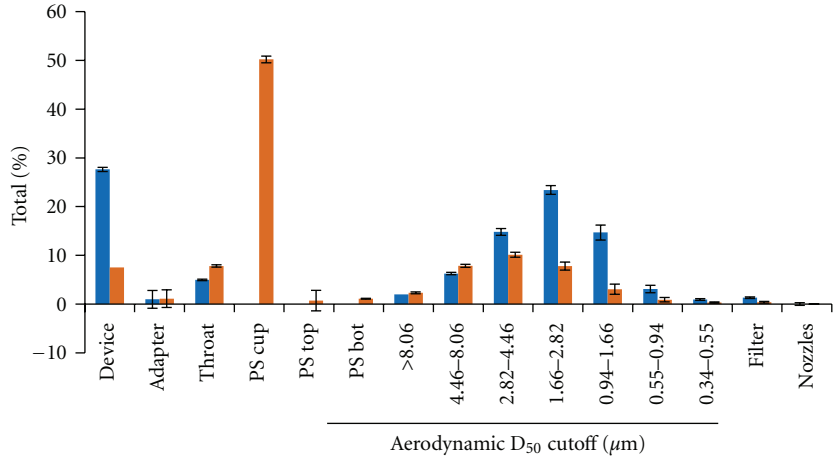
Figure 3: Aerodynamic characterization of PRINT aerosols. (a) SEM micrographs and aerodynamic performance of 1.5 μm, 3 μm, and 6 μm particles by APS. PRINT affords precise control over particle geometric size and aerodynamic size. (b) SEMs and aerodynamic distributions of jet-milled itraconazole aerosols compared to 1 μm PRINT cylinder particles made out of itraconazole. PRINT-itraconazole particles result in a narrower size distribution and higher available respirable fraction.

particles for a particular size and shape. Thus, each batch of particles possesses high uniformity and batch-to-batch consistency, regardless of the batch size. In addition, the uniform particle populations that are produced lend themselves to straightforward in-process characterization using a number of standard particle sizing methods, such as microscopy and light scattering. These features make the PRINT technology attractive from the perspective of compliance with Quality-by-Design directives from the FDA.

From a formulation perspective, PRINT technology has been shown to be a versatile approach to deliver many classes of therapeutic compounds and excipients. Particle size can be controlled over several orders of magnitude, from the sub-100 nm scale to hundreds of microns. In traditional fabrication methods, particle chemical composition and physical characteristics such as geometric or aerodynamic size are inherently coupled, for example, the molecular properties of a small molecule pharmaceutical ingredient are known to impact the particle size distribution of micronized particles, whereas the solubility and drying kinetics of precursor solutions can impact the particle size distribution of spray-dried particles [8]. In contrast, micromolded particle engineering has the ability to define the particle size and shape independent of the input material properties, which was demonstrated by fabricating particles of identical geometry yet comprising hydrophilic and hydrophobic small molecules, proteins, or nucleic acids (Figures 2(d)–2(i)). While particularly relevant for aerosol lung delivery, this ability to independently control particle composition and physical size should find utility in multiple dosage forms and routes of administration.

Small molecule drug compounds can be formulated as drug alone or drug/excipient mixtures with tunable loading. Enlow et al. demonstrated the production of PLGA/docetaxel PRINT nanoparticles with up to 40% chemotherapeutic loading [13]. This finding is in contrast to typical polymer nanoparticle drug delivery systems produced by emulsion [24], nanoprecipitation [25], and ultrasonication [26] that have theoretical drug loading of less than 15% and variable encapsulation efficiency. Furthermore, the authors demonstrated the ability to independently tune particle size, shape, and drug loading. *In vitro* results indicated that potency of these PLGA-docetaxel nanoparticles was up to 10x greater than Taxotere, a commercially marketed micellar formulation of docetaxel. In this work, we highlight the ability of PRINT to fabricate particles of neat small molecule drugs. Figures 2(d)–2(f) show particles composed of 100% itraconazole, prepared by molding an amorphous itraconazole glass. Particles composed of zanamivir were also fabricated (Figure 2(g)), and both itraconazole and zanamivir particles showed good aerosol delivery performance *in vitro* (Figures 3 and 4).

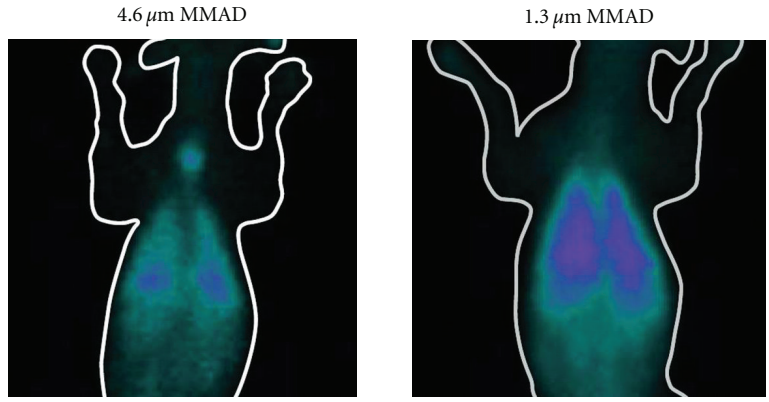
PRINT particles can be prepared from protein and oligonucleotide therapeutic agents as well. Kelly and DeSimone demonstrated the capability to use PRINT technology to fabricate monodisperse particles of albumin and insulin without causing agglomeration of the protein [12]. In this work, we demonstrate molding of DNase, a therapeutic



(a)

Aerosol parameter	Relenza (mean (%RSD))	PRINT (mean (%RSD))
Target Fill wt. (mg)	5	5
MMAD (μm)	3.4 (4.9)	2.3 (0.6)
GSD (μm)	1.8 (1.1)	1.8 (4.3)
FPF (% emitted dose)	25.6 (9.9)	81.7 (2.8)
<1.6 μm (% total dose)	4.6 (13.9)	20.0 (4.0)

(b)



(c)

Figure 4: Favorable properties of PRINT aerosols for dry powder pharmaceutical use. (a, b) Comparison of 1.5 μm torus PRINT-zanamivir particles against the marketed product Relenza (active pharmaceutical ingredient zanamivir) using an NGI. (b) PS: preseparator; RSD: relative standard deviation. (c) Whole lung deposition by gamma scintigraphy in canine shows increased whole-lung deposition of 1.5 μm (right, 1.3 μm MMAD) torus aerosols versus 6.0 μm (left, 4.6 μm MMAD) torus aerosols.

protein for cystic fibrosis (marketed as Pulmozyme). Figure 2(h) shows 1.5 μm torus particles composed of DNase. Size exclusion chromatography of PRINT-DNase microparticles shows minimal agglomeration of the protein, and *in vitro* bioassay measurements demonstrate equivalent enzyme activity to naïve DNase. Oligonucleotide molecules such as siRNA therapeutics were also successfully molded as particles (Figure 2(i)) with retention of chemical structure. Taken together, these data demonstrate that PRINT particles can be formed of biological materials without aggregating/denaturing the molecule or changing its functionality.

Micromolded particles produce high-performance aerosols that possess tunable aerodynamic diameters and narrow aerodynamic size distributions. This control over aerosol characteristics was demonstrated across a wide range of aerodynamic diameters within the respirable range (Figure 3(a)) and through differential *in vivo* lung deposition based on particle size (Figure 4(c)). In addition, PRINT aerosols

achieve an increased respirable dose and decreased MMAD, including the dose fraction below 1.6 μm, compared to aerosols generated by traditional micronization processes (Figures 3(b) and 4(a)). These attributes are expected to translate into more efficient respiratory drug delivery for a wide range of therapeutics that are intended to deposit in the lung periphery. Importantly, the aerosolization of PRINT particle dry powders does not require the use of bulking excipients, such as lactose, for particle dispersion, as is often the case for dry powder products. Elimination of bulking agents potentially simplifies the chemistry, manufacturing, and control processes required to develop dry powder products, as well as mitigating the potential for excipient-induced user side effects.

The micromolding particle fabrication approach presented here also holds the potential to engineer dry powder aerosols optimized for specific disease targets. There are a number of instances where more precise respiratory drug delivery could be useful, as has been demonstrated by others. Particle aerodynamic size and regional drug deposition has been shown to influence pharmacodynamic responses in diseases such as asthma and cystic fibrosis. Usmani et al. demonstrated that 6.0 μm MMAD albuterol aerosols improve forced expiratory volume (FEV1) in asthmatic subjects to a greater degree than 3 μm or 1.5 μm aerosols. The authors correlated the enhancements FEV1 to higher central lung deposition (confirmed by scintigraphy) and postulated that the pharmacodynamic advantage of these 6.0 μm aerosols was related to greater deposition in proximity to conducting airway smooth muscle tissue [27]. In another study in cystic fibrosis patients, improved forced expiratory fraction (FEF_{75}) was observed for DNase aerosols delivered preferentially to the small airways compared to the large airways. This data suggests that enhanced deposition of DNase at the site(s) of disease pathology could benefit patient lung function [28]. In addition, it is reasonable to expect that enhanced deposition in the alveolar region may be favorable for applications such as systemic delivery of therapeutics via the lung [21]. These studies suggest that technologies such as PRINT, which possess the ability to engineer particles with desirable aerosol and deposition characteristics, could ultimately result in inhaled products with enhanced efficacy when applied to the appropriate disease and therapeutic compound. In particular, the benefits of differential lung deposition and efficient lung delivery will be particularly useful for expensive therapeutic agents such as biologics or highly potent, narrow therapeutic index compounds.

Lastly, particle shape is known to influence all stages of pulmonary drug delivery: from entrainment and deagglomeration into a disperse aerosol [21, 29, 30], to aerodynamic characteristics and deposition [8, 30–34], to mucociliary clearance and macrophage uptake [14, 35, 36]. Others have demonstrated that shape has an impact on particle aerodynamic characteristics through studies on simple shapes, such as rods, plates, fibers, and spheres [30, 31]. Though particle shape is known to be a critical factor of aerosol properties, thorough exploration of its effect has been limited by current fabrication methods of aerosol particles [31]. Controlling particle shape thus provides an opportunity to systematically optimize the effect of shape on these stages of drug delivery. Microfabrication techniques such as PRINT offer a promising strategy to control particle shape, and more thorough investigations on the impact of particle shape on lung deposition, clearance, and cellular internalization are currently underway in order to better characterize the specific benefits particle shape may hold for respiratory drug delivery.

5. Conclusion

In summary, coopting the top-down manufacturing capabilities of the microelectronics industry enables the generation of high-precision particle-based drug delivery systems that are compatible with novel and existing formulation strategies and dosage forms. In particular, the PRINT process is well suited for the production of high-performance aerosol particles for respiratory drug delivery. Precise control over size and shape allows for defined aerodynamic properties, which, in turn, leads to enhanced aerosol performance and differential lung deposition *in vivo*. In addition to the benefits imparted by control over particle size and shape, micromolding is presented as a versatile strategy for formulating particle systems of small molecules, biologics, oligonucleotides, and drug/excipient mixtures. Overall, micro-molding is a viable particle design strategy that may address challenges existing for respiratory drug delivery and other dosage forms, thereby constituting a promising opportunity for the development of next-generation therapeutics.

Disclosure

B. W. Maynor, J. M. DeSimone, A. Garcia, P. Mack, J. Tully and S. Williams are all shareholders at Liquidia Technologies.

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Review Article

Cyclodextrin-Containing Polymers: Versatile Platforms of Drug Delivery Materials

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Nanoparticles are being widely explored as potential therapeutics for numerous applications in medicine and have been shown to significantly improve the circulation, biodistribution, efficacy, and safety profiles of multiple classes of drugs. One leading class of nanoparticles involves the use of linear, cyclodextrin-containing polymers (CDPs). As is discussed in this paper, CDPs can incorporate therapeutic payloads into nanoparticles via covalent attachment of prodrug/drug molecules to the polymer (the basis of the Cyclosert platform) or by noncovalent inclusion of cationic CDPs to anionic, nucleic acid payloads (the basis of the RONDEL platform). For each of these two approaches, we review the relevant molecular architecture and its rationale, discuss the physicochemical and biological properties of these nanoparticles, and detail the progress of leading drug candidates for each that have achieved clinical evaluation. Finally, we look ahead to potential future directions of investigation and product candidates based upon this technology.

1. Cyclosert: Rationale and Introduction

Ever since Paul Ehrlich introduced the concept of the "magic bullet"—that is, the combination of an agent conferring selectivity towards a disease-causing organism with a therapeutic agent—scientists have worked towards achieving this vision. One way to achieve selectivity towards certain disease states was to develop a prodrug that would be administered in its inactive and nontoxic form but would be metabolized to its active form once it reached the diseased organ. Prodrug approaches have been used by medicinal chemists to improve the absorption, distribution, metabolism, and excretion (ADME) of many small-molecule drugs. This approach was also important in increasing the selectivity of many small-molecule drugs, especially in the field of oncology. Examples such as irinotecan (a prodrug of the camptothecin analog, SN-38), capecitabine (a prodrug of 5-FU), and etoposide phosphate (a prodrug of etoposide) have shown clinical success and thereby demonstrated the value of this approach. This concept was further expanded through the development of macromolecular prodrugs. The rationale for using macromolecules as drug carriers is that they may be able to incorporate many more functional features than a relatively simple small molecule, therefore enabling them to perform complex functions at the right time and right place within a patient. A nanoparticle drug, one form of a large macromolecular drug, has a hydrodynamic diameter between ~10 and ~100 nm. Many types of nanoscaled drugs, such as antibody conjugates, polymer conjugates, and liposomal drugs, have been developed. The most important functional features of nanoparticle drugs are shown in Table 1.

Here, we discuss the preclinical and clinical development of a class of nanoparticles for the delivery of small-molecule drugs based on linear, cyclodextrin-based polymers (CDPs). CDPs contain alternating repeat units of β-cyclodextrin (CD) and polyethylene glycol (PEG) with two carboxylate groups per repeat unit for drug conjugation (Figure 1). Both components are commonly used in drug delivery applications. Cyclodextrins are cyclical sugar molecules with a hydrophilic exterior and hydrophobic cavity interior. High aqueous solubility and the ability to encapsulate hydrophobic moieties within their cavity through the formation of inclusion complexes enable cyclodextrins to enhance the

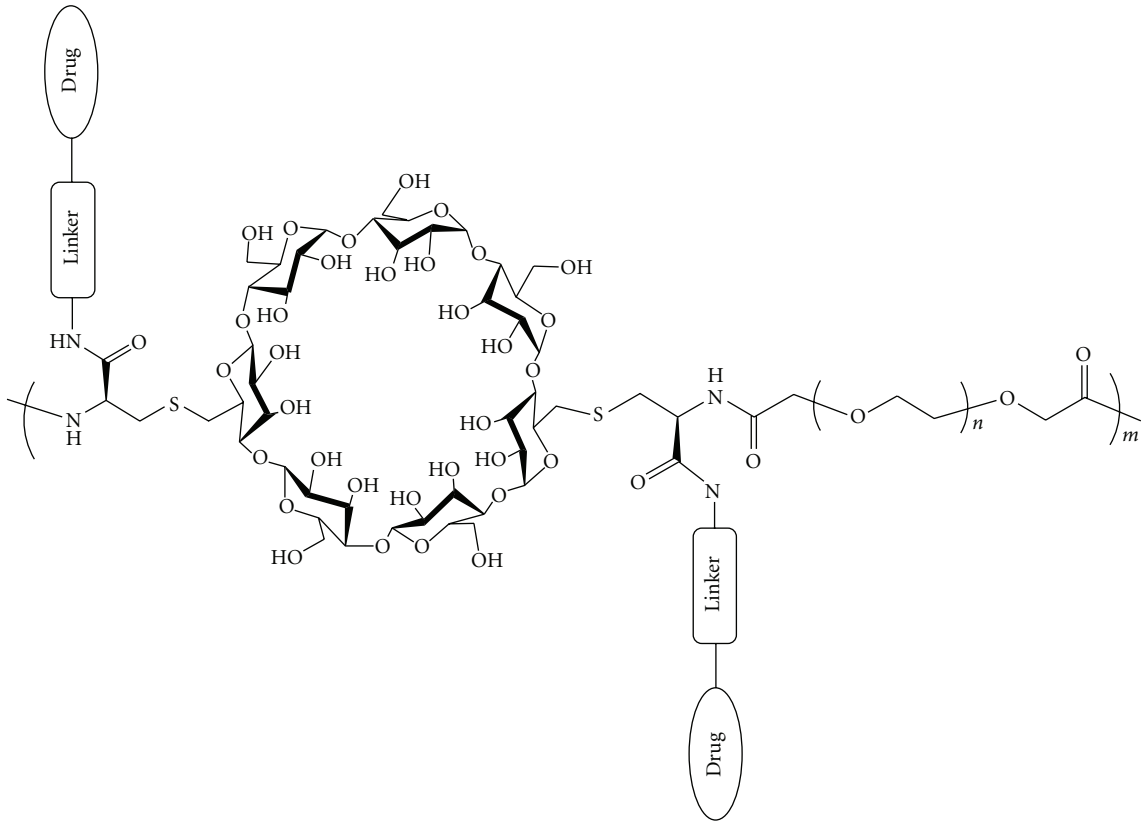


Figure 1: Structure of linear, cyclodextrin-based polymer (CDP) for small molecule delivery. The polymer consists of the cyclical sugar β-cyclodextrin that has been difunctionalized with the natural amino acid cysteine (CDDCys) and polyethylene glycol (PEG). Two small-molecule drugs per polymer repeat unit can be attached via various linker chemistries, resulting in a neutrally charged, highly water-soluble polymer conjugate.

Table 1: Key nanoparticle characteristics and their effect on *in vivo* functionality.

Nanoparticle characteristics	Function
Diameter between 10 and 100 nm	Control over pharmacokinetics and biodistribution
Surface properties (charge, hydrophilicity)	Solubility, protection from aggregation, and interaction with cells and proteins
Core properties	Protection of payload from chemical and enzymatic inactivation, control of release kinetics
Linker chemistry	Protection of drug from chemical or enzymatic inactivation, control of release kinetics
Targeting ligands	Control of cell surface binding and intracellular uptake

solubility, stability, and bioavailability of hydrophobic small-molecule drugs [1]. PEG is often used in pharmaceutical applications to increase the solubility, stability and plasma half-life of drugs [2].

In order to form the CDP polymers, a difunctionalized β-cyclodextrin is reacted with a difunctionalized PEG through condensation polymerization [3]. The resulting polymer is highly water soluble and neutrally charged when fully conjugated with drug through various linkers. This results in a high biocompatibility of the polymer, eliciting no observable side effects or immune responses at intravenous doses up to 240 mg/kg in mice [4]. A number of small-molecule drugs such as camptothecin [3], a natural alkaloid antineoplastic agent, tubulysin [5], a naturally occurring tetrapeptide with antineoplastic activity isolated from strains of myxobacteria, and methylprednisolone [6], an steroid anti-inflammatory drug, have been attached to CDP through various linkers (Table 2). One of the unique features of CDP is that the CD blocks form inclusion complexes with hydrophobic small-molecule drugs through both intra- and intermolecular interactions. Such interactions between adjacent polymer strands are essential for catalyzing the self-assembly of several CD-PEG polymer strands into highly reproducible nanoparticles (Figure 2). Parameters affecting the particle size are the type of drug, the polymer molecular weight, and the drug loading. Covalent attachment of a hydrophobic drug is required to initiate self-assembly, and release of drug from the polymer results in the disassembly

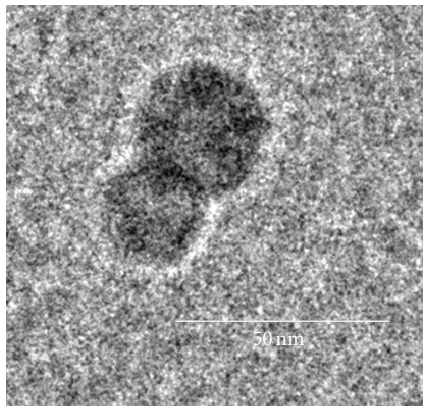


Figure 2: Transmission electron micrograph (TEM) of CRLX101 (from [8]).

into individual polymer strands of 8-9 nm, which have the potential to be cleared through the kidney [5–7].

CDP-based nanoparticles are highly water soluble at concentrations >100 mg/mL, limited by the high viscosity of resulting solutions, increasing the solubility of hydrophobic drugs by more than 100-fold (Table 2). One attractive feature of nanoparticle prodrugs is their ability to protect small-molecule therapeutics from enzymatic and chemical degradation. This was impressively shown in the case of the camptothecin (CPT) drug, CRLX101 (formerly IT-101). The chemical structure of CPT includes an unstable lactone ring that is highly susceptible to spontaneous and reversible hydrolysis, which yields an inactive, but more water-soluble, carboxylate form that predominates at physiologic pH. To form CRLX101, CPT is derivatized at the 20-OH position with the natural amino acid glycine to form an ester linkage for covalent attachment to CD-PEG (Table 2). *In vitro* studies confirmed that this linker strategy successfully stabilizes the labile lactone ring of CPT in its closed, active form. Release of CPT from the nanoparticles was found to be mediated through both enzymatic and base-catalyzed hydrolyses of the ester bond, with observed half-lives of 59 and 41 hours in PBS and human plasma, respectively [3]. Release of methylprednisolone showed similar kinetics, with observed half-lives of 50 and 19 hours in PBS and human plasma, respectively [6]. These release kinetics are substantially slower than what is typically observed with nonnanoparticle ester prodrugs [9, 10] and this is most likely due to the displacement of water from within and reduced access of enzymes to the hydrophobic core of CDP nanoparticles. The disulfide linked ester conjugate was significantly more stable, with minimal release observed in PBS or human plasma over 72 hours [5].

