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DIPLOMARBEIT

Self-Nanoemulsifying Drug Delivery Systems (SNEDDS) - Influences on Cellular Uptake

Ausgeführt am Institut für Angewandte Synthesechemie

der Technischen Universität Wien

unter der Anleitung von Univ.Prof. Dipl.Ing. Dr. Peter Ertl

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durch

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Wien, am 12.06.2017

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Abstract

The field of nanocarriers for drug delivery has developed rapidly over the last decades. Recently the innovative approach of self-emulsifying systems has caught researcher's interest. The aim of this study was to investigate influences on cellular uptake of self-nanoemulsifying drug delivery systems (SNEDDS) with regard to droplet size and the presence of anionic surfactants. In order to determine size-dependence, formulations exhibiting different components and ratios were developed. Especially the oil-to-surfactant ratio determined the droplet size of resulting emulsions. Anionic surfactants were dissolved in a formulation by heating and ultrasonics, thereby achieving a maximum of 1 % loading for all chosen excipients. Emulsification was carried out in water, phosphate buffer or culture medium. The resulting nanoemulsions were characterized with regard to particle size, stability and zeta potential using light scattering methods. Among the screened formulations, two compositions with three different droplet sizes each were chosen, varying between 35 and 150 nm with a PDI below 0.25. Unloaded SNEDDS of roughly neutral zeta potential (-7 to +3 mV) differed to the increasingly negative value of anionic surfactant loading (-4 to -28 mV). CaCo-2 and HEK-293 cells were chosen as *in vitro* cell models. A concentration dependent cytotoxicity of nanoemulsions was found on the cells via resazurin assay. Thus 0.05 % SNEDDS in OptiMEM was used for the cellular uptake studies, which were performed on both cell types through incorporation of FDA (1 %) into the formulations. The amount of entered marker molecules was analyzed by fluorescence. These investigations revealed an improved cellular uptake efficiency for some of the incorporated anionic surfactants (up to 1.6-fold), but no tendency regarding droplet size could be observed. According to these results, cellular uptake of SNEDDS does generally not depend on the size of the nanoemulsions, but rather the uptake is determined by the choice of components and the amount of surfactants.

Kurzfassung

Die Anwendung von Nanocarriern in der Medizin hat sich über die letzten Jahrzehnte rapide weiterentwickelt. Ein innovativer Ansatz dabei sind selbst-emulgierende Systeme. Das Ziel dieser Arbeit war es, Einflüsse auf die zelluläre Aufnahme von sogenannten "self-nanoemulsifying drug delivery systems" (SNEDDS) bezüglich Tröpfchengröße und die Anwesenheit von anionischen Tensiden zu untersuchen. Um eine Größenabhängigkeit feststellen zu können, wurden mehrere Formulierungen mit unterschiedlichen Zusammensetzungen hergestellt. Dabei entscheidet vor allem das Verhältnis von Öl zu Tensid über die Partikelgröße der Nanoemulsionen. Die anionischen Tenside wurden in einer Formulierung durch Anwendung von Hitze und Ultraschall gelöst. Dabei konnte eine maximale Konzentration von 1 % für alle Bestandteile erreicht werden. Die SNEDDS wurden in Wasser, Phosphat Puffer oder Nährmedium emulgiert. Mittels Lichtstreuungs-methoden wurden die entstandenen Nanoemulsionen im Hinblick auf Tröpfchengröße, Stabilität und Zeta Potenzial charakterisiert. Es wurden zwei Arten von getesteten Formulierungen ausgewählt, die je drei verschiedenartige Größen aufweisen, welche zwischen 35 und 150 nm liegen und einen PDI unter 0.25 haben. Die nicht beladenen SNEDDS mit in etwa neutralem Zeta Potenzial (-7 bis +3 mV) unterscheiden sich dabei von den stärker negativen Werten der SNEDDS mit anionischen Tensiden (-4 bis -28 mV). CaCo-2 und HEK-293 wurden als *in vitro* Zell-Modelle angewendet. Mittels Resazurin Assays wurde eine Konzentrationsabhängigkeit der Zytotoxizität festgestellt. Daher wurde als Konzentration 0.05 % SNEDDS in OptiMEM für die zelluläre Aufnahmestudien an beiden Zelltypen gewählt. Dafür wurde 1 % FDA in den Formulierungen gelöst, dessen Absorption in die Zellen über Fluoreszenz quantifiziert wurde. Die Untersuchungen zeigten eine höhere Aufnahmeeffizienz für einige der anionischen Tenside (bis zu 1.6-fach), allerdings konnte keine Tendenz bezüglich der Partikelgröße festgestellt werden. Gemäß dieser Ergebnisse hängt die zelluläre Aufnahmeeffizienz im Allgemeinen nicht von der Größe der entstandenen Nanoemulsionen ab, sondern vielmehr von der Wahl an Bestandteilen und dem Anteil an Tensiden.