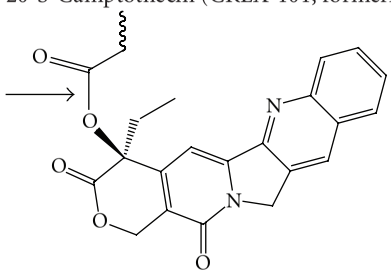
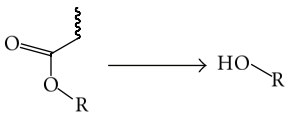
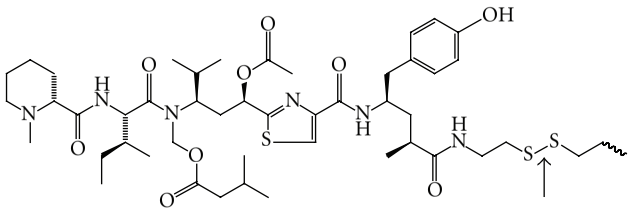
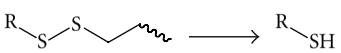
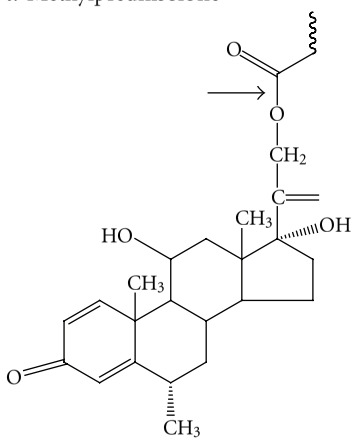
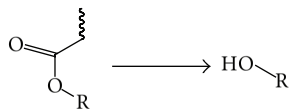
The ability of any nanoparticle therapeutic to deliver the payload to the target cell and release it at the right time and location will be important for its performance. Release of the payload can be triggered by various mechanisms, depending on the linker chemistry. CDP polymers have been used in combination with ester linkages, such as glycine or triglycine, as well as disulfide linkers. While ester linkers are cleaved through pH-dependent and enzymatic hydrolysis, disulfide linkers are cleaved in response to a change in redox potential upon intracellular uptake of the nanoparticle. *In vitro* and *in vivo* studies showed that CDP nanoparticles are taken up by various cell types, including tumor cells and cells of the immune system [4, 7, 11]. Intracellular uptake and release are also directly correlated to the *in vitro* potency of the conjugate. In the case of CRLX101, the *in vitro* potency was found to be between one-half to one-tenth the potency of the unconjugated CPT in a 48-hour MTS assay [12]. In contrast, the *in vitro* potency for the disulfide-conjugated tubulysin nanoparticle was similar to that for the free drug in a 48-hour assay, consistent with a more rapid release after intracellular uptake [5]. The time dependence of *in vitro* potency was studied more extensively in the case of the ester-linked methylprednisolone nanoparticle, for which the potency of the nanoparticle at 5 days in a lymphocyte proliferation assay was higher than that of free drug [6]. In the same assay, the free drug was more potent at 3 days, consistent with the slow release of active drug from the nanoparticle over time.

2. Pharmacokinetics and Pharmacodynamics of Cyclosert-Based Nanoparticle Drugs

The ability of nanoparticles to dramatically change the pharmacokinetics (PK) and biodistribution of drugs on both a macroscopic level (i.e., whole organ) and a microscopic (i.e., cellular) level is key to achieving the desired improvements in pharmacodynamics (PD) and, ultimately, therapeutic index. Plasma PK after intravenous injection was extensively studied for CRLX101 by traditional HPLC assays in rats [13] and by micro-PET/CT in mice using ^{64}Cu-labeled nanoparticles [7]. The nanoparticle PK is characterized by a low volume of distribution approximately equal to the total blood volume and long terminal half-life of 13 to 20 hours in mice and rats, respectively. This result indicates that the nanoparticles are able to avoid first-pass kidney clearance, which is commonly observed for drugs with hydrodynamic diameters below 10 nm [14]. This was in contrast to the PK of CPT alone, which showed a high volume of distribution and short terminal half-life of 1.3 hours.

After intravenous administration, CDP nanoparticles therefore form a circulating reservoir of active drug that is subsequently distributed to multiple organs. Consistently, tumor tissue showed high drug concentrations 24 to 48 hours after injection of nanoparticles. Other tissues with high drug concentrations were liver, spleen, and kidney, while most other organs showed low concentrations. A detailed study of multiorgan PK by PET/CT and histology revealed that CRLX101 nanoparticles were taking advantage of the unique tumor physiology characterized by a high density of abnormal blood vessels, high vascular permeability, and decreased rate of clearance due to a lack of lymphatic drainage, all of which act together to cause accumulation. This phenomenon has also been called the enhanced permeability and retention (EPR) effect [15]. In the same study, intact nanoparticles were found inside cancer cells distributed throughout the tumor tissue, forming an intracellular reservoir of active

Table 2: Linkers and drugs evaluated with the CDP nanoparticle system. The cleavage position is indicated with an arrow.

Drug	Linker Aqueous solubility	Polymer Mw Drug loading Particle size
20-S-Camptothecin (CRLX-101, formerly IT-101) 	Glycine-ester  NP >100 mg/mL ⟶ Drug 0.004 mg/mL	35–85 kDa 6–12% w/w 20–40 nm
Tubulysin A 	Disulfide  NP >100 mg/mL ⟶ Drug 0.1 mg/mL	67 kDa 12–16% w/w 100–130 nm
α-Methylprednisolone 	Glycine-ester  NP >100 mg/mL ⟶ Drug 0.12 mg/mL	137 kDa 12.4% w/w 27 nm

drug. This intracellular accumulation can also explain the finding that tumoral concentrations of CRLX101 and released CPT remained relatively constant for several days after intravenous injection, as opposed to the rapid decline (over several orders of magnitude in less than 24 hours) of irinotecan and its active metabolite, SN-38, in several preclinical lymphoma models [16]. These increased tumor concentrations also correlated with increased inhibition of topoisomerase I enzymatic activity at 48 hours after administration by CRLX101 compared to irinotecan.

Studies of the biodistribution and cellular uptake of fluorescently labeled CDP nanoparticles (NPs) in a syngeneic glioma model in C57BL/6 mice showed an additional mechanism of nanoparticle transport and distribution [11]. Irrespective of route of administration (intravenous versus intracranial), CDP-NPs were more efficiently taken up by tumor-associated macrophages (TAMs), macrophages, and microglia, than tumor cells. These TAMs not only internalized NPs by phagocytosis but also were able to migrate into the circulation after local intracranial CDP-NP injections. Additionally, NP-positive TAMs distributed to distant tumors within the CNS after local intracranial delivery. One unique characteristic of the fluorescently labeled CDP-NPs used for these studies was their slightly positive surface charge, while all of the other CDP-NPs discussed here had a slightly negative to neutral surface charge. Taken together, these observations indicate that CDP-NPs could be tuned to circulate as free NPs in plasma for prolonged periods of time and/or be taken up by immune cells such as TAMs and transported via cell migration. Both of these effects may occur at the same time and are not mutually exclusive. In addition to cancer, these findings of macrophage transport may have implications for the application of CDP-NPs in other indications, such as inflammatory diseases. It is conceivable that some of the enhanced *in vivo* activity of Cyclosert-methylprednisolone [6] was also due to immune cell-mediated transport.

One major benefit of these PK and PD improvements is a dramatic increase in therapeutic index for many small-molecule drugs. For example, CPT essentially has no therapeutic window and its development was abandoned due to excessive toxicity. CRLX101, in contrast, has shown to be highly active in multiple human subcutaneous and disseminated cancer models [16, 17]. In all cases studied, one treatment cycle of 3 weekly doses of CRLX101 resulted in significant antitumor activity that was superior to irinotecan or topotecan, two small-molecule analogs of CPT. In the case of tubulysin A (TubA), the increase in therapeutic index was even more impressive, showing a >100-fold increase in maximum tolerated dose (MTD). Whereas TubA at its MTD was completely inactive, CDP-TubA showed equal or superior efficacy compared with vinblastine and paclitaxel reference treatments with minimal observed toxicity [5]. While cancer is a natural indication for nanoparticle drugs, many other indications may be amenable to treatment with nanoparticle drugs. The common denominator in these diseases is the presence of inflammation resulting in similar physiological changes, such as neovascularization and high vascular permeability. Preclinical studies in models of rheumatoid arthritis showed that this approach can work for anti-inflammatory therapy and may be expanded to other disease indications [6].

Table 3: Nanoparticle-specific independent variables, process control measures, and dependent variables used in setting specifications for Cyclosert drugs.

Independent variable	Process control measures	Dependent variable
Polymer molecular weight and polydispersity	Real-time viscosity determination during polymerization	Particle size
Drug loading	Stoichiometry of coupling reaction	Particle size, release kinetics

3. CRLX101 Clinical Translation

Based on the preclinical activity of CRLX101, clinical development was initiated. This required a significant investment in process improvements and scale-up of nanoparticle manufacturing. Specific process challenges that had to be overcome were the control over the polymerization reaction, consistency of drug loading, and reproducible nanoparticle formation. In order to set appropriate specifications for key parameters potentially affecting the *in vivo* characteristics of the drug, a bracketing approach was chosen. Key nanoparticle specific parameters identified were polymer molecular weight (Mw) and drug loading, both of which are controllable by specific process control measures, as well as the particle size, which is a function of the two independent parameters (Table 3). A series of nanoparticle compounds bracketing each independent parameter were synthesized, their particle sizes determined, and pharmacokinetics and pharmacodynamics evaluated *in vivo*. Results of these studies were then used to set upper and lower specification limits for both independent and dependent variables.

A phase I study of CRLX101 in patients with refractory solid tumors was initiated. The primary objectives of this first-in-man study were to determine the safety, pharmacokinetics, dose-limiting toxicities, and MTD, as well as the recommended dose and dosing schedule for future studies. Secondary objectives of the study included the assessment of potential biomarkers, an estimation of clinical activity by RECIST, and an estimation of progression-free survival in patients receiving multiple cycles of CRLX101 monotherapy. Interim results of that study are available [18].

Patients with refractory solid tumors received CRLX101 using either 3 weekly (Qwkx3) or every other week (Qow) infusions every 28 days. CRLX101 was administered at 6, 12, or 18 mg/m^2 Qwkx3 and 12 or 15 mg/m^2 Qow. The occurrence of adverse events during the first cycle was used to assess the toxicokinetics. As of the interim analysis, eighteen patients had been enrolled; of these, 12 patients received CRLX101 Qwkx3 and 6 Qow. Consistent with preclinical results, CRLX101 showed a long elimination half-life of 31.8 and 43.8 hours for polymer-bound and free CPT, respectively. Volume of distribution of the polymer

conjugate was 4.2 ± 1.1 liters, indicating that CRLX101 is initially primarily retained in the vasculature. An analysis of toxicokinetics in patients that received CRLX-101 either on the Qwkx3 or Qow schedule showed that tolerability was improved on the Qow regimen while maintaining similar per-cycle drug exposures. Hematologic toxicity was dose limiting at 18 mg/m^2 on the weekly schedule. The authors concluded that CRLX101 given intravenously appeared safe when administered between 18 and 30 mg/m^2/month in both Qwkx3 and Qow regimens; however, the Qow schedule was better tolerated. More recently [19], data from additional patients dosed on the Qow regimen highlight observations of stable disease in advanced non-small-cell lung carcinoma (NSCLC) patients. Specifically, the interim data showed that 70% of the NSCLC patients achieved stable disease of greater than or equal to 3 months, and 20% of them achieved stable disease of greater than or equal to 6 months. Accrual of this phase I study has since been completed, and a randomized phase 2 study of CRLX101 in patients with advanced NSCLC has been initiated. Results from these upcoming studies will be critical for establishing the potential of CRLX101 as a new oncology agent.

4. RONDEL: Introduction and Rationale

The development of linear cyclodextrin-containing polymers (CDPs) for nucleic acid delivery traces back to the mid-1990s in the laboratory of Dr. Mark Davis at Caltech (Figure 3). In order to function as delivery agents for polyanionic nucleic acids, of which DNA oligonucleotides and plasmid DNA (pDNA) were most prevalent at that time, cationic polymers were conceived by Dr. Davis as those that would contain several key attributes: (i) assemble with nucleic acids to yield small (~100 nm or below in diameter) colloidal particles, (ii) could be easily modified with a stabilizing agent (e.g., poly(ethylene glycol) (PEG)) and a targeting ligand to facilitate *in vivo* stability and engagement of cell surface receptors on target cells and promote endocytosis, and (iii) respond to vesicular acidification as a trigger to escape the endosome and trigger particle disassembly, thereby releasing the nucleic acid payload within the cytoplasm. Cyclic oligomers of glucose, cyclodextrins were selected as the foundation of these polymers because of their known low toxicity, lack of immunogenicity, and ability to form noncovalent guest-host inclusion complexes with hydrophobic small molecules; the first description of the synthesis of a cationic CDP and characterization of the nanoparticles it formed with pDNA, including their *in vitro* transfection efficiency, was published in 1999 [20]. To overcome the salt-induced aggregation of CDP/pDNA nanoparticles in physiological media, chemistry was developed to conjugate a neutral stabilizing polymer, PEG, to a hydrophobic small molecule, adamantane (AD), which forms strong inclusion complexes with β-cyclodextrin. In this manner, nanoparticles could be noncovalently stabilized, and this approach was extended to allow incorporation of targeting ligands via preparation of AD-PEG-ligand conjugates [21, 22]. Utilizing a small interfering RNA (siRNA) targeting the EWS/Fli1 fusion oncogene and the human transferrin protein as a targeting ligand, the first *in vivo* proof-of-concept experiments were performed shortly thereafter in a disseminated murine model of Ewing's sarcoma [23]. The significant antitumor effect demonstrated in this work motivated the creation of a company, Calando Pharmaceuticals, to further advance this delivery platform (RONDEL) towards therapeutic candidates suitable for clinical evaluation in human cancer patients. The first such candidate, termed CALAA-01, contained an siRNA targeting the M2 subunit of ribonucleotide reductase (RRM2), a protein involved in DNA replication whose function is required to complete cell division. Upon identification of the optimal anti-RRM2 siRNA sequence [24] and evaluation of the *in vivo* nanoparticle performance [25], an IND application was submitted to the Food and Drug Administration (FDA) and Calando received approval to initiate a phase I trial of CALAA-01 in patients with solid tumors in 2008. In 2010, encouraging interim clinical data from this study was published [26, 27] which revealed, in addition to a promising safety profile and multiple dose escalations, the first evidence of the RNA interference (RNAi) mechanism of action in humans and the first dose-dependent tumor accumulation in humans of nanoparticles of any kind upon systemic administration.

In this paper, we describe the development of each of the components of this nucleic acid delivery system. We review the assembly of these nanoparticles, including their physicochemical properties and *in vivo* performance. The development of the CALAA-01 drug product is then discussed, including selection of the gene target and siRNA sequence optimization, safety and efficacy evaluations in animals, and manufacturing/scale-up of the components. The clinical findings of CALAA-01 are then discussed, including characterization of safety parameters (pharmacokinetics (PK), complement activation, cytokine levels, serum chemistry, complete blood counts (CBCs), and adverse events), and efficacy and a discussion of exploratory objectives. Finally, we conclude with a survey of additional explorations conducted with this delivery platform with an eye towards next-generation therapeutic candidates.

5. RONDEL Components

Fully formulated nanoparticles made with the RONDEL (RNAi/Oligonucleotide Nanoparticle Delivery) system, such as the CALAA-01 drug product developed by Calando Pharmaceuticals currently in clinical evaluation, contain a total of four (4) components described below.

The three primary cyclodextrins (CDs)—α, β, and γ—are cyclic oligomers comprised of 6, 7, and 8 glucose moieties, respectively. Functionalization and polymerization efforts were conducted with these cyclodextrin species as part of several studies to assess structure-activity relationships (SARs) of cationic polymers varying in properties such as carbohydrate size, carbohydrate distance from charge centers, and charge center type [28–31]. In general, the cyclodextrins were difunctionalized and reacted with a difunctional comonomer to yield linear, AB-type copolymers (Figure 4). A number of trends emerged from these SAR

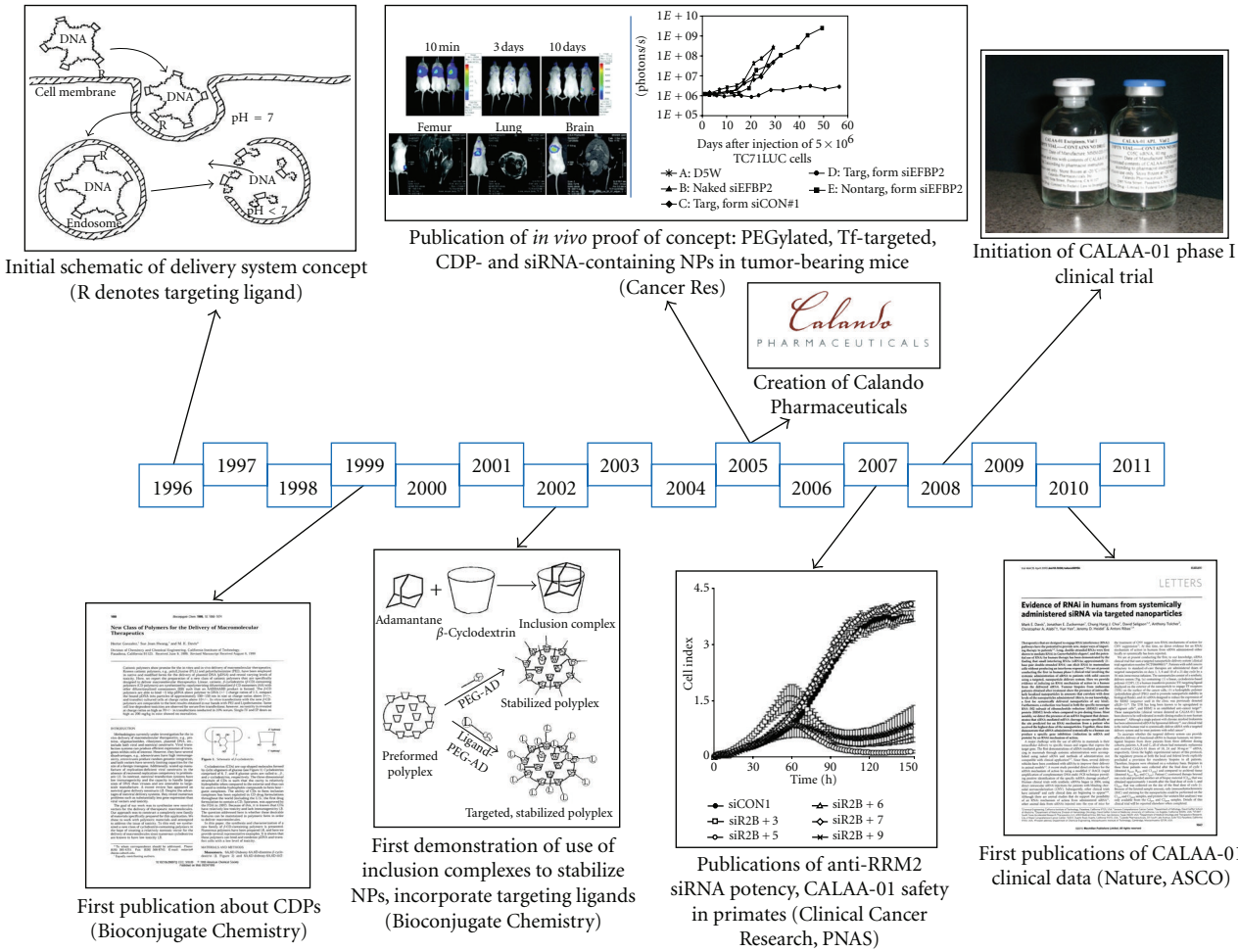


Figure 3: Timeline of the development of cyclodextrin-containing polymers (CDPs) for nucleic acid delivery.

studies (Table 4) which led to the identification of a preferred structure for the CD-containing polymer (CDP) which was the focus of further development (Figure 5). Designated as "βCDP6," "CDPim," or "CAL101" in various publications (hereafter referred to as CAL101), this polymer is made by copolymerization of β-CD diamine and dimethylsuberimidate (which imparts two amidine charge centers separated by six methylene units), and its termini are modified to contain an imidazole derivative. This modification has been shown to facilitate enhanced transgene expression from a plasmid DNA (pDNA) payload and to significantly release intracellular release of siRNA (Figure 6). Nanoparticles made with CAL101 and pDNA yielded significant gene delivery in transfected cultured cells, comparable to that of leading commercially available transfection reagents, with low cytotoxicity. Despite this *in vitro* potency, these charged colloidal CAL101/nucleic acid nanoparticles rapidly aggregate in physiological medium, rendering them unfit for *in vivo* application; this phenomenon motivated investigation into incorporation of a stabilizing agent.

The objectives of addition of a stabilizing agent to CAL101-containing nanoparticles are to minimize self-self (aggregation) and self-nonself (e.g., protein binding) interactions in an animal or human subjects receiving a systemic administration of these nanoparticles. For cancer treatment in particular, it is known that passive targeting of nanoparticles to tumors can occur through a prolonged circulation time which enables extravasation through fenestrated tumor neovasculature (enhanced permeation and retention (EPR) effect). Thus, in order to direct the biodistribution of CAL101/nucleic acid nanoparticles such that tumor uptake is maximized (and the potential for off-target deposition and toxicities are minimized), efforts to incorporate a neutral polymer, PEG, to stabilize these nanoparticles were undertaken.

While PEGylation of cationic polymer-based nanoparticles to extend circulation times and prevent aggregation was widely performed, it typically required covalent attachment of PEG at the same polymer functional sites required for nucleic acid binding. This tradeoff is undesirable, and it was overcome in this case due to exploitation of the β-CD moiety within CAL101 (Figure 7). Forming strong noncovalent inclusion complexes with β-CD (association constant of ~ 10^4-10^5 M^{-1}), adamantane (AD) was conjugated to one terminus of a linear PEG (AD-PEG) and added to CAL101 either before (pre-PEGylation) or after (post-PEGylation)

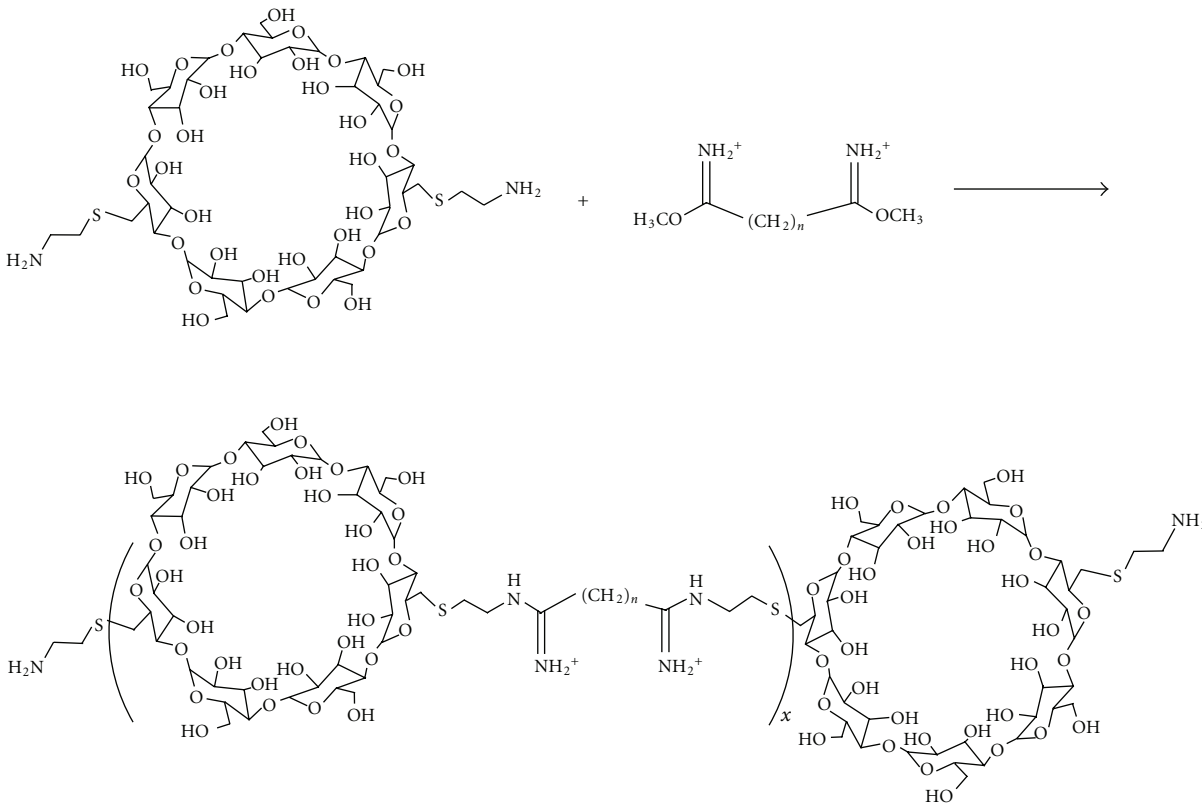


Figure 4: Polymerization scheme to yield amine-terminated CDP (from [32]).

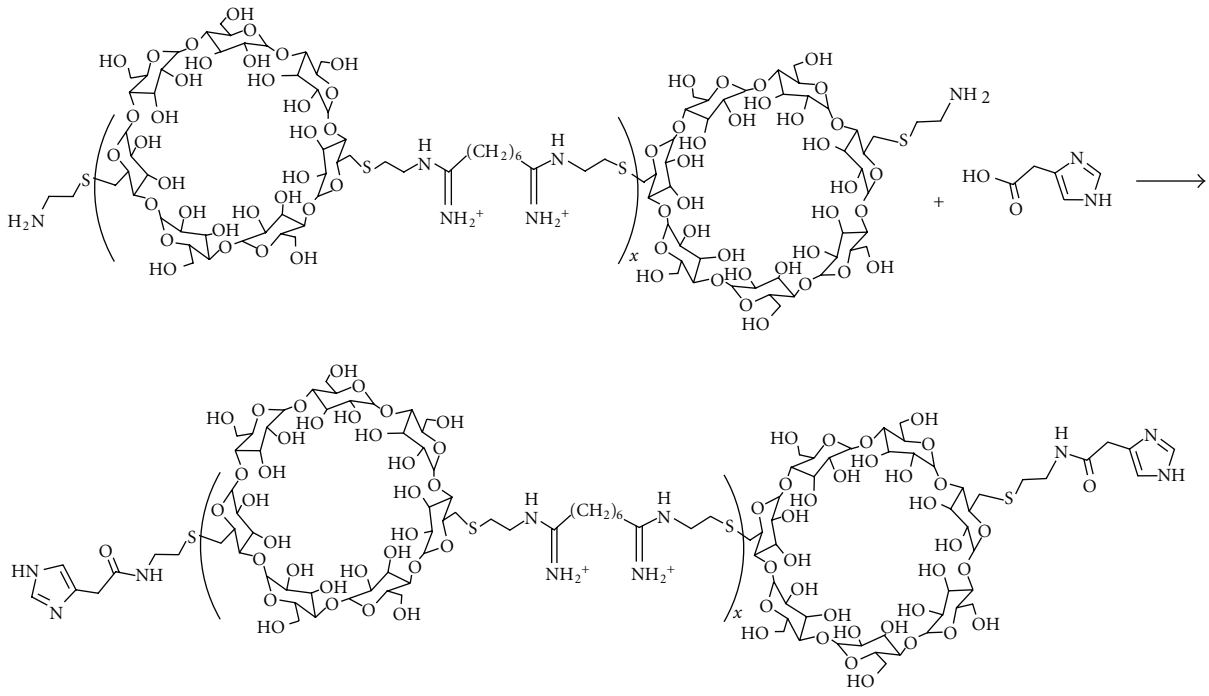


Figure 5: Polymer modification scheme to incorporate imidazole derivative within CDP.

Table 4: Parameters and result summaries for early investigations of polymer structure-activity relationships (SARs).

Parameter	Variants tested	Results/trends	Reference(s)
Carbohydrate size	(i) Trehalose (ii) α-CD (iii) β-CD (iv) γ-CD (v) (hexane)	(i) The absence of a carbohydrate produces high toxicity and reduces water solubility (ii) CD-containing species had comparable properties	[29]
Carbohydrate distance from charge centers (spacer length) and hydrophilicity of the spacer	(i) 4, 5, 6, 7, and 8 versus 10 methylene units (ii) alkyl versus alkoxy spacers	(i) Transfection efficiency is dependent on distance from charge centers, with up to 20-fold difference among β-CD-containing polymers (ii) As the charge center is further removed from the carbohydrate unit, the toxicity is increased (iii) Optimum transfection is achieved with a spacer length of 6 methylene units (iv) Increasing hydrophilicity of the spacer (alkoxy versus alkyl) provides for lower toxicity	[28, 29, 31]
Cyclodextrin functionalization	(i) $6^A, 6^D$-Dideoxy-$6^A, 6^D$-Diamino-β-CD (ii) $3^A, 3^D$-Dideoxy-$3^A, 3^D$-Diamino-β-CD (iii) $3^A, 3^B$-Dideoxy-$3^A, 3^B$-Diamino-γ-CD	(i) The structure of diaminated CD monomers was found to influence both the molecular weight and polydispersity of polycations resulting from reaction of these compounds with dimethylsuberimidate (DMS) (ii) Longer alkyl regions in the polycation backbone increased transfection efficiency and toxicity, while increasing hydrophilicity was toxicity reducing (iii) γ-CD polycations were shown to be less toxic than otherwise identical β-CD polycations	[31]
Charge center type	(i) Amidine (ii) Quaternary ammonium	(i) Quaternary ammonium analogues exhibit lower gene expression values and similar toxicities to their amidine analogues	[30]
Termini	(i) Primary amine (ii) Histidine (iii) Imidazole	(i) Incorporation of pH-buffering moiety to polymer termini increases gene delivery, buffers acidification experienced by nanoparticles, and enhances intracellular release of nucleic acid payload	[33–35]

CAL101 had been combined with the nucleic acid of interest. In this manner, simple physical mixing of these components was sufficient to achieve sufficient interaction and incorporation of AD-PEG into the nanoparticles. A minimum PEG length of 5 kDa was shown to be required to prevent salt-induced aggregation of these nanoparticles [21], and thermodynamic analysis suggests that length-dependent interactions among PEG chains on the surface of nanoparticles contribute significantly to the effective stabilization [36]. This AD-PEG_{5000} conjugate was the focus of future development work for this RONDEL delivery platform as well as clinical translation of the CALAA-01 therapeutic candidate.

Having included CAL101 as a condensing agent to induce nanoparticle formation and AD-PEG as a stabilizing agent to render these nanoparticles suitable for *in vivo* application, a third component was investigated which would facilitate cellular internalization of nanoparticles. Typical candidates for such an agent in nanoparticle formulations are ligands (in the form of peptides, proteins/antibodies, aptamers, or small molecules) whose cognate receptor is expressed on the surface of target cells either exclusively or to a much greater extent than on other (nontarget) cells. For application of these nanoparticles to cancer, the transferrin receptor (TfR) was selected [22] as a target owing to its significant overexpression on a variety of cancer cell types [37]; indeed, TfR is a well-studied surface protein for targeting of cancer therapeutics [38, 39]. The aforementioned AD/β-CD inclusion complex phenomenon was exploited to incorporate the human transferrin (Tf) protein to these nanoparticles. Specifically, Tf was conjugated to the distal end of a functionalized AD-PEG_{5000} to yield an AD-PEG_{5000}-Tf species which could also contribute to nanoparticles via physical mixing with the other components. Owing to the

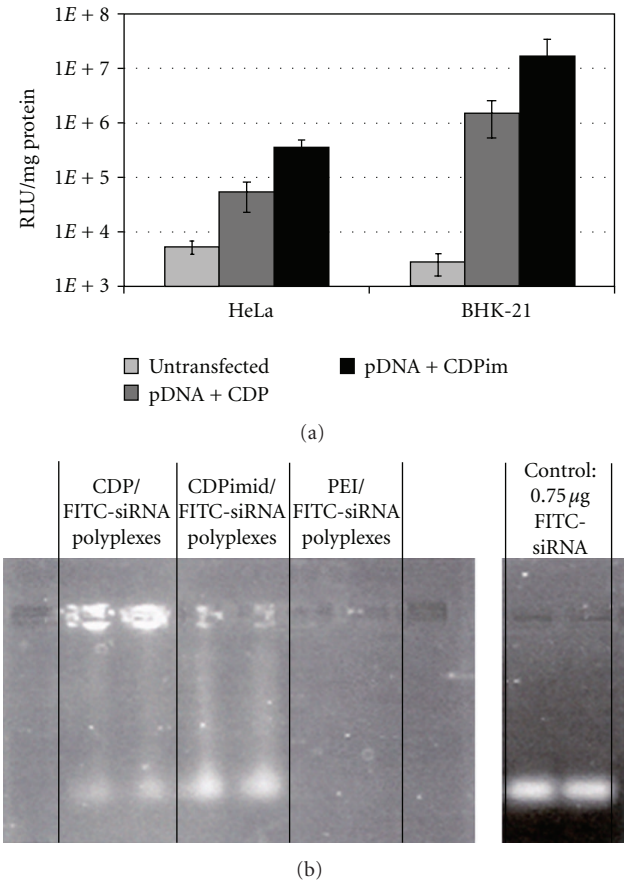


Figure 6: Effect of imidazole incorporation within CDP upon gene delivery efficiency and intracellular siRNA release. (a) Incorporation of an imidazole derivative within CDP (CDPim) leads to a significant increase in transgene (luciferase) expression levels in transfected HeLa and BHK-21 cells (from [33]). (b) Imidazole incorporation (CDPimid) yields a significant (~4x) increase in the fraction of intracellular siRNA that is released from the polymer and able to migrate through an agarose gel when electrophoresed (from [34]).

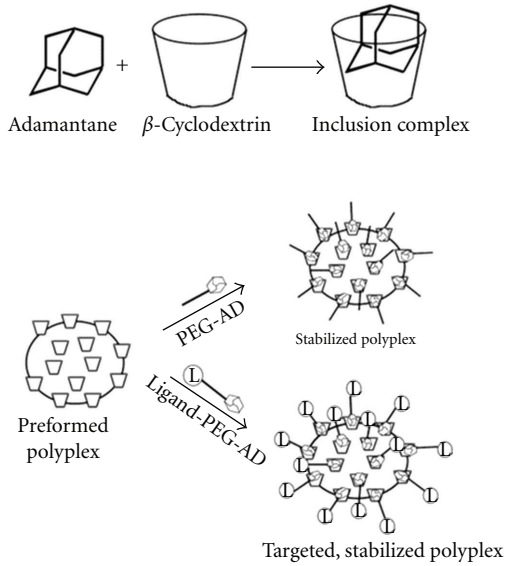


Figure 7: Formation of inclusion complexes between adamantane (AD) and β-cyclodextrin allows straightforward, noncovalent incorporation of stabilizing (via PEG-AD conjugates) and/or targeting (via ligand-PEG-AD conjugates) components to a polymer-nucleic acid nanoparticles (polyplex) (Figure from [21]).

significant size (~80 kDa) and net anionic charge of Tf, the range of stoichiometries which would retain desired nanoparticles size and stability while yielding a biological effect was established (Figure 8). As is discussed below and has been reviewed previously [40], the presence of AD-PEG-Tf within these nanoparticles does not significantly alter their overall biodistribution but appears to enhance activity *in vivo*, presumably through enhanced internalization by cancer cells.

The final component of the nanoparticles, the siRNA, is typically a canonical siRNA (two 21-nucleotide strands sharing 19 nt of Watson-Crick complementarity with 2-nt, 3′ overhangs) although successful formulation with alternative RNAi constructs has been observed. Because protection from serum nucleases is afforded by formulation within CAL101-containing nanoparticles, replacement of native phosphodiester linkages with phosphorothioates (which impart nuclease resistance) was not performed. In addition, because preclinical investigation did not reveal evidence of strong immunogenicity at therapeutically relevant dose levels (as discussed below), siRNA modifications that may reduce cytokine activation via Toll-like receptor (TLR) interaction, such as 2′-OMe and 2′-F, were not imposed. As a result, the siRNA species investigated within these nanoparticles as described in this paper are truly native/unmodified species whose degradation products are naturally occurring and require no special chemistries to synthesize.

The modular nature of these siRNA-containing nanoparticles affords flexibility with respect to the means and order of assembly by which they are formulated. Two distinct orders of assembly ("post-PEGylation" versus "pre-PEGylation") can be employed. For post-PEGylation, CAL101 is combined with siRNA to form polyplexes to which PEG-containing species (i.e., AD-PEG and AD-PEG-Tf) are subsequently added. By contrast, a pre-PEGylation approach involves combining all three delivery system components together to yield a mixture which is then added to siRNA. Both strategies can provide nanoparticles <100 nm in diameter that demonstrate resistance to salt-induced aggregation. Because it involves a single mixing step to create nanoparticles at the time of use, the pre-PEGylation strategy was employed for the nanoparticle investigations described in the remainder of this paper. In addition, because of nearly instantaneous nanoparticle formation with this approach and reproducibility with respect to physicochemical properties, subsequent investigation of these siRNA-containing nanoparticles involved formulation at the time of use—that is, rather than prepare a large quantity of nanoparticles in advance and store them, separate preparations of (i) combined delivery components and (ii) siRNA were provided which were mixed to yielded nanoparticles on the

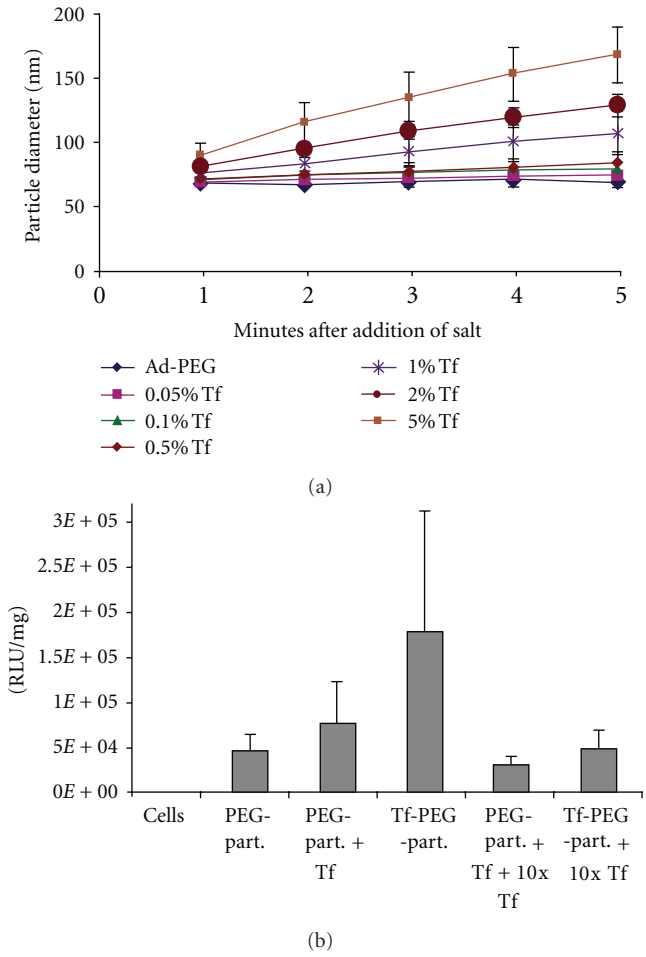


Figure 8: Effect of AD-PEG-Tf incorporation on nanoparticle size, salt stability, and transgene efficiency. (a) Dynamic light scattering (DLS) measurements of nanoparticle size as a function of time after the addition of salt (phosphate-buffered saline) help to define an optimal formulation window above which excessive AD-PEG-Tf leads to salt-induced nanoparticles aggregation. Nanoparticles were prepared containing 0% (Ad-PEG) or the indicated mol% of AD-PEG-Tf (percentage of total cyclodextrins by mole, with the remaining balance to 100% comprised of AD-PEG). (b) When plasmid-containing nanoparticles are exposed to cultured cells, inclusion of AD-PEG-Tf in the formulation increases transgene expression in a manner that can be reversed by addition of soluble Tf as a competitor, suggesting that TfR-mediated endocytosis plays a role in nanoparticle uptake and/or intracellular trafficking. Treatments included cell alone (cells), non-AD-PEG-Tf-containing nanoparticles (PEG-part.), non-AD-PEG-Tf-containing nanoparticles plus 0.05 mol% free soluble Tf (PEG-part. + Tf), AD-PEG-Tf-containing nanoparticles (Tf-PEG-part.), non-AD-PEG-Tf-containing nanoparticles plus 10 equivalents of free soluble Tf (PEG-part. + Tf + 10x Tf), or AD-PEG-Tf-containing nanoparticles plus 10 equivalents of free soluble Tf (Tf-PEG-part. + 10x Tf) (from [22]).

day of administration. This approach eliminated the need to demonstrate long-term nanoparticle storage stability and, owing to a single mixing step, permitted a facile preparation protocol to which it was easy for personnel at animal facilities and hospital/clinic pharmacies to adhere.

6. RONDEL Proof of Concept in Tumor-Bearing Mice: Expanded Nanoparticle Characterization

Having developed small-scale synthetic procedures for the three aforementioned components of the delivery system (CAL101, AD-PEG, and AD-PEG-Tf), an appropriate *in vivo* model was sought for a proof-of-concept investigation of the ability of this system to deliver siRNA to tumor cells in mice. In collaboration with Dr. Timothy Triche and colleagues at Children's Hospital Los Angeles, a disseminated murine model of Ewing's family of tumors (EFT)—mesenchymal malignancies that arise in bone or soft tissue or present as primitive neuroendocrine tumors and typically affect teenagers—was identified and selected. The vast majority (85%) of EFT patients have a unique chromosomal translocation that results in the creation of a chimeric EWS-Fli1 fusion that serves as an oncogenic transcription factor. Accordingly, siRNA species targeted specifically to the region of fusion had been described [32] which could induce apoptosis of EFT cells. A potent published anti-EWS-Fli1 siRNA was utilized within Tf-targeted nanoparticles to investigate the effect of treatment on cumulative tumor burden in mice. To create a disseminated EFT model in mice for which tumor burden could be readily measured, systemic (tail vein) injections were made of EFT cells which constitutively expressed firefly luciferase; this allowed the use of whole-animal bioluminescence imaging to quantify tumor burden. Employing a twice-weekly dosing regimen for four weeks, a statistically significant reduction in tumor burden was observed only for those nanoparticles which contained (i) the anti-EWS-Fli1 siRNA and (ii) the Tf targeting ligand (Figure 9(a)). Importantly, this was achieved in the absence of strong indications of toxicity or immunogenicity in these animals (Figure 9(b)). Together, these findings suggested a strong potential for continued development of this platform of siRNA-containing nanoparticles as anticancer therapeutics.