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1 Introduction

Oral drug administration is of particular interest to drug delivery, but limited gastrointestinal absorption of drugs remains a challenge due to several physiological barriers before entering the target cells including enzymatic degradation,[1] mucus gel layer[2] and membrane barriers.[3] To overcome some of these issues, the application of nanocarriers for drug delivery is of increasing interest to researchers. They are colloidal particles ranging from 1 to 1000 nm in size, where drugs can be encapsulated or attached to the surface. Several types of nanocarriers have been established already, e.g. polymeric nanoparticles, inorganic and lipid-based delivery systems.[4]

An increasingly popular approach is the use of self-nanoemulsifying drug delivery systems (SNEDDS).[5] These are anhydrous isotropic liquid mixtures consisting of oil(s), surfactant(s), co-solvent (solubilizer) and drug molecules,[6] which spontaneously form oil-in-water nanoemulsions (20 - 200 nm) upon contact with aqueous medium under gentle agitation. Therefore, the mild gastrointestinal movement is sufficient for the emulsification process.[7] The improved bioavailability of SNEDDS is a result of large surface area, permeation and absorption enhancement and a protective effect against enzymatic degradation.[7,8] Self-emulsifying systems are mainly investigated for the oral delivery of lipophilic drugs which are easily incorporated into the lipid based carrier, hence improving water-solubility of hydrophobic drugs.[9] Initial cellular recognition of nanoparticles for drug delivery is affected by particle size, surface chemistry and morphology. Especially particle size is known to have a crucial impact on cellular uptake efficiency for nanoparticles[10] and liposomes.[11]

Therefore, it is the aim of this study to investigate the size-dependence on cellular uptake efficiency of SNEDDS. Hence, formulations exhibiting designated droplet sizes were developed. The emulsions were characterised regarding droplet size and zeta potential using dynamic light scattering and electrophoretic light scattering respectively. Two different SNEDDS compositions were chosen, each displaying three distinct droplet sizes. The variation is size was achieved by modifying the ratios of components, mainly oil-to-surfactant proportion. Thereafter, the chosen SNEDDS formulations were further characterised in respect of cytotoxicity and cellular uptake efficiency on two different *in vitro* cell models, namely HEK-293 and CaCo-2 cells. Resazurin cell assay was performed to determine cytotoxicity of formulations and cellular uptake efficiency was investigated by incorporation of fluorescein diacetate into SNEDDS. Both investigations were analysed by fluorescence microplate reader.

2 Theoretical Part

2.1 Nanocarriers for (oral) Drug Delivery

Nanomedicine, which is the application of nanotechnology for medicinal purposes, has become a great advancement in the treatment of diseases in the last decades.[12] Nanocarriers, which are colloidal particles in a size range from 1 to 1000 nm[13,14] are gaining more and more importance as drug delivery systems. They are employed for the transportation of small molecule drugs or therapeutical biomolecules in the body, which outperforms conventional approaches in many cases. Advantages include improved solubility and permeability of poorly water-soluble but also hydrophilic drugs, high stability by protection against degradation and increased blood residence time. Furthermore, target specific delivery can be accomplished, leading to increased target organ drug concentration and reduced side effects by lower off-target effects.[15] Requirements for such nanocarriers are high safety, biocompatibility, efficiency and loading capacity, low aggregation and a simple preparation.[16] One of the most important benefits of nanomaterials compared to larger particles is the high surface-to-mass ratio,[13] along with other properties arising from the nanosized modification of a material.[17]

Among numerous different routes of drug administration, oral administration is the most accepted and widely used. It achieves highest patient compliance as it provides several advantages like being safe, painless and non-invasive.[16] Furthermore, drugs can easily and at low cost be packed into tablets, capsules or other forms of delivery, this offers the possibility of being easily self-administrable. Taken these benefits into account, it is obvious that the oral route is usually the preferred way of administration. The most challenging barrier to overcome is represented by the complexity of the gastrointestinal tract, where the drug is exposed to different enzymes and extreme changes in pH-value, which can lead to its degradation. Therefore, innovative drug delivery systems are required for many oral applications.

One of the major goals is to attain high drug bioavailability, which is defined as proportion of drug reaching systemic circulation as a fraction of the total amount of drug administrated to the body. Bioavailability is influenced by several factors, which are pharmaceutical preparation (formulation of drug), physicochemical interactions (e.g. food or other drugs), patient factors, pharmacokinetic interactions and first-pass metabolism.[18] In order to achieve the demanded therapeutic concentration of poorly bioavailable drugs in the blood system, it is necessary to either raise the dose or to use a suitable nanocarrier system, which enhances bioavailability.

However, 50 % of drugs delivered via the oral route show poor bioavailability due to their low water solubility.[19] As pharmaceutical agents have to be absorbed in the dissolved state, carriers are required that are capable of improving the solubility of such hydrophobic drugs. Amidon *et al.* (1995) introduced the biopharmaceutics classification system (BCS)[20] to evaluate oral bioavailability, which classifies drug molecules into four groups according to their solubility and permeability (*Figure 1*). This categorisation helps to predict whether absorption is limited by physicochemical properties or due to physiological barriers. Drugs belonging to classes 2 and 4 (both poorly water soluble) are those drugs aimed for at bioavailability improvement by delivery via nanocarrier systems.[21]

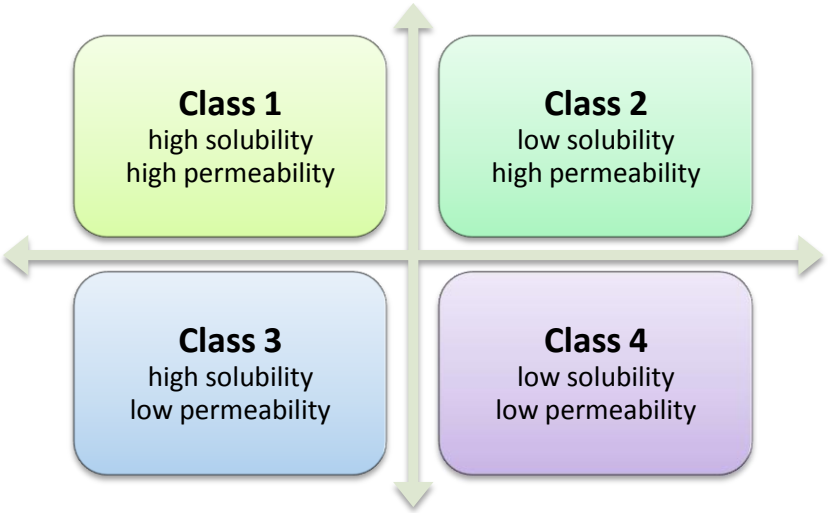


Figure 1: Biopharmaceutics classification system (BCS) according to Amidon.[20]