Even as these proof-of-concept results were obtained and Calando Pharmaceuticals was established (in 2005) to continue development of therapeutic candidates, research into the fundamental nature and behavior of these siRNA-containing nanoparticles continued in the laboratory of Mark Davis at Caltech. Two important publications in 2007 provided a more comprehensive physicochemical and *in vitro* biological characterization of these nanoparticles [36] and examined their biodistribution and pharmacokinetics in mice [41], respectively. A summary of the characterization findings is provided in Table 5. Notably, a combination of multiple experimental methods, including multiangle laser light scattering (MALS), allowed determination of nanoparticle stoichiometry—a 70 nm nanoparticle contains an average of ~10,000 CAL101 molecules, ~4000 AD-PEG molecules, ~100 AD-PEG-Tf molecules, and ~2,000 siRNA molecules. In addition, it was shown that the net ratio of positive (from CAL101) to negative (from siRNA) charges in the nanoparticles is ~1, implying that all additional CAL101 in the formulation remains as "free" (non-nanoparticle-contained). Since it is free components that are likely

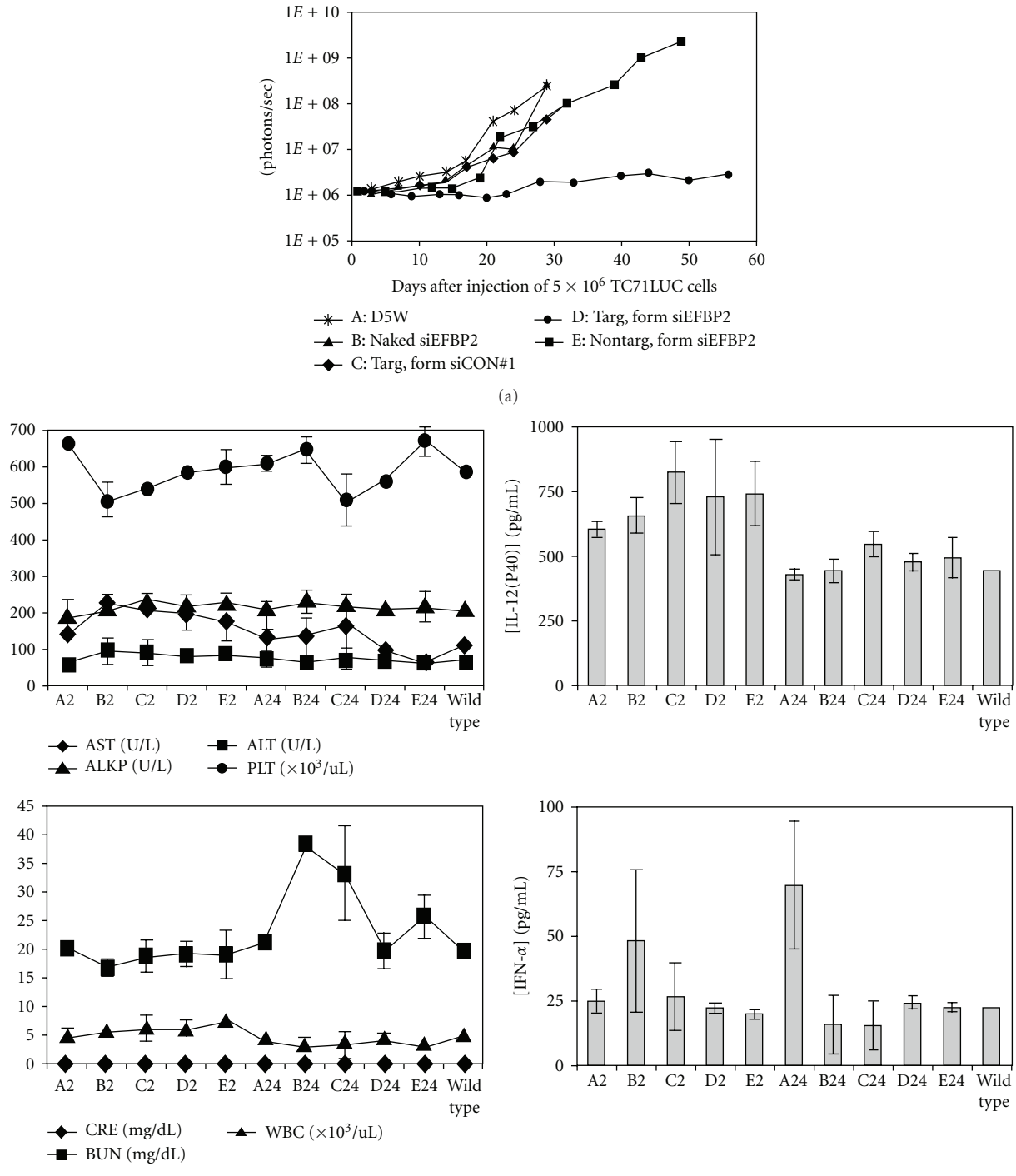


Figure 9: RONDEL-based nanoparticles containing siRNA against EWS/Fli-1 were well tolerated by mice and efficacious in a disseminated murine model of Ewing's sarcoma. (a) When administered twice weekly for four weeks, only nanoparticles containing AD-PEG-Tf and anti-EWS/Fli-1 siRNA (D) were effective in reducing tumor burden, as measured as integrated bioluminescence. Treatment group definitions: (A): vehicle control (D5W, 5 wt% dextrose in water), (B): anti-EWS-Fli1 siRNA without any other nanoparticle components (Naked siEFBP2), (C): nontargeting siRNA within Tf-targeted nanoparticles (Targ. form siCON#1), (D): anti-EWS-Fli1 siRNA within Tf-targeted nanoparticles (Targ. form siEFBP2), (E): anti-EWS-Fli1 siRNA within non-Tf-targeted nanoparticles (Nontarg. form siEFBP2). (b) When administered once to immunocompetent mice, these nanoparticles were well tolerated with respect to liver enzymes (aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALKP), platelets (PLTs), indicators of kidney function (blood urea nitrogen (BUN) and creatinine (CRE)), and cytokine response (IL-12(p40) and IFN-α). Wild type denotes untreated mice; all other results are indicated by treatment group letter (A–E), as defined above, and the time point (2 or 24, in hours) at which blood was sampled (from [23]).

Table 5: Selected physicochemical properties of siRNA-containing, RONDEL-based nanoparticles.

Property	Method	Result
Size (diameter)	Dynamic light scattering	60 to 150 nm
Zeta potential (surface charge)	Electrophoretic mobility	0 to +30 mV
Nanoparticle molar mass	Multiangle laser light scattering (MALS)	~7e7 to ~1e9 g/mol
Stoichiometry (of 70 nm nanoparticle)	Various, including MALS	CAL101: ~10,000 molecules/particle AD-PEG: ~4,000 molecules/particle AD-PEG-Tf: ~100 molecules/particle siRNA: ~2,000 molecules/particle

Table 6: Manufacturers of primary toxicology and initial clinical lots of CALAA-01 components.

Component	Toxicology lot(s)	Initial clinical lot(s)
CAL101	Cambrex	Cambrex/Agilent Technologies
AD-PEG	Sun Bio	Sun Bio
AD-PEG-Tf	Calando Pharmaceuticals	Agilent Technologies
C05C (siRNA)	Agilent Technologies	Agilent Technologies
Fill/Finish	University of Iowa Pharmaceuticals	University of Iowa Pharmaceuticals

responsible for toxicity seen as high nanoparticles doses in animals (as discussed below), this finding suggests a strategy for potentially improving the therapeutic window of these formulations via removal/reduction in the levels of free components. To further examine the *in vivo* properties of these nanoparticles, positron emission tomography (PET)/computerized tomography (CT) was employed to monitor whole-body biodistribution kinetics and tumor localization of nanoparticles while concurrently using bioluminescence imaging to measure the ability of the nanoparticles (which contained antiluciferase siRNA) to downregulate their target in luciferase-expressing tumors. Comparing Tf-containing (targeted) versus non-Tf-containing (nontargeted) analogue formulations, it was revealed that both formulations exhibited similar biodistribution and tumor localization as measured by PET; however, compartmental modeling showed that a primary advantage of targeted nanoparticles was associated with processes involved in cellular uptake by tumor cells, rather than overall tumor accumulation. Thus, as has been discussed before [40], the term "internalization ligand" might well replace "targeting ligand" to describe the role of Tf in these nanoparticles. In addition, as had been shown in the EFT work described above, only targeted nanoparticles in this study were able to achieve a significant reduction in the expression level of the gene target in tumor cells.

7. RONDEL Translation: Calando Pharmaceuticals and CALAA-01

Founded in 2005, Calando Pharmaceuticals' mission is to develop drug delivery solutions to unlock the promise of RNAi therapeutics. The company designated the nanoparticle delivery system comprised of the cyclodextrin-containing polycation and adamantane-based stabilization and internalization components as its RONDEL platform. In addition to focusing on further advancing the analytical methodologies and manufacturing capabilities (as discussed below) for these components, Calando made substantial effort in the early days to identify an initial cancer gene target suitable for eventual clinical application and to optimize an siRNA to downregulate that target. Calando selected the M2 subunit of ribonucleotide reductase (RRM2), an established anticancer target which catalyzes a rate-limiting step in the production of 2′-deoxyribonucleoside 5′-triphosphates which are necessary for DNA replication. Just as TfR overexpression across a variety of cancer types opened the possibility of RONDEL-based nanoparticles to achieve uptake in many different classes of tumors, the demonstrated sensitivity of many cell types to RRM2 inhibition maintained the potential generality of anti-RRM2 siRNA-containing nanoparticles to treat multiple types of cancers. A combination of *in silico* and *in vitro* screening of many siRNA candidates led to identification of a lead sequence (named "C05C"; also referred to as "siRRM2B+5") which was shown to be a potent downregulator of RRM2 in cancer cells of various types and species and induced a concomitant antiproliferative effect in those cells [24].

Having defined the four components of Calando's putative lead candidate formulation (CAL101, AD-PEG_{5000}, AD-PEG_{5000}-Tf, and C05C), named "CALAA-01," campaigns to scale up the manufacturing of each component were made while simultaneously expanding and improving the analytical methods employed to characterize each of them. The manufacturers of the lots of components used for IND-enabling toxicology studies and initial clinical material are listed in Table 6. Improvements in scale of up to three orders of magnitude were achieved for these molecules, and, as is customary for such projects, several challenges were identified and overcome during development. For example, in the case of CAL101, a previously unidentified impurity created in the initial β-CD functionalization step was observed that could be carried through subsequent steps; methodologies for quantifying and removing this species were developed and employed. Ultimately, sufficient quantities of all components were obtained that satisfied all acceptance criteria and were employed for subsequent testing.

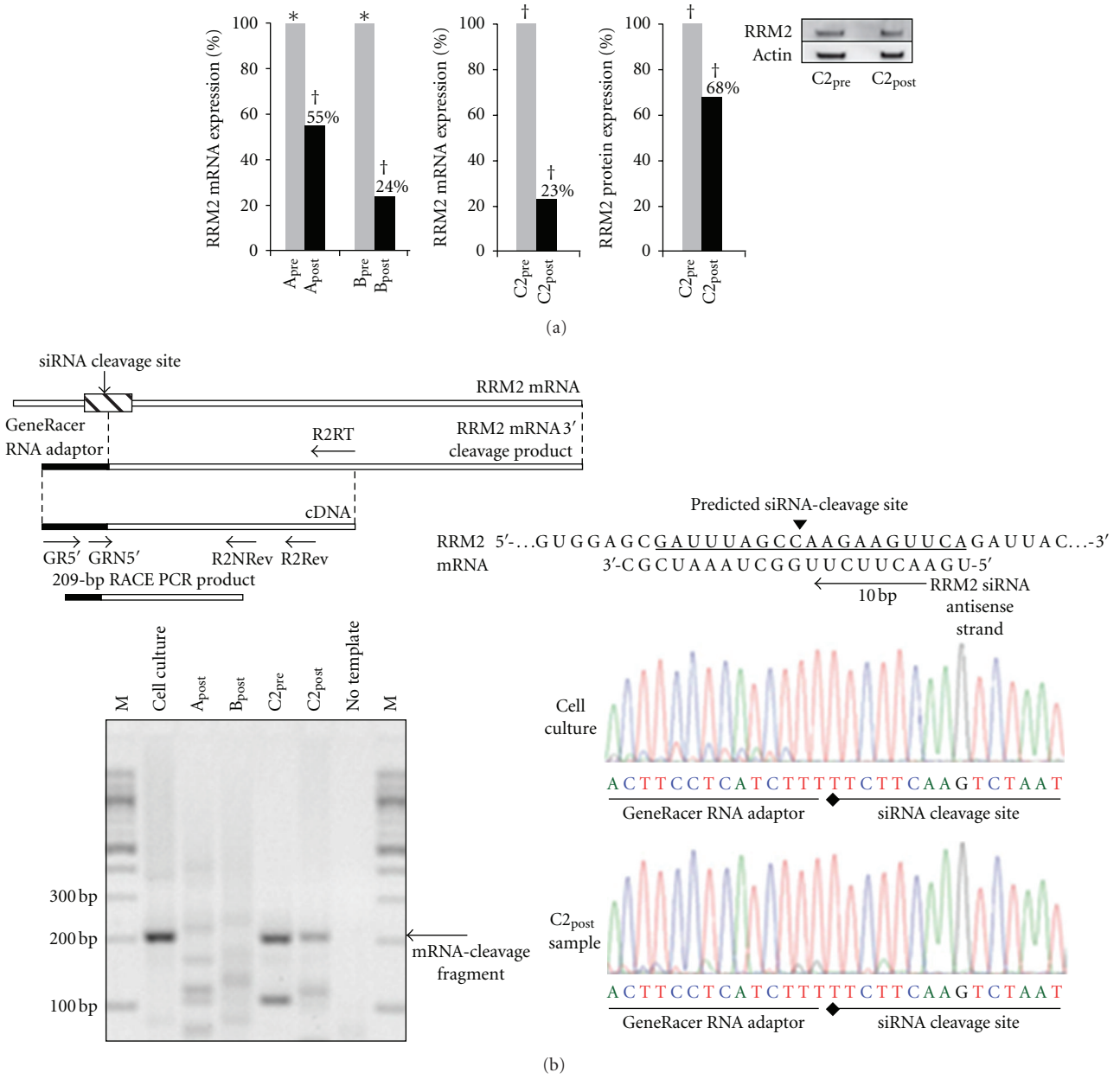


Figure 10: Interim data from a first-in-man, phase I clinical evaluation of CALAA-01 reveals RRM2 down regulation via an RNAi mechanism of action. (a) Measurements of RRM2 mRNA or protein levels in tumor biopsies from three patients (A, B, and C2) obtained before or after CALAA-01 treatment reveal significant reductions in target expression levels. (b) 5′-RLM-RACE analysis of RNA from one patient (C2) reveals evidence of the precise RRM2 mRNA cleavage product expected from RNAi-mediated down-regulation from the C05C siRNA contained within CALAA-01. This was the first such evidence of the RNAi mechanism of action in humans of any kind (from [26]).

Preclinical safety and efficacy testing of CALAA-01 were performed across a number of species and tumor types, respectively. A dose-range-finding study in non human primates [25] provided an early glimpse of the safety profile at each of three different dose levels (3, 9, and 27 mg/kg with respect to C05C). Several key findings were made in this study, including (i) the nature of toxicity at the highest dose levels (elevations in blood urea nitrogen and creatinine, as well as mild transient elevations in transaminase levels, indicative of kidney and liver effects, resp.), (ii) induction of mild levels of antinanoparticle (specifically, anti-Tf) antibodies that did not affect pharmacokinetics, (iii) elevation in IL-6 at the highest (27 mg/kg) dose level, and (iv) identification of relatively fast nanoparticle clearance from circulation ($t_{1/2}$ < 30 min). Importantly, the overall safety profile indicated good tolerability at the 3 and 9 mg/kg dose levels, in the range for which antitumor effects had been observed. Additional (unpublished), more comprehensive

toxicology and safety-pharmacology studies were performed in four species (mouse, rat, dog, and nonhuman primate) which provided a foundation for an initial clinical dose level and anticipated toxicities. In terms of efficacy, a twice-weekly dosing regimen of CALAA-01 yielded a significant reduction in tumor burden in mouse subcutaneous tumor models, including liver and melanoma [42], at dose levels in the range of 2.5–10 mg/kg.

CALAA-01 preclinical evaluation culminated in the submission of an Investigational New Drug (IND) application which received approval in April 2008. Shortly thereafter, a first-in-humans phase I investigation of CALAA-01 in patients having solid tumors was initiated. Patients who were refractory to standard-of-care treatment received four twice-weekly infusions (days 1, 3, 8, and 10) during a 21-day cycle over which numerous safety evaluations were made. CT assessments of tumor burden were performed, and PET assessment of tumor metabolism was also made. For volunteers willing to provide biopsies, assessments of RRM2 levels and investigation of the RNAi mechanism of action were also performed. At the time of this writing, a phase Ib study remains open, but interim clinical data have been published [26, 27]. Several dose level escalations spanning an order of magnitude (3, 9, 18, 24, and 30 mg/m^2) have been tolerated, and key observations of RRM2 downregulation have been made in multiple patients. Pharmacokinetics indicate relatively fast clearance, consistent with preclinical findings, and some transient elevations in cytokines (IL-6, IL-10, and TNF-α) were seen. Importantly, the first evidence of the RNAi mechanism in humans (for any siRNA) and the first evidence of dose-dependent tumor accumulation of nanoparticles administered systemically in humans (for any nanoparticles) have been observed in this study (Figure 10). Taken together, these early indications of safety and efficacy suggest potential for CALAA-01 and the RONDEL platform for continued clinical investigation.

8. RONDEL and CALAA-01: Future Directions

Supported by over a decade of research and development, there are many ongoing and future directions for CALAA-01 and the RONDEL delivery platform. Certainly completion of the CALAA-01 phase I clinical trial, including establishment of a maximum tolerated dose (MTD) and recommended dose level for subsequent trials, is a near-term priority. Thorough evaluation of all of the safety and preliminary efficacy indications from this study will greatly inform the design of a phase II investigation of CALAA-01. Beyond CALAA-01, investigation of additional therapeutic candidates employing the RONDEL system, such as those targeting hypoxia-inducible factor-2α (HIF-2α), has been undertaken. The relatively fast clearance of these nanoparticles that has been observed, as has been described above, suggests that strategies to prolong circulation in an effort to enhance tumor accumulation may warrant investigation. The transient elevations in some cytokine levels seen in interim CALAA-01 clinical data imply that exploration of chemical modifications to the siRNA payload may yield nucleic acids that enhance the nanoparticles therapeutic index. With encouraging interim clinical data in hand, avenues for continued development and improvement of nanoparticles identified, and the emergence of alternative siRNA-containing nanoparticles in the clinic from which all in this field will learn, the future for siRNA-containing nanoparticles based on cyclodextrin-containing polycations appears bright.

9. Conclusions

CDP-based nanoparticles have made the transition from the laboratory to the clinic within the last several years. Two technology platforms have been developed, Cyclosert for small molecule delivery and RONDEL for nucleic acid delivery. Both programs have produced a clinical candidate for oncology, CRLX101 (formerly IT-101), a camptothecin analog, and CALAA-01, an siRNA therapeutic targeting RRM2. While clinical development is still in the early phases, proof of concept was achieved for both technologies. Clinical development is ongoing and it will be interesting to see what patient benefits these innovative drugs can provide.

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Research Article

A Versatile Polymer Micelle Drug Delivery System for Encapsulation and *In Vivo* Stabilization of Hydrophobic Anticancer Drugs

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Chemotherapeutic drugs are widely used for the treatment of cancer; however, use of these drugs is often associated with patient toxicity and poor tumor delivery. Micellar drug carriers offer a promising approach for formulating and achieving improved delivery of hydrophobic chemotherapeutic drugs; however, conventional micelles do not have long-term stability in complex biological environments such as plasma. To address this problem, a novel triblock copolymer has been developed to encapsulate several different hydrophobic drugs into stable polymer micelles. These micelles have been engineered to be stable at low concentrations even in complex biological fluids, and to release cargo in response to low pH environments, such as in the tumor microenvironment or in tumor cell endosomes. The particle sizes of drugs encapsulated ranged between 30–80 nm, with no relationship to the hydrophobicity of the drug. Stabilization of the micelles below the critical micelle concentration was demonstrated using a pH-reversible crosslinking mechanism, with proof-of-concept demonstrated in both *in vitro* and *in vivo* models. Described herein is polymer micelle drug delivery system that enables encapsulation and stabilization of a wide variety of chemotherapeutic drugs in a single platform.

1. Introduction

It was estimated that there were 1,500,000 new cancer cases and approximately 560,000 deaths from cancer in 2010 [1]. The use of chemotherapy has dramatically improved the survival rate of patients for the last several decades; however, stand-alone chemotherapy drugs suffer from numerous problems including rapid *in vivo* metabolism and/or excretion, inability to access and penetrate cancer cells, and nonspecific uptake by healthy cells and tissue. Often, a large percentage of cytotoxic drug administered to the patient does not reach the tumor environment but rather is distributed throughout the body, resulting in the many toxic effects associated with chemotherapy and a narrowing of the drug's therapeutic window. Polymer micelles offer a promising approach to achieving these goals due to their inherent ability to overcome multiple biological barriers, such as avoidance of the reticuloendothelial system (RES) [2]. Due to their unique size range (20–150 nm), micelles are able to avoid renal clearance (typically less than 20 nm) and uptake by the liver and spleen (particles greater than 150 nm). These micelles can also preferentially accumulate in solid tumors via the enhanced permeation and retention (EPR) effect [3, 4]. The EPR effect is a consequence of the disorganized nature of the tumor vasculature, which results in increased permeability of polymer therapeutics and drug retention at the tumor site.

When considering the design of a nanocarrier, several important factors should be addressed. An ideal delivery system should be composed of biocompatible and biodegradable materials, reproducibly assemble into the desired size range, encapsulate a wide range of drugs and drug classes,

maintain particle size in biological media, have the ability to attach cell-specific targeting groups, and release the therapeutic at the site of disease. Polymer micelles have received much attention over the past thirty years as drug delivery vehicle [5–11]. In traditional micelle systems, however, there are no mechanisms in place to keep the micelle intact when it is diluted in the bloodstream, where it is below the critical micelle concentration and interacts with surfactant proteins within the blood. Thus, stability of nano-carriers in biological media remains an issue that needs to be addressed [12]. Some have utilized the approach of chemically conjugating the active drug to a polymer to potentially improve stability. However, this "prodrug" approach is dependent on enzymatic or chemical cleavage of the bond to release the active drug [13–15]. In an attempt to add stability to the micelle, various types of micelles have been developed whereby either the core or shell of the micelle has incorporated crosslinking chemistries, thereby imparting stability at low micelle concentrations [16–22]. However, in many cases, crosslinking is achieved utilizing covalent bonding within the micelle, which does not lend itself to tunable drug release. In addition, in some crosslinked micelles, the crosslinks are physically located with the drug in the core of the micelle, which may interfere with pharmaceutical drug action or drug release from the micelle.

This paper describes a polymer micelle drug delivery system (IVECT) that has effectively addressed the limitations of traditional polymer micelles, by forming micelles that are stable in biological environments. The IVECT triblock copolymer consists of poly(ethylene glycol)-*b*-poly(aspartic acid)-*b*-poly(D-leucine-*co*-tyrosine). The leucine/tyrosine core unit in this polymer is able to encapsulate a wide variety of hydrophobic molecules, which is enhanced by the use of both D and L stereoisomers. The poly(aspartic acid) block was designed to participate in a metal-acetate crosslinking reaction that effectively stabilized drugs inside the core of the micelle and also mediates pH-dependent release of the drug. In this paper, a polymer micelle is described that is composed of biocompatible materials, has the versatility to encapsulate a wide range of therapeutic payloads, is stable to dilution within the blood stream, and has a tunable, highly sensitive, and reversible stabilization mechanism. Data are presented whereby several different hydrophobic molecules are encapsulated and stabilized by crosslinking using a single polymer and without physical manipulation of the drug.

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals were obtained from Aldrich or Fisher unless otherwise specified. N_3-PEG12k-NH-BOC was prepared as described previously [23]. N-carboxy anhydrides (NCAs) were prepared according to previously published procedures. [24, 25]. N-methylpyrrolidone (NMP) was distilled prior to use. BB4007431 and NX-8 were provided by Novartis. Daunorubicin and doxorubicin were obtained from LGM Pharma (Boca Raton, FL). All other drugs were obtained from Yingxuan Pharmaceuticals (Shanghai, China).

2.2. Synthesis of Triblock Copolymer. N_3-PEG12K-NH-Boc (150 g, 12.5 mmol) was dissolved into 1 L of CH_2Cl_2/DFA (70/30) and was allowed to stir at room temperature overnight. The product was precipitated twice in diethyl ether and was recovered as a white powder (yield ~ 90%). ^{1}H NMR (d_6-DMSO) 7.77 (3 H), 5.97 (1 H), 3.83–3.21 (1050 H), 2.98 (2 H) ppm.

N_3-PEG12K-NH_3/DFA (95 g, 7.92 mmol) was weighed into an oven-dried, 2 L-round-bottom flask and was left under vacuum for three hours before adding the NCA. Asp(OBu) NCA (17.04 g, 79.2 mmol) was added to the flask, and the flask was evacuated under reduced pressure and subsequently backfilled with nitrogen gas. Dry NMP (560 mL) was introduced by cannula, and the solution was heated to 60°C. The reaction mixture was allowed to stir for 24 hours at 60°C under nitrogen gas. Then, D-Leu NCA (24.88 g, 158 mmol) and Tyr (OBzl) NCA (47.08 g, 158 mmol) were dissolved under nitrogen gas into 360 mL of NMP into an oven-dried, round-bottom flask, and the mixture was subsequently added to the polymerization reaction via a syringe. The solution was allowed to stir at 60°C for another three days at which point the reaction was complete (as determined by HPLC). The solution was cooled to room temperature, and diisopropylethylamine (DIPEA) (10 mL), dimethylaminopyridine (DMAP) (100 mg), and acetic anhydride (10 mL) were added. Stirring was continued for 1 hour at room temperature. The polymer was precipitated into diethyl ether (10 L) and isolated by filtration. The solid was redissolved in dichloromethane (500 mL) and precipitated into diethyl ether (10 L). The product was isolated by filtration and dried *in vacuo* to give the block copolymer as an off-white powder (134.6 g, yield = 73%). ^{1}H NMR (d_6-DMSO) δ 8.43–7.62 (50 H), 7.35 (100 H), 7.1 (40 H), 6.82 (40 H), 4.96 (40 H), 4.63–3.99 (50 H), 3.74–3.2 (1500 H), 3.06–2.6 (60 H), 1.36 (90 H), 1.27–0.47 (180).

N_3-PEG12K-*b*-poly(Asp(OBu)$_{10}$)-*b*-poly(Tyr(OBzl)$_{20}$-*co*-D-Leu$_{20}$)-Ac (134.6 g, 6.4 mmol) was dissolved into 1 L of a solution of pentamethylbenzene (PMB, 0.5 M) in trifluoroacetic acid (TFA). The reaction was allowed to stir for five hours at room temperature. The solution was precipitated into a 10-fold excess of diethyl ether, and the solid was recovered by filtration. The polymer was redissolved into 800 mL of dichloromethane and precipitated into diethyl ether. An off-white polymer was obtained after drying the product overnight *in vacuo* (111.8 g, yield = 93%). ^{1}H NMR (d_6-DMSO) δ 12.2 (10 H), 9.1 (10 H), 8.51–7.71 (50 H), 6.96 (40 H), 6.59 (40 H), 4.69–3.96 (60 H), 3.81–3.25 (1500 H), 3.06–2.65 (60 H), 1.0–0.43 (180). ^{1}H NMR (d_6-DMSO) δ 171.9, 171, 170.5, 170.3, 155.9, 130.6, 129.6, 127.9, 115.3, 114.3, 70.7, 69.8, 54.5, 51.5, 50, 49.8, 49.4, 36.9, 36, 24.3, 23.3, 22.3, 21.2. IR (ATR) 3290, 2882, 1733, 1658, 1342, 1102, 962 cm^{-1}. The final composition of the polymer is N_3-PEG12K-*b*-poly(Asp)$_{10}$-*b*-poly(Tyr$_{20}$-*co*-D-Leu$_{20}$)-Ac, which is also referred to as poly(ethylene glycol)-*b*-poly (aspartic acid)-*b*-poly(D-leucine-*co*-tyrosine).

2.3. Micelle Production. All formulations were prepared using oil-in-water emulsion techniques involving dissolving

the polymer in water and the drug in an organic solvent. An exemplary formulation technique for daunorubicin follows. The IVECT triblock copolymer (3 g) was dissolved in wa-ter (500 mL). Daunorubicin (301 mg) was dissolved in dichloromethane (48 mL) and methanol (12 mL). Just prior to use, triethylamine (0.28 mL) was added to the organic solution to complete the dissolution of the daunorubicin. The aqueous solution was mixed with a Silverson LRT-4 shear mixer (fine emulsor screen, 10,000 RPM). Daunorubicin was added to the mixed solution in a single portion over ~10 s. The solution was mixed for an additional minute and then stirred at room temperature overnight. The resulting solution was then filtered through a 0.22 μm PES filter (Millipore Stericup). Iron (II) chloride solution was added to the concentrated micelle solution at a concentration of 10 mM, and the pH was adjusted to 8.0 and stirred overnight. This solution was frozen on a shell freezer at -40°C and then lyophilized on a Labconco 6 L Plus manifold lyophilization system operating at a pressure of 0.050 Torr and a collector temperature of -85°C. After 48 h, crosslinked, daunorubicin-loaded micelles were recovered as a purple powder (3.22 g, 93% yield).

2.4. Drug Weight Loading by HPLC. The mass percentage of active drug within the formulation was determined by HPLC. An exemplary procedure for daunorubicin follows. The daunorubicin-loaded micelle was analyzed by a Waters Alliance separations module (W2695) equipped with Waters Novapak C18, 4 μm column (no. WAT086344) coupled with a Waters Photodiode Array Detector (W2998). Daunorubicin was detected at an absorbance of 480 nm. Mobile phase consisted of a 10 : 70 : 20 ratio of methanol : 10 mM phosphate buffer pH 2.0 : acetonitrile over a 10-minute gradient. Known standards of free daunorubicin were used to determine the percentage by weight of daunorubicin in the formulation (wt/wt%).

2.5. Particle Size Analysis. Particle sizes were determined using dynamic light scattering on a Wyatt DynaPro (Santa Barbara, CA). Following lyophilization, micelles were dissolved at 1 mg/mL in 150 mM NaCl and were centrifuged at 2,000 RPM prior to analysis to remove dust.

2.6. Encapsulation, Crosslinking, and pH-Dependent Release Dialysis. To test drug encapsulation, the uncrosslinked formulation was dissolved at a concentration of 20 mg/mL in water, which is above the critical micelle concentration of the polymer. Two milliliters were dialyzed in a 3500 MWCO dialysis bag in a volume of 300 mL of 10 mM phosphate buffer, pH 8.0. After dialysis for six hours, the pre- and postdialysis samples from inside the bag were quantified for drug concentration by HPLC. Encapsulation retention was calculated by dividing the postdrug concentration by the preconcentration.

To test crosslinking, the crosslinked formulation was dissolved in water at a concentration of 0.2 mg/mL, which is below the critical micelle concentration. Three milliliters were dialyzed in a 3500 MWCO dialysis bag in a volume of 300 mL of 10 mM phosphate buffer pH 8. After dialysis for six hours, the pre- and postdialysis samples from inside the bag were quantified for drug concentration by HPLC. Crosslinking retention was calculated by dividing the postdrug concentration by the preconcentration. For pH-dependent release, samples were treated the same as for crosslinking dialysis except for dialysis in 10 mM phosphate buffer pH 3, 4, 5, 6, 7, 7.4, or 8.

2.7. In Vivo Pharmacokinetic Studies. Female Sprague-Dawley rats weighing about 220 g with jugular vein catheters were obtained from Harlan. Rats were randomly divided into groups of four and were given a single injection of free drug, uncrosslinked drug loaded micelles, or crosslinked, drug loaded micelles dissolved in 150 mM NaCl. Daunorubicin micelles were injected at 10 mg/kg daunorubicin-equivalent dosing, and BB4007431 micelles were injected through the catheter at 25 mg/kg BB4007431 drug-equivalent dosing. Free BB4007431 was dissolved in 0.33 M lactic acid/1.67% dextrose and then diluted in 5% dextrose in water for injection. About 0.25 mL of blood was collected through the catheter at 1, 5, 15 min, 1 h, 4 h, 8 h, and 24 h. Samples were centrifuged at 2000 RPM for 5 minutes to separate plasma. Plasma was then diluted 1 : 4 in cold 0.1% phosphoric acid in methanol with an appropriate internal standard, vortexed for 10 minutes, and centrifuged for 13,000 RPM for 10 minutes. The supernatant was then analyzed by HPLC to determine the drug concentration for each sample. Plasma concentrations were plotted in Microsoft Excel to determine AUC values. Animals were maintained in accordance with *The Public Health Service Policy on Humane Care and Use of Laboratory Animals,* and *the Institutional Animal Care and Use Committee's (IACUC) Principles and Procedures of Animal Care and Use.*

3. Results

The IVECT triblock copolymer consists of poly(ethylene glycol)-*b*-poly(aspartic acid)-*b*-poly(D-leucine-*co*-tyrosine), in which each segment is biodegradable or biocompatible and plays a very important role (Figure 1). Hydrophobic drugs that are loaded into the micelle reside in the encapsulation block (yellow), forming the core of the micelle. The poly(aspartic acid) middle block (green) is the crosslinking block that stabilizes the micelle. In contrast to crosslinking in the core or periphery of the micelle, Intezyne has developed pH-reversible crosslinking technology in the middle block of the triblock copolymer. Crosslinking of this middle layer of the micelle is advantageous since it does not interfere with the core region, which is where the drug resides. The chemistry utilized to crosslink the polymer chains together, and thus stabilizes the micelle, is based on metal acetate chemistry (Figure 2). It is well known that a number of metal ions can interact with carboxylic acids to form metal-acetate bonds [26]. It is also understood that these ligation events form rapidly when the carboxylic acid is in the carboxylate form (e.g., high pH, pH ~ 7-8) yet only weakly interact when the carboxylic acids are fully protonated (e.g., low

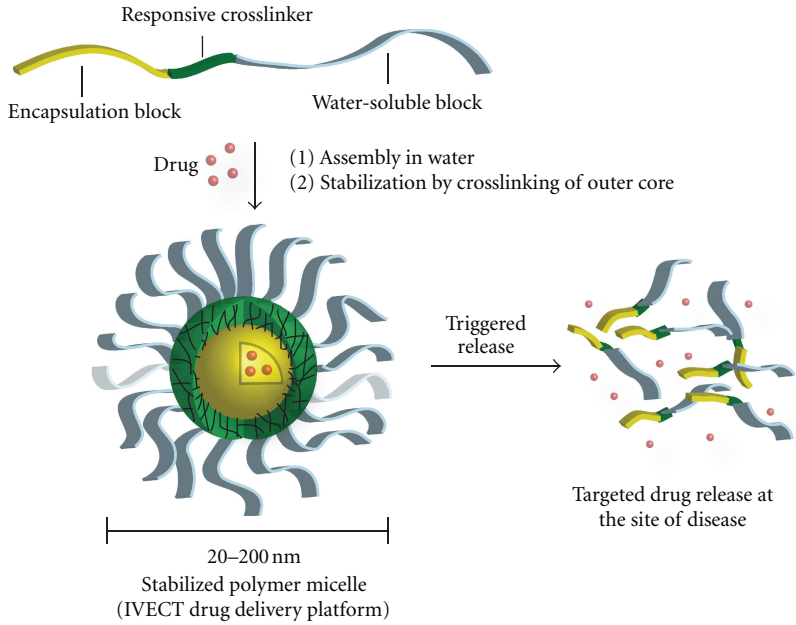


Figure 1: The IVECT polymer micelle. Drugs are loaded into the core hydrophobic block (yellow). The crosslinking block (green) provides stability to the micelle by forming pH-reversible metal-acetate bonds that allow for triggered drug release near the tumor. The PEG block (gray) gives the micelle aqueous solubility and stealth properties *in vivo*.

pH, pH 4-5), therefore allowing release of the drug in low-pH environments, such as regions surrounding the tumor, and the endosomes of tumor cells following endocytosis of micelles. The poly(ethylene glycol) block (Figure 1, shown in gray) allows for water solubility and provides "stealth" properties to the micelle in order to avoid protein opsonization and the reticuloendothelial system [2].

As an initial study, the triblock copolymer was used to encapsulate several different small molecule drugs with varying hydrophobicities. A trend was discovered such that the ability of the triblock to encapsulate a drug was dependent on the drug's Log *P* value. Effective encapsulation was achieved with molecules having a Log $P > 1.4$ (Figure 3). The weight loadings of the formulations ranged between 1 and 20%. Molecules that were encapsulated were subsequently crosslinked by the addition of iron chloride. The addition of iron chloride to the micelle did not affect the drug and did not result in generation of polymer-drug conjugates. To test stability of the crosslinked micelle, the *in vitro* stability of the micelle below the CMC was determined using a dialysis assay. In contrast to the encapsulation retention, there was no clear correlation between the Log *P* value and crosslinking retention (Table 1). The particle sizes of crosslinked micelles, as determined by dynamic light scattering, also did not seem related to the Log *P* value. These results demonstrate that the hydrophobicity of the drug influences its ability to be encapsulated within the micelle, but does not influence crosslinking retention or particle size.

To determine whether crosslinked micelles exhibited pH-dependent release, different micelles were dialyzed at concentrations below the CMC in 10 mM phosphate buffer of different pHs. Crosslinked micelles containing BB4007431 demonstrated pH-dependent release of the drug, with increased retention of the drug within the micelle at pH 8, and near total release of the drug after incubation at pH 3 (Figure 4(a)). In contrast, uncrosslinked micelles containing BB4007431 showed nearly complete release of the drug at all pHs, reflecting the instability of the uncrosslinked micelle. To assess the effect of salt in the stability of the micelle, crosslinked BB4007431 was diluted below the CMC and dialyzed in 10 mM phosphate buffer or phosphate-buffered saline (PBS) at different pHs (Figure 4(b)). This experiment

Table 1: Drug formulation properties. The encapsulation retention percentage, crosslinking retention percentage, and particle sizes are shown for eleven compounds tested for loading within the polymer micelle.

Drug	Log *P*	Encapsulation retention (%)	Crosslinking retention (%)	Particle size (nm)
5-Fluorouracil	−0.58	0	NA	ND
Caffeine	−0.24	0	NA	ND
Melphalan	−0.22	0	NA	ND
Gemcitabine	0.14	0	NA	ND
Etoposide	0.73	12	NA	ND
Doxorubicin	1.41	80	63	30
Daunorubicin	1.68	85	78	30
BB4007431	1.94	79	90	55
Paclitaxel	3.2	93	60	36
NX-8	4.18	86	52	86
Vinorelbine	4.39	87	37	47

NA: not applicable, ND: not determined.

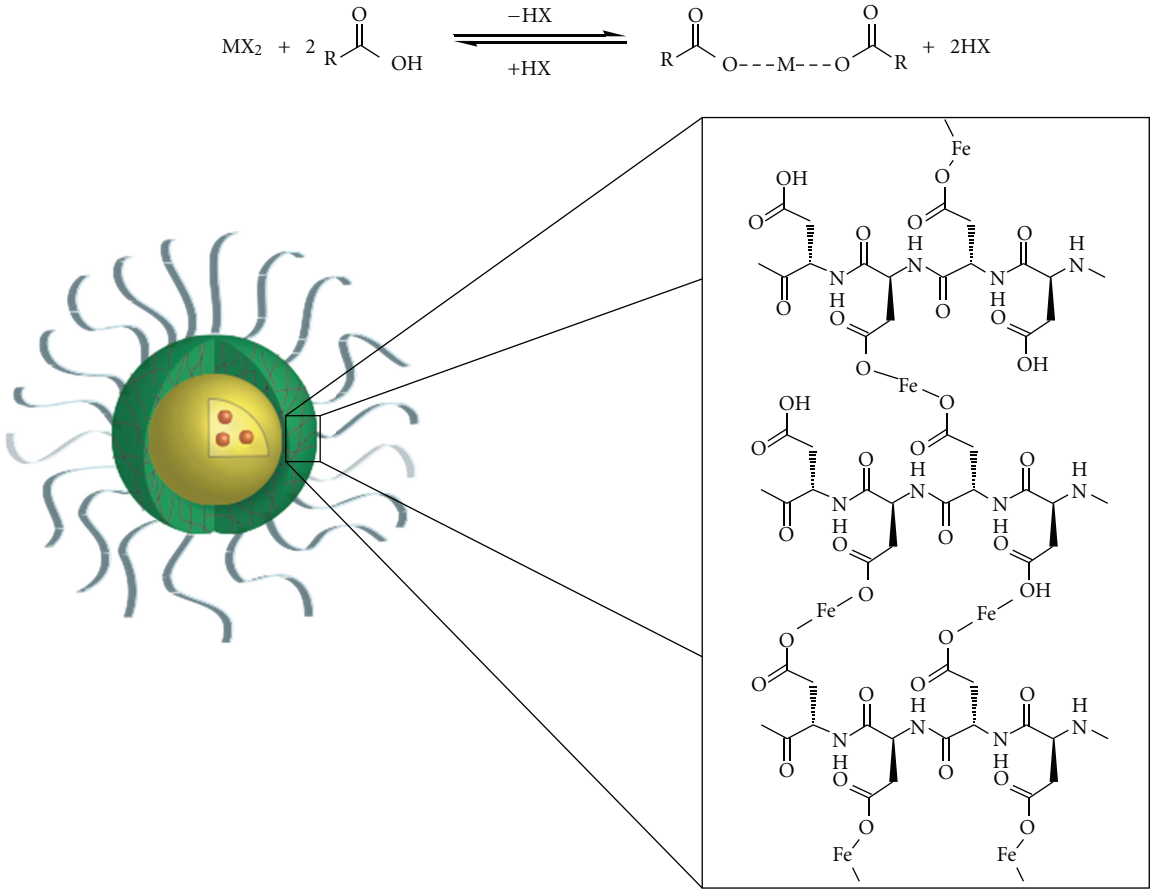


Figure 2: Metal-acetate crosslinking chemistry for stabilization of polymer micelles. While the drug is localized in the core block, the poly(aspartic acid) block of the middle block reacts with metals to form metal acetate bonds. Bonds are formed at high pH and are dissociated at low pH. M represents metal, and X represents a halogen.

showed that salt did destabilize the crosslinked micelle to some degree, but a pH-dependent release was still exhibited.

In order to test the stability of the micelle *in vivo*, a crosslinked, daunorubicin-loaded micelle was assessed in a pharmacokinetic study. Rats were intravenously injected with 10 mg/kg of free daunorubicin, uncrosslinked daunorubicin micelle, or crosslinked daunorubicin micelle, and the concentration of daunorubicin in plasma was determined over the course of twenty four hours (Figure 5). Results demonstrated that the crosslinked daunorubicin micelle exhibited 90-fold increase in plasma in AUC compared to free daunorubicin and 78-fold increase in AUC compared to uncrosslinked daunorubicin. Crosslinked daunorubicin also exhibited a 46-fold higher C_{max} than free daunorubicin and a 59-fold increase compared to uncrosslinked micelle. These data demonstrate significantly higher *in vivo* micelle stability with the crosslinked daunorubicin micelle compared to the free drug. A similar study was repeated with a crosslinked formulation of compound BB4007431. Rats injected with crosslinked BB4007431 micelle displayed a vastly superior increase in C_{max} (20-fold) and AUC (202.4-fold) compared to free drug (Figure 6). Similar increases in stability were also obtained with crosslinked doxorubicin and paclitaxel-loaded micelles (data not shown), demonstrating the wide applicability of this crosslinking technology to provide increased drug stability *in vivo*.

4. Discussion

Improving stability of therapeutic molecules is a well-established aim in the field of drug delivery. An ideal drug-loaded nanoparticle would be stable to dilution in biological media, possess stealth-like properties to avoid uptake by the RES, and release the drug only in the area of diseased tissue. The data presented in this paper describe a versatile polymer micelle drug delivery system that has been engineered to efficiently encapsulate a wide variety of hydrophobic drugs. In addition, the stabilization technology built-in to the micelle is dependent on pH, such that the micelle is stable at physiological pH, and unstable at low pH, thus providing a mechanism to release the drug in the tumor microenvironment or in endosomes, which are both slightly acidic environments.

A vast number of drugs exist today that possess potent anticancer activity; however, many of them are unable to be utilized in the clinic due to their inability to be dissolved

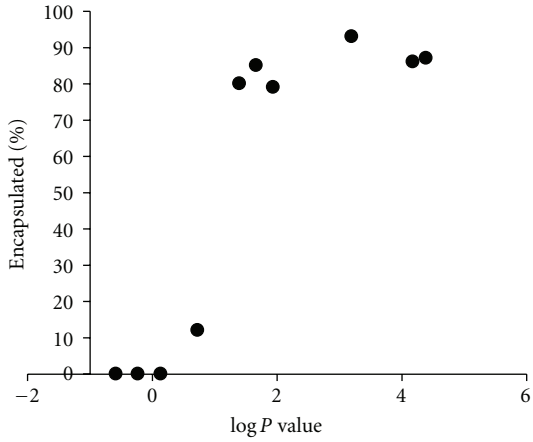


Figure 3: Encapsulation retention of drugs within the micelle is correlated to Log *P* value. The encapsulation retention of the drug, based on an *in vitro* dialysis assay, is plotted compared to its Log *P* value.

in aqueous solutions [27]. Some hydrophobic drugs can be solubilized with excipients; however, such vehicles have been shown to cause toxicity to the patient [28]. The core block of the triblock copolymer (poly(D-leucine-*co*-tyrosine)) was rationally designed and chosen to encapsulate hydrophobic molecules. A key factor leading to the versatility arises from the use of both D and L stereoisomers of amino acids in the core block, which disrupts the secondary structure of the polypeptide. Replacing the rod-like helical nature of the polypeptide with the flexibility of a random coil allows for significant increases in drug loading efficiency. The ability of drugs to be encapsulated within the triblock copolymer was related to its Log *P* value, such that only hydrophobic drugs could be encapsulated. This result is logical as hydrophilic molecules would prefer to associate with the hydrophilic part of the polymer versus the hydrophobic core, leading to inefficient drug encapsulation.

Crosslinking was performed using metal acetate chemistry, specifically, iron (II) chloride. The crosslinking dialysis assay determined that 40–90% of the drug remained in the crosslinked micelle after six hours. Typically, 10% of the drug or less was retained in uncrosslinked micelles examined using the same crosslinking dialysis assay. Although there was a correlation between Log *P* and encapsulation ability, there was no clear correlation between Log *P* and the crosslinking retention or the particle size. Therefore, it is hypothesized that while hydrophobicity is a strong predictor of success for encapsulation, other variables such as chemical functionality and drug crystallinity play a significant role in micelle size and crosslinking efficiency.