The spreading of a material through the body and its residence time within the body's tissues is combined in the term biodistribution. It is influenced by different parameters such as method of administration and dose, as well as size, shape, surface charge, surface hydrophobicity, degree of stabilizing agents like poly(ethylene glycol) (PEG), presence and density of targeting ligands and ability to repel adsorption of proteins.[15,22]

As orally taken drugs are resorbed in the stomach or intestine, many of them need to be protected from degradation in the harsh milieu of the stomach (highly acidic and gastric enzymes). The enteric coating of capsules or tablets protects drugs in the stomach, allowing for a drug release only in the intestine, where the coating can be degraded by intestinal enzymes. The mucus gel layer of the intestine forms a barrier for orally administrated drugs, thereby strongly limiting their bioavailability. The particles that cannot pass the mucus layer are cleared from the body without being absorbed.

Drugs absorbed from the gut arrive via the portal vein to the liver where they may be subject to first-pass metabolism,[18] thereby decreasing the available amount of active compound. To prevent that hepatic first-pass effect, drug delivery systems should protect and avoid metabolization in the liver. Alternatively, drugs or nanocarriers can bypass the liver if they are reaching systemic circulation by the intestinal lymph.[23] Finally, the active compounds need to cross one or more cell membranes to get to the demanded site of action, which is situated inside the target cells.[18]

Degradation of many drug molecules in the presence of numerous enzymes radically decreases bioavailability, but nanocarriers beneficially show a protective effect against enzymatic attack.[16] In the case of solid nanoparticles, the metabolic enzymes are too bulky to penetrate the carriers. Regarding the lipid-based carriers, enzymes are too hydrophilic to get into the lipophilic phase.

Moreover, it is crucial that drug delivery systems show a prolonged release of the cargo to provide the requested bioavailability. Due to therapy requirements, a suitable release profile must be provided, ideally in the fashion of minimal release while in circulation but then effective release of drug at the target site.[16,22]

Despite all the mentioned advantages, only a few nanocarriers have been clinically approved yet. This can be attributed to the fact that their applicability is limited by *in vivo* due to many factors.[14] Though, there are some being evaluated in clinical trials at the moment.[24] One of the most promising applications of nanotechnology is the use in cancer therapy,[25] specifically for tumor targeting and as imaging agents. Targeting can be either passively by means of enhanced permeability and retention (EPR) effect of tumors, or actively, by attached targeting ligands on the carrier, which are molecules that specifically bind to receptors.[24] Other than the potential oral applications, it was shown that transdermal applications of liposomes[26] and self-microemulsifying systems[27] are promising candidates for enhanced drug delivery via the skin.

2.2 Types of Nanocarriers

The materials nanocarriers can be made of are numerous, possibly natural or synthetic and are classified into polymeric, inorganic and lipid-based. Various forms are investigated for drug delivery such as polymeric nanoparticles, nanoshells, dendrimers, nanosuspensions, nanoemulsions, solid lipid nanoparticles, liposomes and micelles. *Figure 2* (reprinted from Conniot J. *et al.* (2014)[28]) gives an overview on several different types of nanocarriers.