While stability is important, equally important is the ability to release the drug in a controlled fashion at the site of disease. *In vitro* release assays demonstrated progressive release of drug from the core of the micelle as the pH decreased, which has physiological relevance for delivering drugs to tumors. While passive targeting of nanoparticles within tumor tissue is accomplished by the EPR effect, an additional

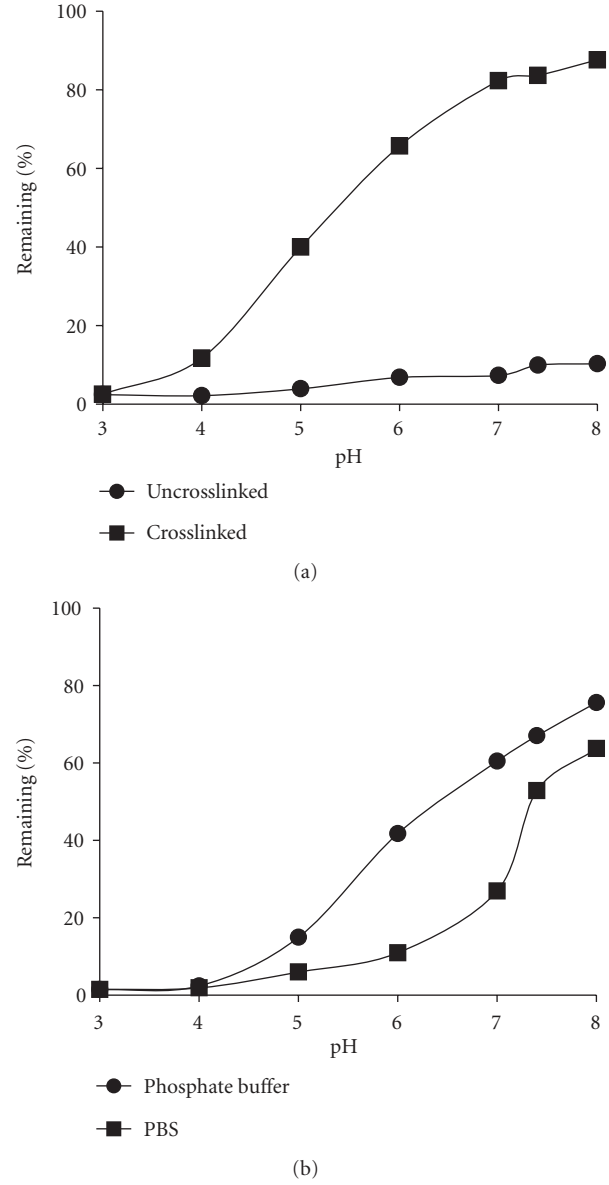
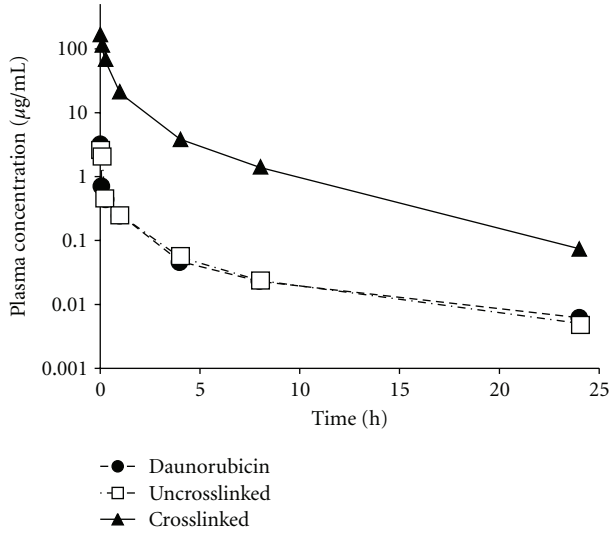


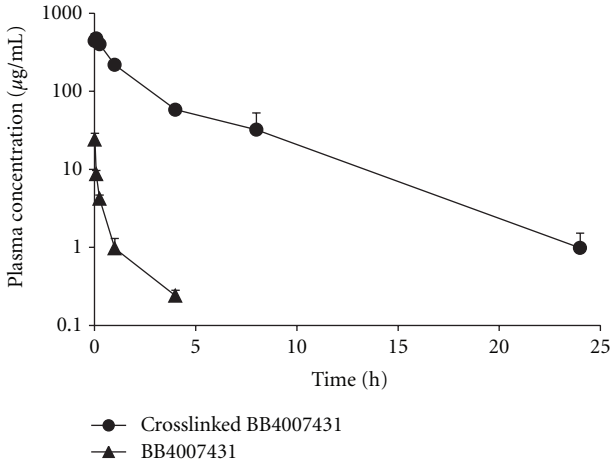
Figure 4: pH-dependent release of drug-loaded micelles. (a) Crosslinked and uncrosslinked BB4007431 micelles were diluted below the CMC and dialyzed for 6 hours in 10 mM phosphate buffer at different pHs. The amount of drug retained before and after dialysis was quantified by HPLC. (b) Crosslinked BB4007431 micelles were diluted below the CMC and dialyzed for 6 hours in either 10 mM phosphate buffer, or PBS, at different pHs. Drug content remaining was quantified by HPLC as above.

layer of targeting is possible by employing active targeting strategies, such as decorating the surface of nanoparticles with targeting ligands [29–33]. It is logical to conclude, however, that the ability to target a nanoparticle to tumors is dependent on the stability of the nanoparticle *in vivo*. In pharmacokinetic experiments, superior AUC and C_{max} were obtained with several crosslinked micelles, including daunorubicin and BB4007431, compared to their free drug or uncrosslinked micelle counterparts. These data suggest that



Administration	AUC (μg-h/mL)	C_{max} (μg/mL)
Crosslinked	116.39	153.82
Uncrosslinked	1.48	2.61
Daunorubicin	1.3	3.29

Figure 5: Pharmacokinetics of daunorubicin-loaded micelles in rats. Sprague-Dawley rats were given a single intravenous administration of crosslinked daunorubicin micelle, uncrosslinked daunorubicin micelle, or free daunorubicin at a 10 mg/kg dose. Plasma was analyzed for daunorubicin concentration at various timepoints. The table depicts the area under curve (AUC) and C_{max} values for each test article.



Administration	AUC (μg-h/mL)	C_{max} (μg/mL)
Crosslinked BB4007431	1196.38	476.46
BB4007431	5.91	24

Figure 6: Pharmacokinetics of crosslinked BB4007431 micelles in rats. Sprague-Dawley rats were given a single intravenous administration of crosslinked BB4007431 micelle, or free BB4007431 at a 25 mg/kg dose. Plasma was analyzed for BB4007431 concentration at various timepoints. The table depicts the area under curve (AUC) and C_{max} values for each test article.

higher tumor accumulation, and correspondingly improved antitumor efficacy, would be achieved following administration of crosslinked micelle compared to free drug in mouse biodistribution experiments. This would primarily be due to passive targeting by the EPR effect although active targeting has the potential to even further improve delivery of crosslinked micelles.

Polymer micelles hold great promise as drug delivery agents. Indeed, many polymer micelles carrying chemotherapeutic drugs are currently in clinical trials [6, 34]. The utility of a single platform to encapsulate and systemically deliver hydrophobic cancer drugs allows for faster drug screening and facilitated manufacturing processes. In addition to improving the delivery of current anticancer drugs, the polymer micelle system presented herein holds promise for the development of potent, but insoluble novel anticancer drugs. It is envisioned that this new technology will ultimately provide superior treatment options for patients with cancer.

5. Conclusions

A polymer micelle drug delivery system was developed that demonstrated encapsulation and stabilization of a wide variety of hydrophobic anticancer drugs. Drug release from stabilized micelles was determined to be pH dependent *in vitro*. *In vivo* pharmacokinetic studies validated increased stability of crosslinked micelles in biological media and demonstrated improved AUC and C_{max} compared to uncrosslinked micelles or free drug. These data demonstrate the utility and versatility of a single platform to enable delivery of hydrophobic anticancer drugs to solid tumors.

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Research Article

A New Application of Lipid Nanoemulsions as Coating Agent, Providing Zero-Order Hydrophilic Drug Release from Tablets

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The objective of the present investigation was to evaluate potential of nanoemulsions as a coating material for the tablets. The nanoemulsion of size less than 100 nm was prepared using a simple and low-energy spontaneous emulsification method. Conventional tablets containing theophylline as a model hydrophilic drug were prepared. The theophylline tablets were coated with the nanoemulsion using a fluid bed coater. The effect of different levels of the nanoemulsion coating on the theophylline release was evaluated. The theophylline tablets containing different levels of the nanoemulsion coating could be successfully prepared. Interestingly, the coating of tablet with the nanoemulsion resulted in zero-order release of theophylline from the tablets. The noncoated theophylline tablets release the entire drug in less than 2 minutes, whereas nanoemulsion coating delayed the release of theophylline from tablets. This investigation establishes the proof of concept for the potential of nanoemulsions as a coating material for tablets.

1. Introduction

The design and development of simple systems with the aim of delivery and controlled release of hydrophilic drugs administered through oral route are still a challenge. Compared to classical dosage forms, the goals for the development of such systems include maintaining of blood levels for the drug in a therapeutic window for a desired period. Such controlled drug-delivery systems present considerable advantage over conventional dosage forms, but they involve carrying out specific and complex technologies [1–12]. The most widespread systems giving modified releases are hydrophilic matrix carriers or hydrophilic coating matrix (e.g., on tablets). Pharmaceutically available polymers such as polymethacrylates (Eudragit RS100 and Eudragit S100), ethyl cellulose (EC), and hydroxypropyl methylcellulose (HPMC), as a single or mixed composition, are largely studied and used for this purpose [13–18]. The modified drug releases are actually a combination of several physical processes including, diffusion, polymer swelling, dissolution, or erosion [19–22].

The literature generally reports investigations on the impacts of the formulation parameters—for example, coatings levels, nature of solvent, nature of polymer and plasticizer, polymer particle size, polymer weight, degree of substitution and polymer concentration [5, 16–18, 23–26], and the processing parameters—air pressure and temperature on the physicochemical properties of the coated film, that is to say, on the drug release profiles. In this context, it has been shown that the drug release is mainly related to the physical behavior of the coating materials with regards to the release media (for instance, tensile strength, contact angle, and solubility) [5, 17, 27, 28]. It is easily understandable, since the drug release, in these coated systems, arises after the drug solvation and diffusion, and thus after the gradual swelling (i) firstly of the coating polymer and (ii) secondly of the vehicle (like a tablet). Accordingly, the solvated drug is released (e.g., by diffusion) through this swollen system towards the bulk phase. It is to be noted here that the swelling kinetics of the coating polymer is of prime importance and must be fast enough to prevent the tablet disintegration during this first phase of the process.

The particular case of zero order is of real interest, since it confers to the system, the ability to deliver a drug at a constant rate. Hence, a steady amount of drug is released over time, which, on the one hand, minimizes potential peak/trough fluctuations and side effects, and on the other hand, maximizes the time for which the drug concentrations remain within the therapeutic window. With the examples of hydrophilic matrix presented above, zero-order release profiles are the direct consequences of the Fickian diffusion of the drugs through a membrane (Fick's first law).

The zero-order release can also be induced by a specific swellable polymer coating technology. The numerous studies reported on these domains are focused on the formulation and processing parameters described above, for a single polymer or blend of various polymers. However, as a constant factor, these technologies still use polymers to create such a barrier between the drug and release media. This is precisely the novelty of our approach, since herein, we propose a new method, applicable to tablets to provide zero-order drug release profiles, by using lipids instead of polymers. This paper presents tablet lipid coating, based on a specific nanotechnology (lipid nanoemulsions), followed by a study of hydrophilic drug releases (theophylline), disclosure, and modeling the release mechanisms. The idea was to coat the tablets, by a lipid species, in order to create a lipid coating or lipid adsorbed layer, serving as barrier against the hydrophilic drug leakage. This was originally carried out by using a fluid-bed apparatus for spray-coating the tablets with an aqueous suspension of lipid nanodroplets, so-called nanoemulsions. Now, a question arises: why to use lipid nanosuspension for this purpose? The answer is simple, since (i) the lipid nanosuspension is able to penetrate the tablet microporous matrix, (ii) the huge homogeneity of these nanoemulsified dispersions will provide a very homogeneous coating, (iii) lipid nanoemulsions are very stable, easy to prepare and are fully compatible with the spray-coating technologies, and finally, (iv) the nanoemulsions formulated by low-energy methods (the case here) are very simple systems adaptable to industrial scaling-up and purposes.

Nanoemulsions are emulsions, in which the size of oil-in-water droplets are typically in nanorange, ranging between 20 and 300 nm [29–31]. The main advantage of nanoemulsions, as in our case, is their stability. Actually, due to their small size, the oil droplets behave typically as Brownian particles and do not interact with each others, resulting in their stability, for up to several months [32–34]. Accordingly, nanoemulsions are considered as particular tools for chemical and pharmaceutical applications, for example, allowing poorly soluble species in water to disperse in a stable way. Another application of nanoemulsion is their use as drug and/or contrast agent nanocarriers, potentially associated with surface functionalization for targeting applications.

In this context, the present study actually constitutes a novel and original application of nanoemulsions, along with a novel approach for the fabrication of oral modified drug-release systems. To summarize, this work presents a new technology for modifying the drug release of tablets. We describe the structures obtained and their links with the drug release kinetics, together with the physical processes involved.

Table 1: Tablets composition (g).

	Tablets (A)	Tablets (B)
Lactose monohydrate	113.8	113.8
Microcrystalline cellulose	222.35	214.45
Corn starch	19.7	27.6
Magnesium stearate	5	5
Colloidal silica	5	5
Talc	2.5	2.5
Carmine red	0.05	0.05
Anhydrous theophylline	131.6	131.6

2. Materials and Methods

2.1. Materials. Lactose monohydrate was provided by Danone (Paris, France) and microcrystalline cellulose (Emcocel 90 M) from JRS Pharma (Rosenberg, Germany). Corn starch, magnesium stearate, talc, and carmine red were obtained from Cooper (Melun, France). Colloidal silica (silica dioxide, Aerosil)was purchased from Evonik (Essen, Germany). Anhydrous theophylline was provided by Fagron (Saint-Denis, France). Food grade nonionic surfactants from BASF (Ludwigshafen, Germany), that is, Cremophor RH40 (polyoxyethylated-40 castor oil, hydrophilic-lipophilic balance, HLB ~14–16) were kindly provided by Laserson (Etampes, France) and used as received. Labrafil M1944CS used as oil phase in the formulation of nanoemulsions was obtained by Gattefossé (Saint-Priest, France). Finally, ultrapure water was obtained using the MilliQ filtration system, Millipore (Saint-Quentin-en-Yvelines, France).

2.2. Methods

2.2.1. Tablets Fabrication. The formulation process and the composition of tablet followed classical pathways. In this study, two formulations named (A) and (B) were studied, differing in the proportions of binding (crosslinked microcrystalline cellulose) and disintegrating (corn starch) compounds. The quantities were as reported Table 1.

Once mixed (lactose, cellulose, starch, carmine red, and theophylline), the powders were homogenized in a Turbula universal mixer (Basel, Switzerland) during 15 min. This was followed by the addition of magnesium stearate, and colloidal silica and the powder were further homogenized in the Turbula mixer for 30 seconds. Next, the powder is sieved through 1 mm meshes sieve and is then pressed with an alternative Frogerais press (Vitry-sur-Seine, France), using a 10 mm diameter hemispherical punch. The tablets thus formed are weighted, their hardness was measured and controlled with a durometer Erweka (Heusenstamm, Germany), and their friability evaluated with a specific apparatus PTF 10E, Pharma Test (Hainburg, Germany). For both formulation (A) and (B), the aimed tablet weight was

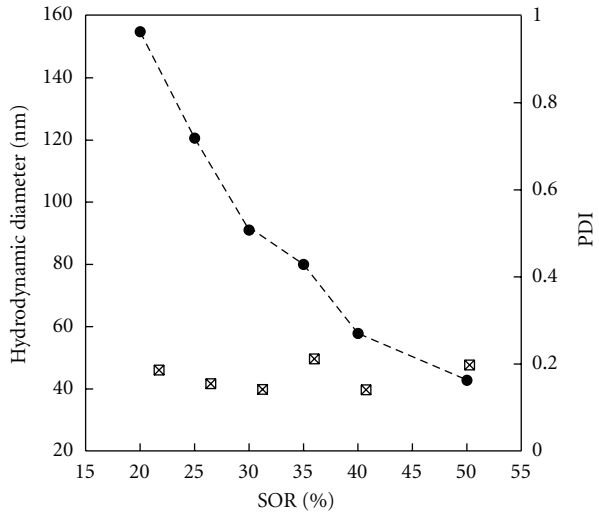

Figure 1: Nanoemulsions formulated with low-energy spontaneous emulsification. Surfactant = Cremophor RH40 oil = Labrafil M1944CS. Hydrodynamic diameter (filled circles) and polydispersity index (open squared) are plotted against the surfactant/oil weight ratio (SOR).

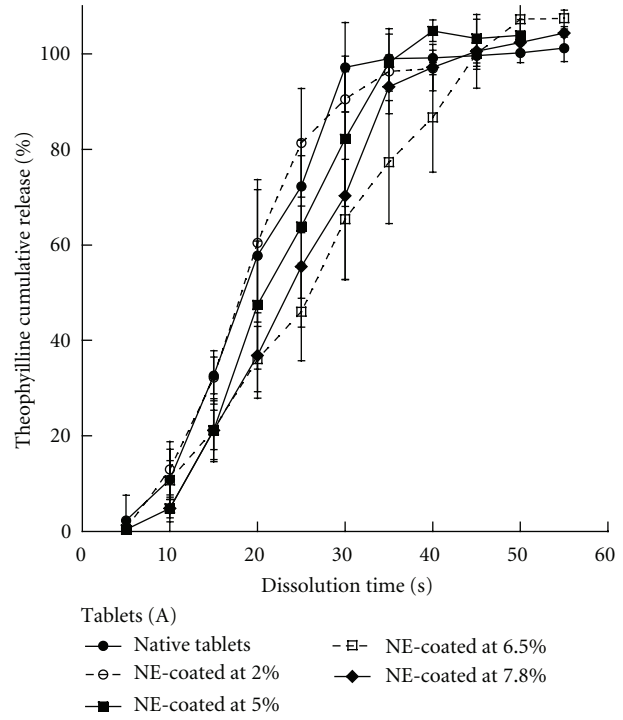

Figure 2: Theophylline release profiles from tablets (A) for different levels of nanoemulsion coating: 2%, 5%, 6.5% and 7.8%, and without coating (noncoated tablets).

fixed at 380 mg, and the aimed hardness was 90 and 190 N for the tablets (A) and (B), respectively.

2.2.2. Nanoemulsion Formulation. Lipid nanoemulsions were formulated according to the low-energy emulsification process published elsewhere [33]. The nanoemulsion droplets were spontaneously formed by bringing into contact two phases: (i) the first was composed of lipid (liquid oil, Labrafil M1944CS) and a hydrophilic surfactant, both totally miscible in each other and gently homogenized at room temperature and (ii) the second phase was aqueous (pure water). Once these two liquid phases were mixed, the hydrophilic species were immediately solubilized by the aqueous phase, inducing the demixing of the oil following a spinodal decomposition, resulting in the nanoemulsion droplets. The nanoemulsion properties, that is, size and polydispersity, have been shown [33] to be closely related to the relative proportions between oil and surfactant. This parameter, so-called surfactant oil weight ratio (SOR = $w_{\text{surfactant}}/(w_{\text{surfactant}} + w_{\text{oil}}) \times 100$) allows the droplet size and polydispersity index to be precisely controlled. In the present study, SOR was fixed at 40% as a representative formulation. Actually, in all the experiments presented here, the SOR (i.e., nanoemulsion droplets size) has no significant influence on the results as well as the release behavior. On the other hand, the relative proportion of water does not influence the nanoemulsion physicochemical properties or their size and PDI. This parameter is given by SOWR = $w_{\text{surfactant}} + w_{\text{oil}}/(w_{\text{surfactant}} + w_{\text{oil}} + w_{\text{water}}) \times 100$, which was also fixed to 40%. The exact composition of the nanoemulsion used for coating of tablets is: oil: 24%; surfactant: 16%; water: 60%. The size distribution and polydispersity of nanoemulsions were assessed by dynamic light scattering (DLS) using a Malvern Nano ZS instrument (Malvern, Orsay, France). The Helium-Neon laser (4 mW) was operated at 633 nm with the scatter angle fixed at 173°, and the temperature was maintained at 25°C. The polydispersity index (PDI) is a measure of the broadness of the size distribution derived from the cumulants analysis of DLS. For a single Gaussian population with standard deviation σ, and mean size x_{PCS}, then PDI = $\sigma^2/x_{\text{PCS}}^2$ is the relative variance of the distribution. The PDI discloses the quality of the dispersion, from values lower than 0.1 for acceptable measurements and good-quality colloidal suspensions, to values close to 1 for poor-quality samples, either with droplet sizes out of the colloidal range or with a very high polydispersity. Measurements were performed in triplicate, before and after the spray drying process (filtered at 0.45 μm in the above case).

2.2.3. Tablets Nanoemulsion Coating. The tablet coating was performed in a fluid bed "bottom spray" apparatus, Innojet Ventilus 2.5 (Steinen, Germany). 50 g of tablets are introduced in the chamber in which is also the rotating spray nose. The experiment was carried out according to the following experimental parameters: air flow: 76 m^3/h; flux: 13%; temperature: 40°C. The weight increase due to the coating is regularly controlled, and the experiment is stopped when the desired nanoemulsion weight coating is obtained.

The upper coating level possible reached in these experiments was around 8%.

2.2.4. Drug Release Profiles. Dissolution tests were performed in an automatized basket apparatus, Dissolutest Caleva BIODIS RRT 9 (Frankfurt, Germany). The basket volume is

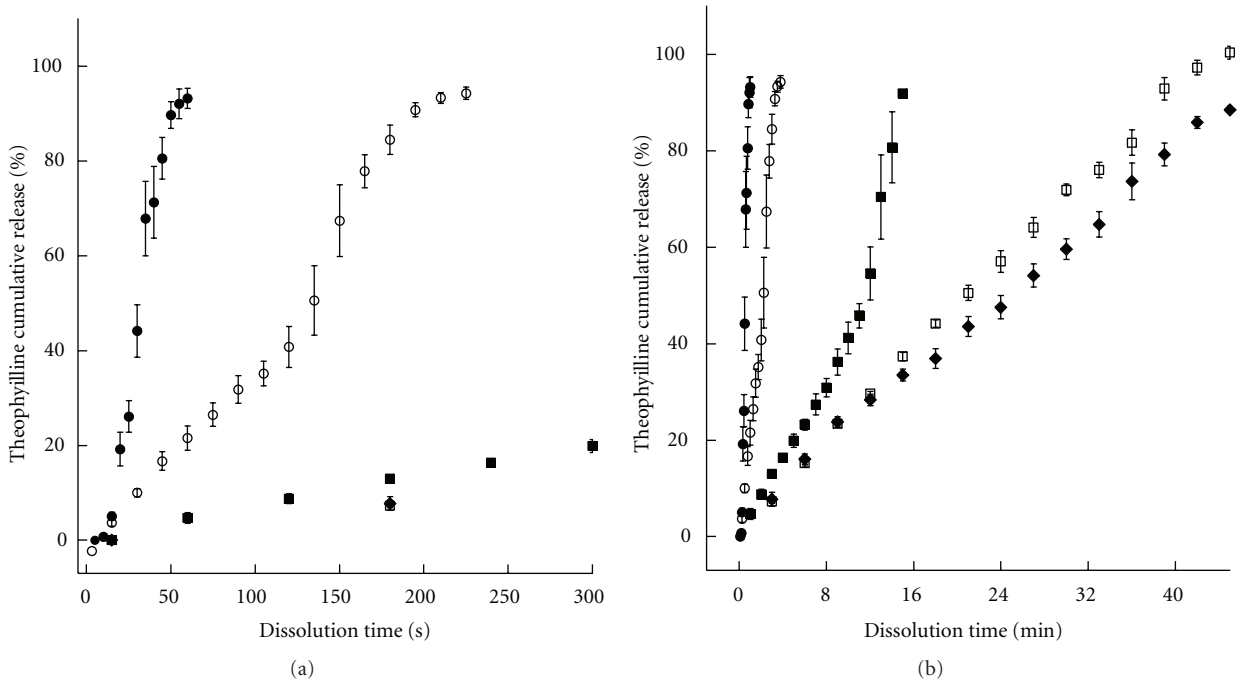

Figure 3: Theophylline release profiles from tablets (b) for different levels of nanoemulsion coating: 2%, 5.5%, 6%, and 7.6%, and without coating (noncoated tablets). The two graphs show the same results with different time scale, in order to emphasize the different release regimes arising for 2% and 5% (for which the frontiers between both are indicated by the arrows).

250 mL, and the dissolution media was an aqueous solution of HCl 0.1 M, maintained at 37°C during 2 hours, as described in the European Pharmacopoeia (7th Ed.) for the delayed release dosage forms.

Aliquots are collected at regular time intervals fixed in function of the release kinetics. Then, the theophylline concentrations, and thus cumulative drug release, are measured at 288 nm by UV spectrophotometry, UV-2401 PC Schimadzu (Kyoto, Japan).

Before performing the measurements, the samples were filtered and diluted, which inhibits the absorption of the various excipients used. In that way, we prevented interference between the theophylline quantification and the absorption of the components of the nanoemulsions or of the tablets. Moreover, a blank test was also performed at 288 nm in absence of theophylline to validate of the measurements.

2.3. Scanning Electron Microscope (SEM). The morphology of tablets (surface and interior) was evaluated by a scanning electron microscopy (Philips XL20, University of Strasbourg, plateforme de microscopie électronique, Institut de Génétique et de Biologie Moléculaire et Cellulaire). The specimens were mounted on the carbon support, coated with a palladium layer and analyzed at 20 kV.

3. Results

The first results concerns the tablet characterization, notably the controls described in the European Pharmacopoeia (7th Ed.).

Table 2: Tablets characterization and Pharmacopoeia controls.

	Tablets (A)	Tablets (B)
Weight (mg)	383 ± 2	382 ± 3
Hardness (N)	84.2 ± 0.8	177.2 ± 0.5
Friability (%)	0.18 ± 0.04	0.12 ± 0.05
Desegregation (s)	19 ± 4	19 ± 2

These results are summarized in Table 2 and validate the dosage forms, compositions, and formulation processes.

The main difference between the two formulations arises in their hardness, and as expected, a higher amount of disintegrating compound reduces the hardness.

Another aspect of the earlier characterization lies in the study of the nanoemulsion formulation process. Hydrodynamic diameter and PDI were measured in function of the surfactant to oil ratio (SOR) defined above. The results are reported in Figure 1.

The global profile of the curves appears coherent with the ones expected for such self nanoemulsifying systems, with relatively monodisperse size distributions (PDI < 0.2). Accordingly, the representative formulation selected for the tablet coating was SOR = 40%, corresponding to d_h = 57.9 nm and PDI = 0.14.

Once the tablets (A) and (B) coated with the nanoemulsion suspension, and at different given proportions, the followup of the theophylline release was performed. These results are reported in Figures 2 and 3, for the tablets (A) and (B), respectively.

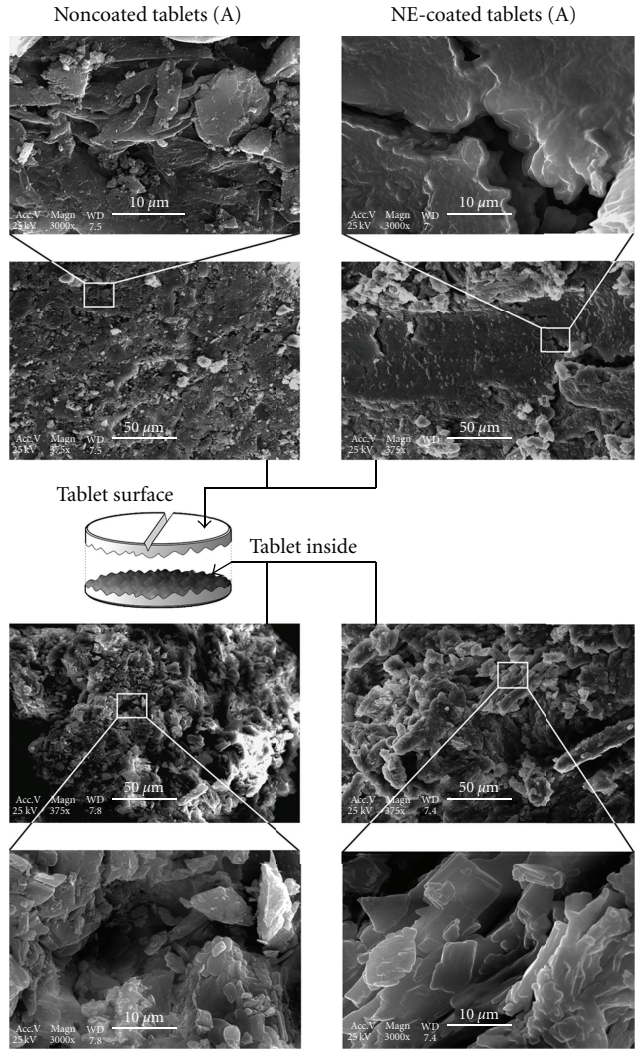

Figure 4: SEM micrographs of the tablets formulation (A). Observations performed on the tablet surface (top) and inside (bottom), for noncoated tablets and nanoemulsions coated (NE-coated).

It clearly appears that the theophylline release can be significantly modified by the intrinsic physical properties of the tablets associated with the lipid coating. In all the experiments, drug release from tablets (A) (Figure 2) was found to be independent of any coating, resulting in fast dissolutions within a minute. On the other hand, drug release from tablets (B) (Figure 3) were very sensitive to the amount of lipid coating. In addition, the curves for the coated tablets (B) show a linear release corresponding to the zero-order kinetics. This regimes, which is followed by a second nonlinear regime for 2.0% and 5.5%. The profiles are entirely linear up to the full release for higher coating amount, 6.0 and 7.6%, providing a zero order during 46 min and 1 h for these examples, respectively. For 2.0% and 5.5% the release profiles show that two regimes follow one another, one exhibits a zero-order release, while the other appears as a transitional drug release similar to the one in noncoated tablets (see details below). Arrows in the figure indicate the location of the frontier between both regimes.

In order to characterize the fine structure on the micrometric scale, the tablets were observed by scanning electron microscopy. The surface and interior of both coated and uncoated tablets, (A) and (B), were analyzed. The pictures are reported in the Figures 4 and 5, for the tablets (A) and (B), respectively.

In both cases (A) and (B), it clearly appears that the lipid coating creates a "smooth" layer on both the tablets surface and the tablets inside. The edges generated by the compression fully disappear after the coating. It means that the nanoemulsions are very homogeneously spread onto the available surface and also can penetrate the microporous tablet matrix during the spray-coating process, which can both be due to the nanometric scale of such a dispersed system. Another point lies in the difference between the formulations of (A) and (B), where the second one (B) was found to be more compact. This actually corroborates their difference in hardness (see Table 2) and contributes to explain the fundamental differences in

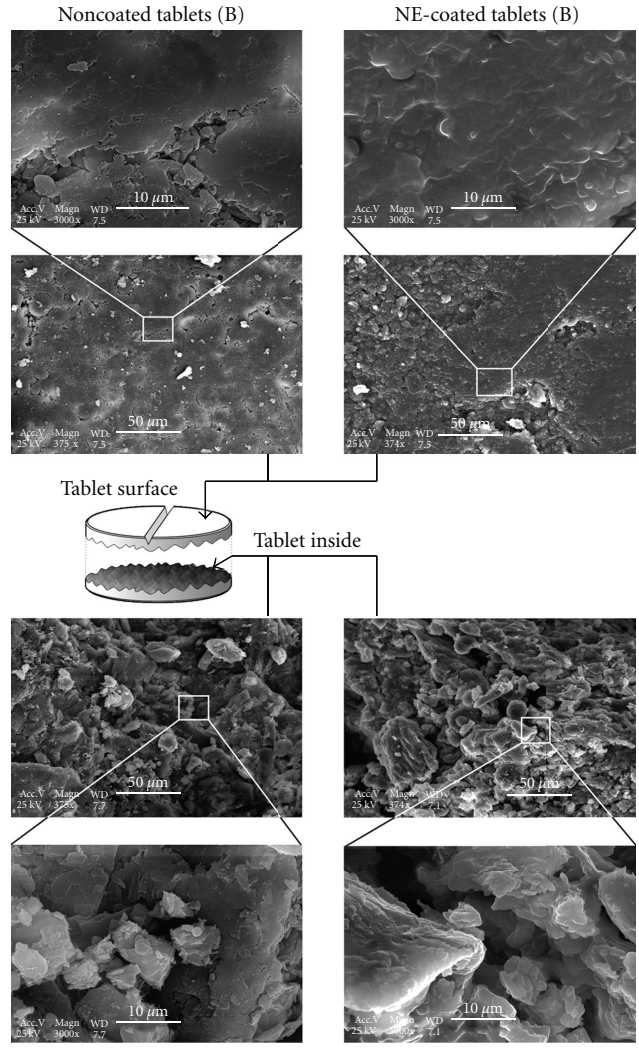

Figure 5: SEM micrographs of the tablets formulation (B). Observations performed on the tablet surface (top) and inside (bottom), for noncoated tablets and nanoemulsions coated (NE-coated).

the release profiles between both formulations (A) and (B).

4. Discussion

The main point of this study lies in the new and simple possibilities offered by lipid nanoemulsions (i) to integrate the microporous matrix of tablets (corroborated by the SEM pictures Figure 5), (ii) to homogeneously coat the surface, and (iii) to create a lipid barrier inducing a zero-order release mechanism in the formulation (B). One interpretation of this zero-order drug release could be the Fickian diffusion-based mechanism, considering that the lipid will create a "filter" or a membrane-like barrier against hydrophilic molecules. As a result, the theophylline molecules leakage from the tablet followed a linear release behavior as long as this lipid barrier is intact. This zero-order release process can be described as a constant regime, also called steady state diffusion. Considering the case of ideal thermodynamic system having a diffusion coefficient D which is independent from the concentration C, and having an unidimensional diffusion, this diffusion regime can be best described by the Fick's first law

$$J = \frac{dM_t}{Sdt} = -D\frac{dC}{dx}, \tag{1}$$

where J is the flux, S the surface of the diffusion plane, and x is the distance of diffusion. Accordingly, this unidimensional equation can easily be adapted for the case of a spherical drug-delivery system of radius R_e, composed of a diffusion-limiting barrier of thickness $R_e - R_i$, giving the drug mass of the released M_t in function of time t, as reported in

$$M_t = \frac{R_e R_i \times 4\pi DKC_0}{R_e - R_i} \times t, \tag{2}$$

where K is the partitioning coefficient between the lipid barrier and water, C_0 is the difference in concentration

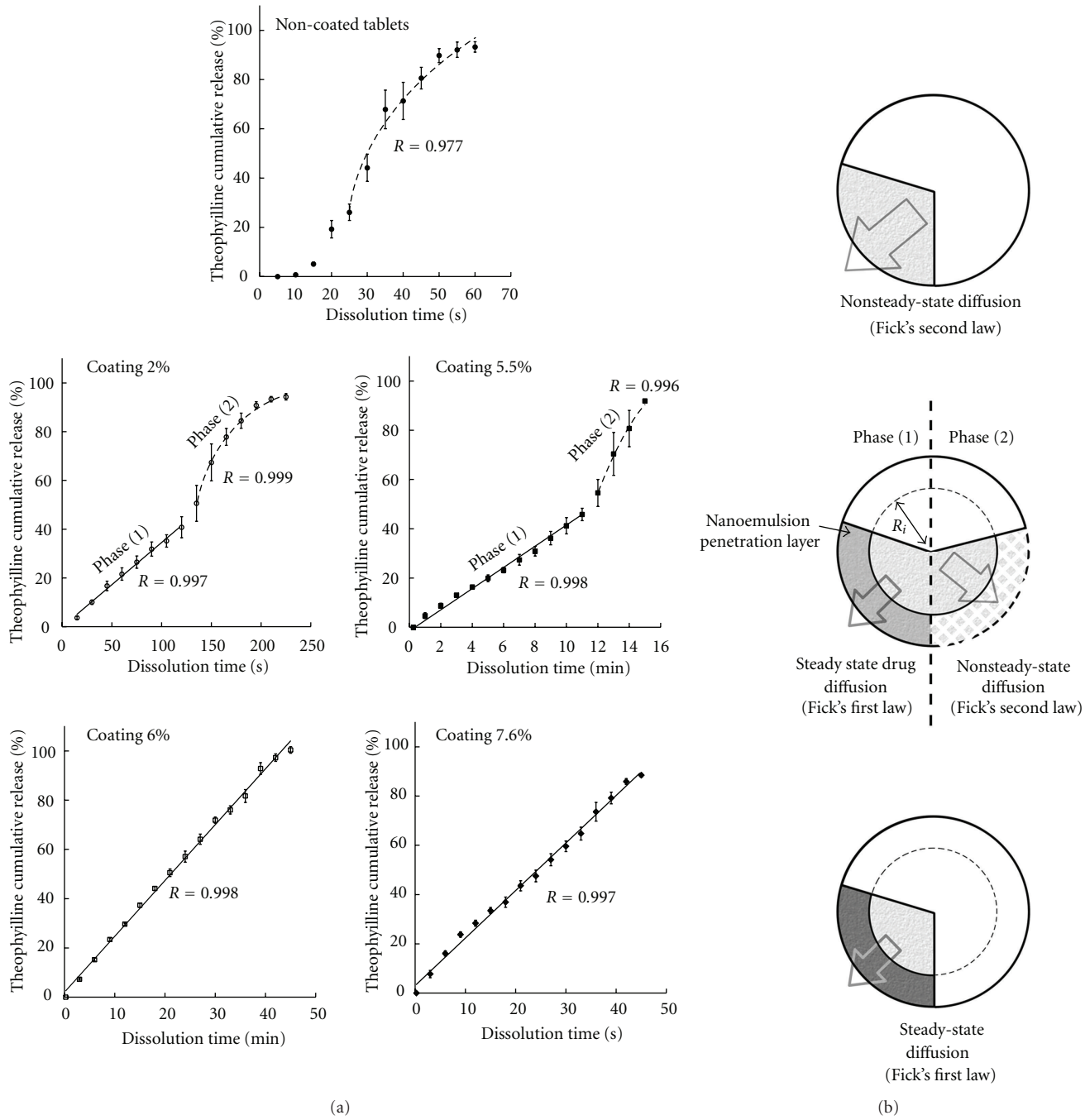

Figure 6: Interpretations of the drug release behaviors from Figure 3. Theophylline release from tablets (b), for different levels of nanoemulsion coating: 2%, 5.5%, 6%, and 7.6%, and noncoating tablets.

between the both sides of the lipid barrier. When the amount of lipid is sufficient (e.g., Figure 3 cases 6 and 7.6%), this barrier appears to be strong enough to allow this linear behavior until the release of all the encapsulated drug amount. However, for intermediate concentrations (as observed in Figure 3 cases 2 and 5.5%), after a given time t_α, this diffusion-limiting layer is dissolved or disaggregated, and a second phase of drug release occurs. This phase follows a "nonsteady state" diffusion regime for which the concentration gradient varies with time. This process is described in the general case by the Fick' second law, reported below:

$$\frac{dC}{dt} = D\frac{d^2C}{dx^2}. \tag{3}$$

In the case of a spherical drug delivery matrix, this equation is adapted as shown below:

$$\frac{M_t}{M_\infty} = 6\left(\frac{D(t-t_\alpha)}{\pi R^2}\right)^{1/2} - \frac{3D(t-t_\alpha)}{R^2}, \tag{4}$$

where M_∞ is the mass of the drug released at infinite time, t_α is the delay induced by the first zero-order release, and R is the sphere radius. This behavior is also found for the noncoated tablets, with a lag time t_α around 19 seconds due to the tablet hydration. It is interesting to note that the zero-order release profiles exhibit slopes (i.e., release speeds quantified below), decreasing with increasing amount of coating lipid. This detail confirms that the diffusion-based mechanism can be a correct interpretation of the zero-order phenomena compared to the other physical possible processes, for example, zero-order homogeneous erosion for which the release speed should be constant in similar experimental conditions. All the release profiles of the formulation (B) are fitted following these two models, and schematic illustrations of the mechanisms and tablets structures are reported in Figure 6.

The main results of a quantitative comparison of the different cases are reported in Table 3.

The theoretical models appear quite well in accordance with experimental results, which confirms the hypothesis ventured regarding the structures and the release processes. The higher the nanoemulsion coating level, the lower the release speed. If the coated lipid layer is considered globally constant, this behavior can be attributed to the decrease of the diffusion coefficient D, and thus to the decrease of the permeability $P = DK/(R_e - R_i)$. On the other hand, the time t_α in which this lipid layer is broken up also appears related to the coating amount. It follows therefrom that t_α indicates the transition between the two diffusion regimes (1) and (2) highlighted in Figure 6. The higher the coating amount, the more stable is the layer, being definitively stable for the examples of 6 and 7 wt.%. Finally, the last parameter D/R^2 characterizing the unsteady-state regimes, shows a gradual increase between the three first cases. As the natural trend for D is a decrease, the observed increase of D/R^2 emphasize a lowering of R, and thus of R_i with the lipid amount. To conclude, coating tablets with lipid nanoemulsions results in the fabrication of a surrounding lipid layer within the tablet, which is able to limit the drug diffusion, similar to a membrane. With the increase of the lipid coating wt.%, this layer become thicker and more stable. Compared now to the hydrophilic matrix discussed above, these systems, made from a fundamentally different technology, appear to present very similar properties.

As a last remark, let us focus on the formulation (A). Even if the coating process and tablet characterization are similar between (A) and (B), the drug release profiles do not have any similarities (Figure 2). Compared with the (B), the tablets (A) show much lower hardness (about half of that of B), which results in higher porosity. The impossibility to create an impermeable lipid layer results in identical drug release profiles whatever may be the coating amount. This can also be observed in the SEM pictures, of the tablet surfaces, which appear to be more compact and robust in the case of the formulation (B).

To finish, such a technology not only appears innovative under the fundamental point of view, since it is the first time that a zero-order release is obtained with a lipid coating, but also it appears interesting in term of industrial scaling up. On the one hand, the nanoemulsion generation method is extremely simple and can be performed only by mixing two liquids, and on the other hand, the method also appears cost effective since it avoids using very specific and expensive polymers for results which can be comparable.

Table 3: Experimental parameters obtained from the kinetics drug release of tablets (B) (see Figure 6). The release speeds reported (dM_t/dt) correspond to the linear diffusion regime.

NE-coating	$v = dM_t/dt$ (10^{-5}g · s^{-1})	t_α	D/R^2(s^{-1})
non-coated	—	19 s	0.011
2 wt.%	38.9	122 s	0.236
5.5 wt.%	7.6	11 min	2.757
6 wt.%	4.2	—	—
7.6 wt.%	3.6	—	—

5. Conclusion

This study presents for the first time the application of lipid nanosuspensions as coating agent for inducing a zero-order hydrophilic drug-release profile. To date, this result was only obtained by using hydrophilic polymeric matrix, and we showed here the proof of concept of this new technology. Lipid nanoemulsions generated by spontaneous nanoemulsifications were used as coating agent. The lipid nanodroplets were able to enter the lipid matrix, to coat the microporous network of the tablet, and to finally create a layer acting as barrier against the diffusion of hydrophilic drugs. This technology is simple, cost effective, and efficient, and we believe that it can open new perspectives for the fabrication of pharmaceutics and oral modified release-dosage forms.

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Review Article

Current State-of-Art and New Trends on Lipid Nanoparticles (SLN and NLC) for Oral Drug Delivery

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Lipids and lipid nanoparticles are extensively employed as oral-delivery systems for drugs and other active ingredients. These have been exploited for many features in the field of pharmaceutical technology. Lipids usually enhance drug absorption in the gastrointestinal tract (GIT), and when formulated as nanoparticles, these molecules improve mucosal adhesion due to small particle size and increasing their GIT residence time. In addition, lipid nanoparticles may also protect the loaded drugs from chemical and enzymatic degradation and gradually release drug molecules from the lipid matrix into blood, resulting in improved therapeutic profiles compared to free drug. Therefore, due to their physiological and biodegradable properties, lipid molecules may decrease adverse side effects and chronic toxicity of the drug-delivery systems when compared to other of polymeric nature. This paper highlights the importance of lipid nanoparticles to modify the release profile and the pharmacokinetic parameters of drugs when administrated through oral route.