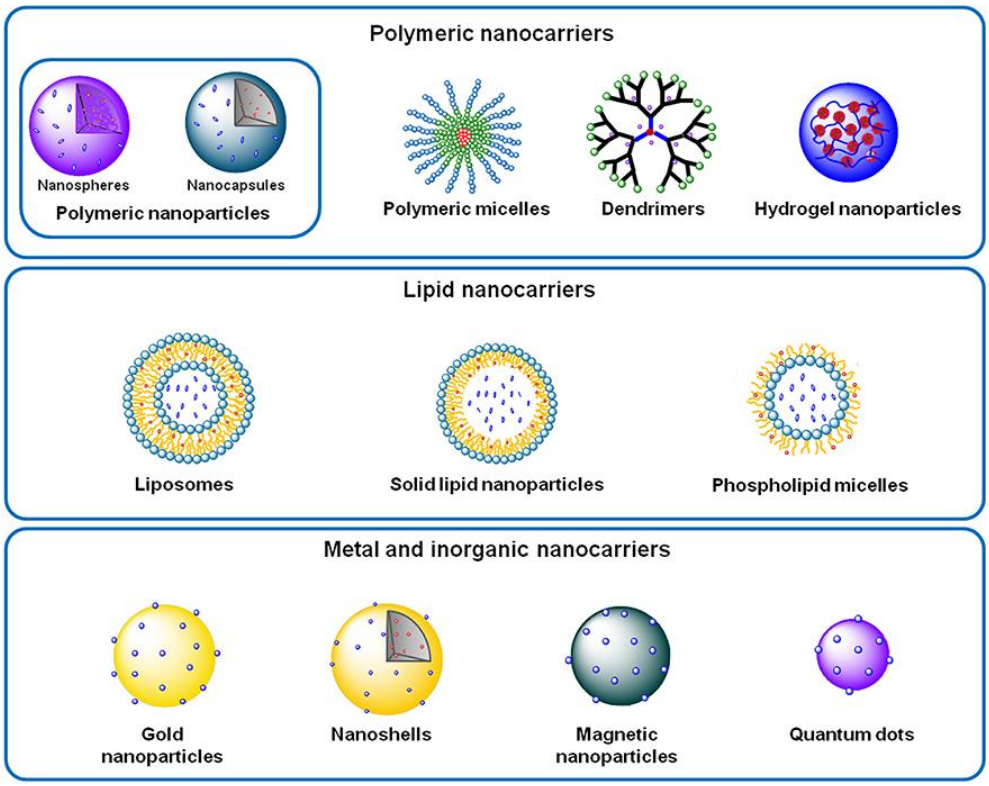


Figure 2: Examples of polymeric nanoparticles, lipid-based carriers and inorganic materials.[28]

2.2.1 Polymeric Nanocarriers

Natural materials for polymeric nanoparticles include chitosan, dextran, gelatin, alginate and agar. Examples for synthetic materials are poly(lactide) (PLA), poly(glycolide) (PGA), poly(lactide-coglycolide) (PLGA), poly(cyanoacrylate) (PCA), polyethylenimine (PEI) and polycaprolactone (PCL).[13,14,29] Drug molecules are either adsorbed on the surface or encapsulated on the inside of particles, depending on the method of preparation and can be either nanocapsules or nanospheres. Drug molecules are dispersed in the matrix and released through degradation, swelling or erosion of the polymer matrix or simply via diffusion down the concentration gradient.[13] The use of biodegradable nanoparticles is of great interest, as they are not accumulated in the body and show good properties to control sustained release of incorporated drugs.[12]

Surface coating of nanoparticles with polyethylene glycol (PEG) is a widespread method of improving several characteristics.[13,15] The increase of physical stability by steric hindrance avoids particle aggregation and prolongs circulation half-life. It also prevents from unfavoured adsorption of proteins. Furthermore, the flexible PEG chains improve water-solubility and are well-hydrated in water where they behave as if they were free in solution.

Special structures include polymeric micelles, single polymer chains and cross-linked hydrogels. Polymeric micelles are made of amphiphilic block co-polymers with surfactant properties, consisting of hydrophilic and hydrophobic segments that result in nanosized liquid colloids in the aqueous medium. As equilibrium structures, micelles are determined by the critical micelle concentration (CMC) above which micellization occurs.[12,15] Another form of polymeric nanoparticles with a tree-like structure is dendrimers, which show relatively low molecular weight, small size (10 - 20 nm) and low polydispersity index.[30] The well defined, regularly hyperbranched architectures are synthesized stepwise, thereby drug molecules can be incorporated in-between branches. Alternatively, drugs can be conjugated chemically or adsorbed onto the surface after preparation of dendrimeres.[4] This allows for good encapsulation properties and high adjustable functionality. The most commonly used material is polyamido amine (PAMAM),[12,15] showing a positive surface charge due to the primary amine groups.

2.2.2 Inorganic Nanocarriers

The primarily metal-based inorganic materials can be produced as almost perfectly monodisperse. Possible candidates as nanocarriers are carbon nanotubes, gold nanoparticles, nanoshells, magnetic nanoparticles or quantum dots. Porous materials like silica, calcium silicate, zeolites etc. can be used as adsorbent particles, releasing the drug molecule as soon as they get into contact with the GI-fluids. Several inorganic nanocarriers were developed not only for treatment of various diseases, but also for their detection and imaging. One of the main reasons for the choice of those materials would be their high stability compared to polymeric nanoparticles or liposomes. One critical drawback of inorganic nanomaterials is that toxicity of accumulating non-biodegradable material in the body may arise.[31]

2.2.3 Lipid-based Nanocarriers

Lipid-based drug delivery systems have gained considerable interest after the commercial success of Sandimmune Neoral (Cyclosporine A), Fortovase (Saquinavir) and Norvir (Ritonavir).[32] This category includes lipid solutions, lipid suspensions, solid lipid nanoparticles (SLN), micelles, liposomes, microemulsions, nanoemulsions and self-emulsifying drug delivery systems.[33] Beneficial biological properties of lipid-based carriers include general biocompatibility, biodegradability, isolation of drugs from the surrounding aqueous environment and the ability to entrap hydrophobic as well as hydrophilic drugs.[24]

Liposomes are spherical versicles made of amphiphilic lipids that self-enclose to hydrophobic spheres of phospholipid bilayers holding an aqueous core.[12] The formation of these thermodynamically stable architectures is due to the hydrophobic effect of the acyl-chains that are surrounded by aqueous medium. The size of vesicles can be controlled by processing conditions, ranging from 30 nm to several micrometers with relatively monodisperse size distributions. Furthermore, they can be created as uni- or multilamellar, which, just as size, depends on the method of preparation. Employed materials can be of natural or synthetic origin, typical components are phosphatidylcholine, cholesterol, diacetylphosphate-o-steroylamylopectin and monosialogangliosides among others.[13,34]

The lipid bilayer can be used to carry lipophilic drugs, whereas hydrophilic molecules can be dissolved in the aqueous core which makes liposomes versatile drug delivery systems. They are beneficial drug delivery systems with regard to drug solubility, versatility (simple surface modification), protection from degradation (under storage conditions and while administration), good biocompatibility and reduced off-target effects.[35] Some of the drawbacks of liposomes involve poor chemical stability and degradation by serum. Moreover, the release of drugs from the liposome carriers cannot be controlled, as the structures fall apart all at once, which leads to a fast burst release.[15] Furthermore, lipophilic drugs can relatively easily escape the liposomes[22] and rapid clearance from the body can be a problem,[24] as well as accumulation of liposomes by macrophages.[36] Therefore even empty liposomes are suspected to cause toxicity to spleen and liver when administrated chronically.

Another type of nanocarrier that has a similar architecture to that of liposomes are the so-called polymersomes which are composed of synthetic polymeric amphiphiles.[24] They are not well established yet, though having similar properties to liposomes. Solid lipid nanoparticles (SLN) consist of a solid core made of lipids or waxes that entrap hydrophobic drug molecules and additionally surfactants to stabilize the suspension. Because of their rigid core, they are more stable than other liquid lipid-based systems.[12,13] Micelles are composed of amphiphilic molecules that aggregate to spherical shapes as they get into contact with water. Typically, the hydrophobic tail regions are packed together in the centre and hydrophilic regions are facing the surrounding aqueous medium. In SLNs and micelles, lipophilic drugs can be incorporated into the hydrophobic core.

Furthermore, lipid-based carriers can be emulsions, subdivided into macro-, micro- and nanoemulsions, which differ physically in terms of droplet size. Smaller size leads to optically transparent dispersions, low viscosity, very high interfacial area and stability against sedimentation. Whereas nanoemulsions are equilibrium systems, microemulsions are thermodynamically stable.[5] Anyway, nanoemulsions are non-sensitive to dilution while microemulsions are.

A special class of emulsions are self-emulsifying drug delivery systems (SEDDS), that can be further differentiated into self-*micro*emulsifying systems (S*M*EDDS) and self-*nano*emulsifying systems (S*N*EDDS), according to the droplet sizes of the resulting emulsions. These systems are opposed to the "normal" emulsions, which are prepared (meaning emulsified) prior to application.

2.3 Self-Nanoemulsifying Drug Delivery Systems

Self-nanoemulsifying drug delivery systems (SNEDDS) are anhydrous forms (preconcentrates) of nanoemulsions. They are isotropic mixtures of oil(s), surfactant(s), solubilizer and drug that spontaneously form heterogeneous oil-in-water (o/w) nanoemulsions as they get into contact with aqueous medium upon gentle agitation. A simplified scheme of a nanoemulsion-droplet is displayed in *Figure* 3. Mean droplet sizes are in the nanometric scale (typically 20 - 300 nm), leading to a high surface area and a transparent appearance. These systems are intended to disperse *in vivo,* to form nanoemulsions as they get into contact to GI-fluids under the digestive motions of the gastrointestinal tract. Optionally, solubilizers can be added to the preconcentrates to improve drug solubility.

Compared to pre-prepared nanoemulsions, the self-emulsifying form offers several advantages like improved physical and chemical stability and it can be filled into capsules which also minimizes palatability-related issues.[5,16,21] The liquid SNEDDS preconcentrates can be administered exemplary encapsulated into gelatin or hydroxypropylmethyl cellulose capsules.[16,33] In various *in vitro* and *in vivo* studies permeation enhancing properties of SNEDDS were demonstrated, which strongly depend on their composition and can be further increased by the use of permeation enhancing fatty acids[37] and other adjuvants. It has to be noticed that an effect merely based on the permeation enhancing properties of used surfactants can be ruled out, as control experiments showed a significantly lower effect in all cases.[16,37]

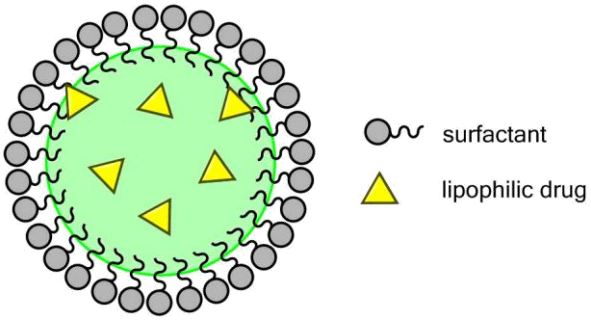


Figure 3: Scheme of a nanoemulsion-droplet. The inner circle represents the oil phase, containing the lipophilic drugs (triangles), enclosed by amphiphilic surfactants, surrounded by aqueous medium (not displayed).

2.3.1 Components of SNEDDS

Most lipid excipients for SNEDDS formulations are esters such as glycerides, propylene glycol esters, PEG-esters of fatty acids and polysorbates. It should be pointed out here that ester-bonds are generally potential substrates of lipolytic enzymes, augmenting biodegradability.[33] Moreover, all the components used in SNEDDS have to be non-toxic and safe. SNEDDS are usually composed of triglycerides together with non-ionic surfactants, the concentration of the latter is always higher than 25 % and can reach up to 60 %.[6] The self-emulsification process was shown to be specific to nature and concentration of compounds (especially oil-to-surfactant ratio) and also to the temperature at which emulsification occurs.[23]

The choice of lipid excipients is determined by several factors, the most important of which is toxicity, especially if nanocarriers are intended for chronic administration. Other factors that have to be taken into account when choosing the oil phase are solvent capacity, melting point, digestibility, capsule compatibility, chemical stability, purity, miscibility and their role in promoting self-dispersibility.[33] Higher lipophilicity of surfactants leads to an enhanced drug loading capacity, but then self-emulsifying properties are decreased. Hence it is necessary to evolve balanced compositions for the best overall performance.[33] Components are selected based on objectives such as achievement of maximal drug loading, minimization of self-emulsification time, protection from drug degradation and reduction of PDI and variation in droplet size.[5]

Oils

The oil phase is crucial to drug solubilisation thus determining the possible dose, and is an important factor in the self-emulsification process. It is known that oils of long-chain fatty acids are more difficult to nano-emulsify compared to medium- or short-chained oils.[5] Examples of commonly used oil phases are reported in *Table 1*, among which synthetic as well as natural oils can be found.[38]

Table 1: Examples of commonly used natural and synthetic oil phases.