1. Introduction

The use of lipid particles in pharmaceutical technology has been reported for several years. The first approach of using lipid microparticles was described by Eldem et al. [1], reporting the production by high-speed stirring of a melted lipid phase in a hot surfactant solution obtaining an emulsion. Solid microparticles are formed when this emulsion is cooled to room temperature, and the lipid recrystallizes. The obtained products were called "lipid nanopellets", and they have been developed for oral administration [2]. Lipospheres were described by Domb applying a sonication process [3–5]. To overcome the drawbacks associated to the traditional colloidal systems [6], such as emulsions [7], liposomes [8], and polymeric nanoparticles [9], solid lipid nanoparticles (SLN) [10, 11] have been developed for similar purposes [12].

SLN are biocompatible and biodegradable and have been used for controlled drug delivery and specific targeting. These colloidal carriers consist of a lipid matrix that should be solid at both room and body temperatures, having a mean particle size between 50 nm and 1000 nm [13, 14].

A clear advantage of the use of lipid particles as drug-carrier systems is the fact that the matrix is composed of physiological components, that is, excipients with generally recognized as safe (GRAS) status for oral and topical administration, which decreases the cytotoxicity. SLN have been already tested as site-specific carriers particularly for drugs that have a relatively fast metabolism and are quickly eliminated from the blood, that is, peptides and proteins [15].

The cytotoxicity of SLN can be attributed to nonionic emulsifiers and preservative compounds which are used in

the production of these systems [16]. SLN prepared up to concentrations of 2.5% lipid do not exhibit any cytotoxic effects *in vitro* [17]. Even concentrations higher than 10% of lipid have been shown a viability of 80% in culture of human granulocytes [18]. In contrast, some polymeric nanoparticles showed complete cell death at concentrations of 0.5%. In addition, a high loading capacity for a broad range of drugs can be achieved, especially if they have lipophilic properties [12, 19].

Due to their physiological and biodegradable properties, SLN have been tested for several administration routes [20, 21], including the oral [22, 23] and peroral [24, 25] routes.

SLN can be obtained by exchanging the liquid lipid (oil) of the o/w nanoemulsions by a solid lipid [19]. In general, a solid core offers many advantages in comparison to a liquid core [26]. Emulsions and liposomes usually show lack of protection of encapsulated drugs, and drug release as a burst (emulsions) or noncontrolled (from liposomes). SLN possess a solid lipid matrix identical to polymeric nanoparticles. In addition, SLN are of low cost [27], the excipients and production lines are relatively cheap, and the production costs are not much higher than those established for the production of parenteral emulsions [28].

At the turn of the millennium, modifications of SLN, the so-called nanostructured lipid carriers (NLCs), have been introduced to the literature, and these NLC represent nowadays the second generation of lipid nanoparticles. These carrier systems overcome observed limitations of conventional SLN [29]. The main difference between SLN and NLC is the fact that the concept of these latter is performed by nanostructuring the lipid matrix, in order to increase the drug loading and to prevent its leakage, giving more flexibility for modulation of drug release. This approach is achieved by mixing solid lipids with liquid lipids in NLC instead of highly purified lipids with relatively similar molecules in SLN. This mixture has to be solid at least at 40°C. The result is a less-ordered lipid matrix with many imperfections, which can accommodate a higher amount of drug [11].

2. Role of Lipids in Oral Delivery

A limiting factor for *in vivo* performance of poorly water-soluble drugs for oral administration is their resistance of being wetted and dissolved into the fluid in the GIT (apart from potential drug degradation in the gut). Thus, the increase in the dissolution rate of poorly water-soluble drugs is relevant for optimizing bioavailability. Over the last 10 years, poorly water-soluble compounds are formulated in lipid nanoparticles for drug administration [30]. The features of lipid nanoparticles for oral and peroral delivery are related with their adhesive properties. Once adhered to the GIT wall, these particles are able to release the drug exactly where it should be absorbed. In addition, the lipids are known to have absorption-promoting properties not only for lipophilic drugs, such as Vitamin E, repaglinide [22], and puerarin [23].

Hydrophilic drugs can also be incorporated in SLN; nevertheless, the affinity between the drug and the lipid needs to be analysed. Therefore, loading hydrophilic drugs in SLN is a challenge due to the tendency of partitioning the encapsulated molecules in the water during the production process of nanoparticles [31]. Successful examples are zidovudine [31], insulin [32], tretinoin [33], and diminazene [34]. There are even differences in the lipid absorption enhancement depending on the structure of the lipids. For example, medium-chain triglycerides (MCT) lipids are more effective than long-chain triglycerides (LCT) [35]. Basically, the body is taking up the lipid and the solubilized drug at the same time. It can be considered as a kind of "Trojan horse" effect [36, 37].

Oral administration of SLN is possible as aqueous dispersion [38] or alternatively transformed into a traditional dosage forms such as tablets, pellets, capsules, or powders in sachets [25, 39]. For this route, all the lipids and surfactants used in traditional dosage forms can be exploited. In addition, all compounds of GRAS status or accepted GRAS status can be employed as well as from the food industry [40]. Since the stomach acidic environment and high ionic strength favour the particle aggregation, aqueous dispersions of lipid nanoparticles might not be suitable to be administered as dosage form. In addition, the presence of food will also have a high impact on their performance [41].

The packing of SLN in a sachet for redispersion in water or juice prior to administration will allow an individual dosing by volume of the reconstituted SLN. For the production of tablets, the aqueous SLN dispersions can be used instead of a granulation fluid in the granulation process. Alternatively, SLN can be transferred to a powder (by spray-drying or lyophilization) and added to the tabletting powder mixture. In both cases, it is beneficial to have a higher solid content to avoid the need of having to remove too much water. For cost reasons, spray drying might be the preferred method for transforming SLN dispersions into powders, with the previous addition of a protectant [42].

For the production of pellets, the SLN dispersion can be used as a wetting agent in the extrusion process. SLN powders can also be used for the filling of hard gelatine capsules. Alternatively, SLN can be produced directly in liquid PEG 600 and put into soft gelatine capsules. Advantages of the use of SLN for oral and peroral administration are the possibility of drug protection from hydrolysis, as well as the possible increase of drug bioavailability. Prolonged plasma levels has also been postulated due to a controlled, optimized released [22] in combination with general adhesive properties of small particles [43]. The advantage of colloidal drug carriers described above is that they are generally linked to their size in the submicron range. Therefore, the preservation of particle size of colloidal carrier systems after peroral administration is a crucial point. The gastric environment (ionic strength, low pH) may destabilize the SLN and potentially lead to aggregation. However, it is possible to produce stable SLN dispersions by optimizing the surfactant/mixture for each lipid *in vitro* [44].

The drug release from SLN in the GIT is also dependent on the lipase/colipase activity for the GIT digestion of the lipid matrix. The lipase/colipase complex leads to a degradation of food lipids as a prestep of the absorption. *In vitro*

degradation assay based on pancreas lipase/colipase complex have been developed to obtain basic information about the degradation velocity of SLN as a function of lipid and surfactant used in the production process [45, 46].

Lipid nanoparticles show great promise to enhance oral bioavailability of some of the most poorly soluble drugs. The physical/chemical characteristics of lipid particulate systems are highly complex due to the existence of a variety of lipid assembly morphologies, the morphology-dependent solubility of drug, the interconversion of assembly morphology as a function of time and chemical structure, and the simultaneous lipid digestion [47].

3. Lipid Nanoparticles as Drug Carriers

Lipid nanoparticles show interesting features concerning therapeutic purposes. Their main characteristic is the fact that they are prepared with physiologically well-tolerated lipids [48]. During the last ten years, different substances have been entrapped into lipid nanoparticles (Table 1), ranging from lipophilic [23, 49] and hydrophilic molecules, including labile compounds, such as proteins and peptides [50].

3.1. Lipid Materials for Oral Administration. The term lipid is used here in a broader sense and includes triglycerides, partial glycerides, fatty acids, steroids, and waxes. However, it is required that matrix maintains the solid state at room temperature, and for this purpose, the selection of lipids is based on the evaluation of their polymorphic, crystallinity, miscibility, and physicochemical structure [11]. Table 2 shows the main lipids employed for the preparation of lipid nanoparticles.

Furthermore, the use of mono- and diglycerides as lipid matrix composition might increase drug solubility compared to highly pure lipids, such as monoacid triglycerides. Naturally occurring oils and fats comprise mixtures of mono-, di-, and triglycerides, containing fatty acids of varying chain length and degree of unsaturation [25, 86]. The melting point of these lipids increases with the length of the fatty acid chain and decreases with the degree of unsaturation. The chemical nature of the lipid is also important, because lipids which form highly crystalline particles with a perfect lattice (e.g., monoacid triglycerides) lead to drug expulsion during storage time. Physicochemically stable lipid nanoparticles will be obtained only when the right surfactant and adjusted concentration have been employed [25].

3.2. Determination of Optimal Hydrophile-Lipophile Balance (HLB) Values for Lipid Nanoparticles Dispersions. Emulsifiers are essential to stabilize lipid nanoparticles dispersions and prevent particle agglomeration [87]. The choice of the ideal surfactant for a particular lipid matrix is based on the surfactant properties such as charge, molecular weight, chemical structure, and respective hydrophile-lipophile balance (HLB). All these properties affects the stability of the emulsion [10]. The HLB of an emulsifier is given by the balance between the size and strength of the hydrophilic and the lipophilic groups. All emulsifiers consist of a molecule that combines both hydrophilic and lipophilic groups. Griffin [88] defined the lipophilic emulsifiers as low HLB values (below 9), and hydrophilic emulsifiers as high HLB values (above 11). Those in the range of 9–11 are intermediate [89].

The HLB system is a useful method to choose the ideal emulsifier or blend of emulsifiers for the system, that is, if its required an oil-in-water (o/w), water-in-oil (w/o) [90], or a double (w/o/w) emulsion. Matching the HLB value of the surfactant with the lipid will provide a suitable *in vitro* performance [91]. Table 3 depicts the mainly surfactants employed in the production of lipid nanoparticles.

Severino et al. [10] determined the HLB value for stearic acid and stearic acid capric/caprylic triglycerides to reach the best combination of surfactants (trioleate sorbitan and polysorbate 80) to obtain a stable lipid nanoparticles emulsion. The HLB value obtained for stearic acid was 15 and for stearic acid capric/caprylic triglycerides was 13.8. Sorbitan trioleate has an HLB value of 1.8 and polysorbate 80 of 15, when used in the ratio 10 : 90, respectively. The surfactant mixtures prepared with different ratios provided well-defined HLB values. Polysorbate 80 is often used in combination with sorbitan trioleate due to their appropriate compatibility attributed to the similar chemical structure (same hydrocarbon chain length) for the production of stable emulsions.

4. Biopharmaceutic and Pharmacokinetic Aspects

Pharmacokinetic behaviour of drugs loaded in lipid nanoparticles need to differentiate if the drug is present as the released free form or as the associated form with lipid nanoparticles [106]. However, the poor aqueous solubility of some drugs turns difficult the design of pharmaceutical formulations and leads to variable bioavailability [107].

Xie et al. [108] reported a significant increase in the bioavailability and extended the systemic circulation of ofloxacin formulated in SLN, which could be attributed to a large surface area of the particles, improving the dissolution rate and level of ofloxacin in the presence of GIT fluids [109, 110], leading to shorter T_{max} and higher peak plasma concentration. In addition, lipid nanoparticles may adhere to the GIT wall or enter the intervillar spaces due to their small particle size, increasing their residence time [111]. Moreover, nanoparticles could protect the drug from chemical and enzymatic degradation and gradually release drug from the lipid matrix into blood, [112] resulting in a several-fold increase mean residence time compared with native drug. Han et al. [113] demonstrated that 5 oral doses of tilmicosin loaded in lipid nanoparticles administered every 10 days provided an equivalent therapeutic benefit to 46 daily doses of oral free drugs. *In vitro* release profile demonstrated that tilmicosin loaded in lipid nanoparticles followed a sustained release profile, and *in vivo* results showed that nanoparticles remained effective for a longer period of time, which was

Table 1: Examples of drugs, miscellaneous active ingredients and macrocyclic skeletons incorporated into lipid nanoparticles.

Incorporated drug or substance	Lipid	Advantageous	System	References
3′-Azido-3′-deoxythymidine palmitate	Trilaurin	Stable after autoclaving, and can be lyophilized and rehydrated	SLN	[51]
5-Fluorouracil	Dynasa 114 and Dynasan 118	Prolonged release in simulated colonic medium	SLN	[52]
Apomorphine	Glyceryl monostearate, polyethylene glycol monostearate	Enhanced the bioavailability in rats	SLN	[20]
Ascorbyl palmitate	Imwitor 900 and Labrafil M1944	Viscoelastic measurements is appropriate for topical/dermal application	NLC	[53]
Baclofen	Stearic acid	Significantly higher drug concentrations in plasma	SLN	[54]
Benzyl nicotinate	Dynasan 116	Increased oxygenation in the skin	SLN	[55]
Calcitonin	Trimyristin	Improvement of the efficiency of such carriers for oral delivery of proteins	SLN	[56]
Camptothecin	Monostearin and Soybean Oil 788	Stable and high performance delivery system	NLC	[57, 58]
Clozapine	Trimyristin, tripalmitin, and tristearin	Improvement of bioavailability	SLN	[59]
Cyclosporin A	glyceryl monostearate, and glyceryl palmitostearate	Controlled release	SLN	[60, 61]
Dexamethasone	Compritol 888 ATO	Drug delivery topical use	SLN	[62]
Diazepam	Compritol ATO 888 and Imwitor 900 K	Prolonged release	SLN	[63]
Doxorubicin	Glyceryl caprate	Enhanced apoptotic death	SLN	[64]
Gonadotropin release hormone	Monostearin	Prolonged release	SLN	[65]
Hydrocortisone	Monoglyceride, chain length of the fatty acid moiety	SLN stable with release properties	SLN	[66]
Ibuprofen	stearic acid, triluarin, tripalmitin	Stable formulation and negligible cell cytotoxicity	SLN	[67]
Idarubicin	Emulsifying wax	Potential to deliver anticancer drugs	SLN	[68]
Insulin	Stearic acid, octadecyl alcohol, cetyl palmitate, glyceryl monostearate, glyceryl palmitostearate, glyceryl tripalmitate, glyceryl behenate	Promising for oral delivery of proteins	SLN	[50]
Ketoprofen	mixture of beeswax and carnauba wax	SLN with beeswax content exhibited faster drug release as compared carnauba wax	SLN	[69]
Lopinavir	Compritol 888 ATO	Bioavailability enhanced	SLN	[70]
Nimesulide	Glyceryl behenate, palmitostearate, glyceryl tristearate	Sustained drug release	SLN	[71]
Penciclovir	Glyceryl monostearate	Provide a good skin targeting	SLN	[72]
Progesterone	Monostearin, stearic acid and oleic acid	Potential drug delivery system for oral administration	NLC	[73, 74]
Repaglinide	Glycerol monostearate and tristearin	Toxicity study indicated that the SLN were well tolerated	SLN	[22, 49]
Salbutamol sulphate	Monostearin and PEG2000	Formulation accelerate release of hydrophilic small molecule drugs	SLN	[75]
Tetracycline	glyceryl monostearate and stearic acid	Sustained release	SLN	[76]

Table 2: Lipids used for lipid nanoparticles production.

Lipids	References
Triglycerides	
Trimyristin (Dynasan 114)	[11]
Tripalmitin (Dynasan 116)	[77]
Tristearin (Dynasan 118)	[11]
Mono, di and triglycerides mixtures	
Witeposol bases	[78]
Glyceryl monostearate (Imwitor 900)	[22]
Glyceryl behenate (Compritol 888 ATO)	[79]
Glyceryl palmitostearate (Precirol ATO 5)	[80]
Waxes	
Beeswax	[81]
Cetyl palmitate	[82]
Hard fats	
Stearic acid	[10]
Palmitic acid	[83]
Behenic acid	[84]
Other lipids	
Miglyol 812	[11]
Paraffin	[85]

Table 3: Emulsifiers used for the production of lipid nanoparticles.

Emulsifiers/coemulsifiers	HLB	References
Lecithin	4–9	[92, 93]
Poloxamer 188	29	[94]
Poloxamer 407	21.5	[56, 95]
Tyloxapol	13	[96]
Polysorbate 20	16.7	[92]
Polysorbate 60	14.9	[97]
Polysorbate 80	15	[10, 11]
Sodium cholate	18	[98]
Sodium glycocholate	14.9	[99]
Taurodeoxycholic acid sodium	13-14	[100]
Butanol and Butyric acid	7–9	[101]
Cetylpyridinium chloride	~15	[102]
Sodium dodecyl sulphate	40	[103]
Sodium oleate	18	[99]
Polyvinyl alcohol	15–19	[104]
Cremophor EL	12–14	[105]

attributed to sustained release of the drug and also to enhanced antibacterial activity by the SLN.

Pandita et al. [114] developed paclitaxel loaded in SLN with the aim at improving the oral bioavailability of this antineoplastic drug. *In vitro* studies of SLN formulation exhibited an initial low burst effect within 24 h followed by a slow and sustained release. Statistical analysis of *in vivo* experiments concluded that the oral bioavailability of paclitaxel loaded in SLN was significantly higher than the control group.

Yuan et al. [115] produced stearic acid-SLN with a fluorescence marked for evaluation of *in vivo* pathway by oral administration. About 30% of SLN transport was efficient, where particles were absorbed following linear mechanism in the GIT. The release profile in plasma increased with the increasing of dosage depicting two concentration peaks. The first peak of SLN in blood took place during 1-2 h, attributed to the fast uptake of SLN from the GIT into systematic circulation. Drug concentration began to decrease attributed to the uptake by and the distribution of SLN among particular organs. The second peak occurred at about 6–8 h, and the maximum concentrations were lower than that of the first peak.

5. Toxicology

Lipid nanoparticles are well tolerated in living systems, since they are made from physiological compounds leading to the metabolic pathways [22, 28]. For this purpose, studies focusing on nanotoxicology comprise cytotoxicity and genotoxicity analysis [116]. However, such effects often occur first at rather in high concentrations and the subtler effects that arise at lower concentrations, without necessarily causing cell death, also need to be considered. One the most important effect is DNA damage, since an increased genetic instability is associated with cancer development [117]. The interaction with proteins and cells are an essential focus in assessing and understanding compatibility and toxicity. Cell and nanoparticle reactions of interest include cellular uptake and processing of nanoparticle in various routes, effects on cell signalling, membrane perturbations, influence on the cellular electron transfer cascades, production of cytokines, chemokines, and reactive oxygen species (ROS), transcytosis and intercellular transport, gene regulation overt toxic reactivity, no observable toxicity, and cell necrosis or apoptosis. *In vitro* culture of cell lines or primary cells on plastic plates are employed in a wide varieties of assays and reflect the variety of possible physiologic responses to nanoparticles *in vivo* and all possible cell processing routes and natural reactions [118].

Silva et al. [119] studied the toxicity of SLN and risperidone loaded SLN with Caco-2 cells by (4,5-dimthylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay. The results suggest that all formulations evaluated are biocompatible with Caco-2 cells and well tolerated by the GIT. Similar results have been reported elsewhere [120, 121]. This test evaluates the mitochondrial function as a measurement of cell viability, which allows the detection of dead cells before they lose their integrity and shape. The amount of viable cells after SLN exposure was performed by the MTT assay with Caco-2 cell models, which are a well-established *in vitro* model that mimics the intestinal barrier and is often used to assess the permeability and transport of oral drugs [122]. Other authors have also reported that SLN

show biocompatibility, which increase their attractiveness for drug-delivery applications [120].

6. Marketed Products and Current Studies

Since early nineties, researchers turned their attention to lipid nanoparticles because of their nontoxicity and cost/effectiveness relationship [12]. In spite of the advantages, formulating with lipid nanoparticles has been suffering some drawbacks. Because of the GIT conditions, most of promising drugs do not reach clinical trials. The stability of particles must be comprehensively tested due to pH changes and ionic strength as well as the drug release upon enzymatic degradation [123]. Lipid nanoparticles absorption through GIT occurs via transcellular (through M cells or enterocytes) or paracellular (diffusion between cells). If the major drug uptake occurs through M cells, the portal vein to the liver is bypassed, resulting in higher drug concentrations to the lymph rather than to plasma [124]. Despite the low number of lipid nanoparticles formulations on the market for drug delivery, Mucosolvan retard capsules (Boehringer-Ingelheim) is a story of success [125]. Mucosolvan retard capsules was the first generation. It was produced by high-speed stirring of a melted lipid phase in a hot surfactant solution obtaining an emulsion. This emulsion was then cooled down to room temperature obtaining the so-called "lipid nanopellets for oral administration" [126].

Successful *in vivo* studies also include rifampicin, isoniazid, and pyrazinamide that are used in tuberculosis treatment. These drugs achieved higher bioavailability when incorporated into SLN compared to the free solutions. Rifampicin has poor cellular penetration which requires high doses to reach effective concentrations. Rifamsolin is a rifampicin-loaded SLN under preclinical phase by AlphaRx. The methodology employed for production is acceptable by the regulatory agencies and has been addressed by various papers and patents [127].

Poor water-soluble drugs, as camptothecin, vinpocetine, and fenofibrate, can have their solubilization improved if incorporated into SLN [124, 128]. Another example is insulin, commonly administered parenterally in the treatment of diabetes mellitus. Injections are often painful and must be administered daily, which result in low patient compliance [129]. Unfortunately, oral administration of insulin, produced by solvent emulsification-evaporation method based on a w/o/w double emulsion, has limitations such as low bioavailability due to degradation in the stomach, inactivation and degradation by proteolytic enzymes, and low permeability across the intestinal epithelium because of lack of lipophilicity and high molecular weight [124, 129]. The main advantages of incorporate insulin into SLN would be the enhancement of transmucosal transport and protection from the degradation in the GIT.

7. Conclusions

Lipids and lipid nanoparticles are promising for oral and per-oral administration route for drugs, proteins, and peptides. Theses matrices are able to promoting controlled release of drugs in GIT and reducing absorption variability. In addition, these matrices can be absorption as food lipids together with drugs improving the bioavailability. These systems present several advantages, including drug protection and excipients of GRAS status, which decreases the danger of acute and chronic toxicity. In addition, the oral administration of lipids nanoparticles is possible as aqueous dispersion or alternatively transformed into a traditional dosage forms such as tablets, pellets, capsules, or powders in sachets.

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