Type of oil	Examples (including trade names)
Vegetable oils	Corn oil, soyabean oil, olive oil, castor oil
Medium chain triglycerides	Glyceryl tricaprylate/caprate (e.g. Captex 200)
Mono-, diglycerides	Glyceryl caprylate/caprate (e.g. Capmul MCM)
Propylene glycol esters	Propylene glycol monolaurate (Capmul PG 8)
Fatty acids	Oleic acid

Solubilizers

To enhance drug solubility and thus loading capacity of SNEDDS, co-solvents (solubilizers) are added to formulations at up to 10 % (m/m). Common examples are propylene glycol, glycerol, ethanol, PEG 400 or Transcutol.[39]

Surfactants

Surface active agents, termed surfactants, act as emulsifiers by lowering the interfacial tension which consequently enables oil-in-water (o/w) but also water-in-oil (w/o) emulsification. In SNEDDS, typically 30 - 60 % of non-ionic surfactants are used to stabilize the nanoemulsions. These amphiphilic excipients consist of a hydrophilic part, which shows hydrogen bonds and dipole interactions with the hydration layer of water, and a hydrophobic alkyl chain showing van-der-Waals interactions with the oil phase. Mostly, a combination of two different surfactants is used, where the main surfactant forms the interfacial film and the co-surfactant ensures flexibility of interfacial layer and further reduces the interfacial tension.

Rather lipophilic amphiphiles are generally regarded as safe, whereas more hydrophilic surfactants are considered more toxic, as they increasingly fluidize or solubilise biological membranes. Concerning surfactant charges, the cationic are more toxic than the anionic and the non-ionic are the least toxic. In general, esters are considered less toxic than ethers. Furthermore, more bulky surfactants seem to be less toxic than those with single chains.[33,38] Some examples for commonly used surfactants are given in *Table 2*. Many non-ionic surfactants, such as the castor oil derivative Cremophor EL, are known to have the ability to well enhance permeability and uptake of drugs. While Cremophor EL is non-hydrogenated and therefore has unsaturated alkyl chains, its close relative, Cremophor RH40 is a typical example for a surfactant with saturated alkyl chains. The saturation is achieved by hydrogenation after ethoxylation of the vegetable oil.[23] A beneficial feature of these two surfactants is that they are generally well tolerated on oral administration.[5]

Table 2: Examples of commonly used surfactants and corresponding HLB values.

Trade name	Chemical name	HLB
Cremophor RH40	PEG-40 hydrogenated castor oil	14-16
Cremophor EL	PEG-35 castor oil	12-14
Capryol 90	Propylene glycol monocaprylate	5
Tween 80	Polyoxyethylen(20)-sorbitan-monooleate	15
Span 20	Sorbitan monolaurate	9
Labrasol	Caprylocaproyl macrogol glyceride	12

HLB value

The classification for non-ionic surfactants by Griffin[40] can be done according to the hydrophilic lipophilic balance. The so called HLB value expresses the ratio of hydrophilic and lipophilic regions of a molecule. The dimensionless value is ranging from 0 (most hydrophobic and completely oil-soluble) to 18 (most hydrophilic and entirely water soluble). A more detailed categorization can be found in *Figure 4*.

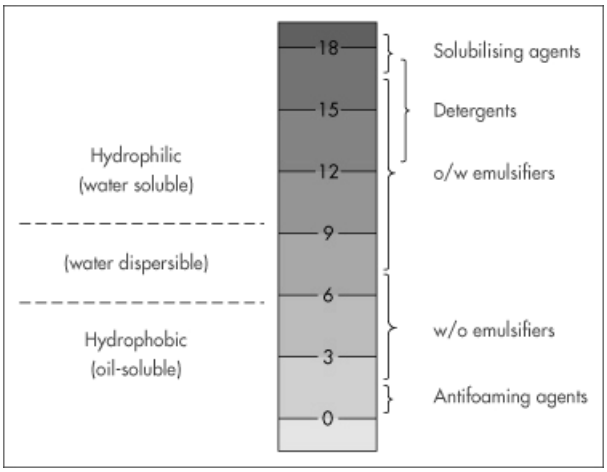


Figure 4: Hydrophilic lipophilic balance (HLB value).[42]

The HLB value of a surfactant is important to the self-emulsification process, as higher numbers (> 12) show faster dispersibility and therefore, those are normally used for stabilising oil-in-water emulsions.[41] However, the compatibility with other excipients and drug solubility has to be taken into account when choosing the desired hydrophilicity of surfactants. The corresponding HLB values for some of the commonly used surfactants are listed in *Table* 2.

2.3.2 Drug Solubility

As 30 % of top marketed drugs in the USA and 70 % of all new drug candidates are lipophilic and thus poorly water soluble,[21] different approaches for solubilisation are followed. SNEDDS are mainly being investigated for the oral delivery of lipophilic drugs as they can easily be incorporated into the lipid based carrier, where the drug is maintained in the solubilised form. As SNEDDS comprise a mixture of more lipophilic and more hydrophilic excipients, they offer high solubilisation capacity to a wide range of drug compounds exhibiting different degrees of lipophilicity.[43] Numerous studies reported an increased dissolution rate of poorly water soluble drugs by incorporation into SNEDDS, for example lacidipine's *in vitro* solubility was significantly increased.[44]

Hydrophilic drugs on the other hand have the tendency to migrate into the aqueous phase during the emulsification process.[45] However, not only hydrophobic but also hydrophilic molecules were reported to be successfully incorporated into SNEDDS. This was achieved by complexation of hydrophilic substances with amphiphilic molecules such as surfactants, phospholipids or fatty acids. Hintzen *et al.* (2014) showed that the model peptide drug leuprorelin whose complex was prepared by hydrophobic ion pairing, displayed a 17-fold improved bioavailability in comparison to a leuprorelin control solution.[2] Following this strategy of complexation by hydrophobic ion-pairing, highly water soluble pharmaceutical agents such as DNA for gene therapy were successfully incorporated into SNEDDS.[37,46]

2.3.3 Permeability through Biological Membranes and Mucus Layer

The improved oral bioavailability of SNEDDS application may be a result of their increased transcellular permeability. This might be based on interactions of SNEDDS components with the phosphilipid cell membrane, leading to higher plasma membrane fluidity and thus enhanced passive transport. In addition, reduction of drug efflux pump activity is another proposed mechanism to increase cellular uptake. Several non-ionic surfactants such as Tweens, Spans and Cremophors have been reported to inhibit efflux transport.[21]

The surface of mucosal tissues is protected by a highly viscoelastic and adhesive mucus layer. From a biological point of view, the mucus layer prevents exogenous substances to enter the circulation, but this characteristic can become a problem to pharmacy. Thus, many of the orally administrated particulate drug delivery systems are caught in the mucosa and become subsequently removed and excreted. This strongly limits the efficiency of such systems and therefore, nanocarriers must be able to quickly permeate the mucus gel layer in order to reach the absorption membrane. So far, several strategies to break down the mucus gel layer were developed. Since destroying the mucus gel layer causes toxicity, more recent strategies rather focus on permeating. Thereby, the use of SNEDDS is one of the approaches to improve diffusion through the intestinal mucus layer, which is achieved by keeping interactions low.[2]

2.3.4 Impact of Physicochemical Properties of SNEDDS

The efficiency of oral absorption and self-emulsification process depends on many formulation-related parameters: surfactant concentration, oil-to-surfactant ratio, polarity of the emulsion, droplet size and charge.[23] The addition of drug can have an influence on the process, depending on its hydrophilicity, pK_a and polarity. Furthermore, pH and temperature of the aqueous phase play a role in the emulsification process.[5]

Droplet Size

The accurate determination of emulsions droplet size is of great importance as it was shown to have a direct impact on *in vitro* evaluations (stability, release kinetics, etc.) as well as on *in vivo* performance.[47] It has to be pointed out that the droplet size of self-emulsifying systems only depends on the composition of formulation, regardless the method of preparation. Reduction in droplet size resulted in an enhanced oral absorption of Cyclosporine A,[48] which was dissolved in microemulsions with exactly the same composition, size was varied by the method of preparation. Thus, it is reasonable to conclude that particle size is of great importance to drug delivery in general and possibly also to self-emulsifying systems.

It was found that mucus permeability was size dependent too, thus increasing with decreasing droplet size. SNEDDS of 12 nm in droplet size showed 70 % permeation compared to 8 % for SNEDDS of 456 nm.[2] Since smaller particles exhibit higher interfacial area to the aqueous environment, a larger surface area is available to the enzymes for hydrolysis. Therefore, smaller droplets lead to a more rapid breakdown of lipids and hence the drug is released quicker from the vehicle.[42]

Zeta Potential

Stability of dispersions strongly depends on the zeta potential. Particles characterised by high values (over ± 30 mV) exhibit strong electrostatic repulsive forces which prevent aggregation and sedimentation. Nanoparticles with a highly positive surface charge (zeta potential ≥ +15 mV) are more likely to be cleared by macrophages, while negatively charged (≤ -15 mV) are rather phagocytosed but interact less with plasma proteins. Still, it appears that a relatively neutral surface charge (zeta potential 0 ± 10 mV) shows the most desirable biodistribution, even though the dispersions are more prone to aggregation.[15] Anyhow, charge-dependent interactions with the biochemical environment in the human body occur inevitably. Positively charged particles are taken up by the cells more effectively. But as the intestinal epithelium has a negative surface charge, positively charged particles are immobilized due to high electrostatic interactions. Thus, neutral or negatively charged particles can permeate the mucus layer better.[2,21] To overcome this conflict, zeta potential changing SNEDDS are being investigated, as an improved overall bioavailability could already be achieved for zeta potential changing nanoparticles.[49] This strategy is based on the assumption that particles pass the mucus layer as negatively charged and subsequently transform into positive to enhance cellular uptake.[2]

2.3.5 Advantages and Disadvantages of SNEDDS

Nanoemulsions in general offer the following advantages: long-term colloidal stability, improved solubility and stability of drugs, high permeation rate, enhanced bioavailability and simplicity of manufacture and scale-up.[5,21] The straightforward one-pot preparation does not require any organic solvent consumption and as the emulsification process happens spontaneously, no additional working step is needed. In addition, SNEDDS reduce inter- and intra-patient variability and food effects when administrated orally and show a quick onset of action via facilitated absorption of the drug. The improvement of bioavailability allows reduction in drug dose and minimizes dose-related side effects of many drugs.[5,21] The formulations exhibit an extended shelf-life as anhydrous preconcentrates, therefore SNEDDS are highly thermodynamically stable in contrast to conventional emulsions or suspensions.[9] The question of long-term stability of nanoemulsions is thus not of crucial relevance, because SNEDDS are stored and applied as preconcentrates.

Thanks to their miniscule globule size, SNEDDS can be easily absorbed through lymphatic pathways, thereby avoiding the hepatic first-pass effect.[50]

Limitations of nanoemulsions, including self-emulsifying systems, are the impossibility to control drug release and compatibility issues among the different excipients and their ratios.[5] As mentioned before, preliminary solubility investigations of chosen drug in most used SNEDDS excipients is necessary, in order to reach a high drug payload. Furthermore, precipitation of the drug might occur in the gastrointestinal tract despite good *in vitro* solubility and evaluation.[51]

2.3.6 Applications

Several potential uses of SNEDDS were reported by Date *et al.* (2010).[5] However, some are currently undergoing clinical trials, but none is ready for the market yet. Some examples are Cyclosporine A,[48,52] Tacrolimus[53] or Ibuprofen.[54] Micro- and nanoemulsions, as well as self-emulsifying systems, are not limited to oral drug administration, but are also investigated for transdermal, ocular, nasal and intravenous drug delivery systems.[33,38]

2.4 Characterisation of Nanoparticles

Adequate characterisation is of great importance to develop reproducible and well defined nanoparticles for drug delivery. The measurement of particle size, shape, zeta potential and chemical properties (e.g. surface functionalisation, impurity, crystallinity) is required for the sufficient description of nanoparticulate systems. It was shown that surface charge and particle size constitute the most important determinants for a variety of biological effects, including cellular uptake, toxicity and dissolution rate.[55–57] Other than that, exemplary scanning electron microscopy (SEM) or transmission electron microscopy (TEM) can be used to characterize surface morphology and structure of nanoparticles.[25] Furthermore, structure and dynamics of emulsions can be studied by nuclear magnetic resonance (NMR).[23]

2.4.1 Size Distributions

Dynamic light scattering (DLS),[58] also known as photon correlation spectroscopy, is one of the most powerful tools to measure various parameters like size distributions and PDI of dispersions. During the measurement, a monochromatic laser beam is passed through the sample which scatters the light in all directions. This Rayleigh scattering occurs if the diameter of particles is smaller than the wavelength of the incident light, as in the case of nanoparticles.

In this way, the random movement (Brownian motion) of particles is measured, its speed depends on size and viscosity of the medium. These motions lead to time dependent intensity fluctuations in the scattered light, leading to positive and negative interferences, which are then transferred into a digital correlator that generates the autocorrelation function for the sample. By applying an algorithm, the size distributions are obtained.

Regarding particle size, different types of values can be obtained. The z-average diameter is the intensity-weighted mean diameter, which is very sensitive to the presence of aggregates or other large particles. The primary measurement result is the intensity size distribution, which is mainly utilized by researchers. Other values that can be obtained via transformations are size by volume (equivalent to mass or weight distributions) and number size distributions.

The polydispersity index (PDI) is a dimensionless value indicating the broadness of size distribution, ranging from 0 (perfectly monodisperse) to 1 (highly polydisperse). Generally, a PDI under 0.3 can be considered as sufficiently monodisperse, whereas 0.7 may already be too poldisperse for the measurement by DLS.

2.4.2 Zeta Potential

The zeta potential or electrokinetic potential is defined as the electrical potential difference between the slipping plane of colloid particles and the dispersion medium. This surface charge, which most colloidal systems in aqueous medium carry, depends on the nature of particles and the surrounding medium. The charge is determined by the ionisation of surface groups, loss of ions from the particle's surface and adsorption of ions to the surface.

The measurement of zeta potential can be done by electrophoretic light scattering (ELS),[58] thereby electrophoresis is triggered by the application of an electric field to the dispersion. The electrophoretic movement is determined by the surface charge of particles and measured by a laser beam via light scattering. Factors that affect the zeta potential are pH, viscosity, conductivity, concentration of dispersion and presence of components like surfactants or polymers. Thus, measurements should be performed in deionized water or a simple buffer system. Dispersants like blood or other complex media cannot be used for this method.

The zeta potential is important for the stability of dispersions because electrostatic interactions lead to higher stability if the value of zeta potential is higher. The stability dividing line is usually situated at around ± 30 mV in aqueous systems. Another fundamental mechanism that affects dispersion stability is steric hinderance, which is simple and irreversible, but requires an extra component.

2.5 In vitro Cell Cultures

2.5.1 Cell cultures

Cell cultures are maintained under sterile conditions at 37 °C, 95 % relative humidity and 5 % CO_2 in an incubator. In order to keep the environment clean of external microorganisms, enzymes and nucleic acids, all work has to be carried out under laminar air flow (LAF). Cells are grown on the bottom wall of culture flasks or plates, in contrast to growth in suspension. Therefore, minimum essential medium (MEM) is used, either with or without antibiotics. Commonly, media contain carbohydrates (energy source), amino acids (building blocks of proteins), proteins and peptides, fatty acids and lipids, vitamins, inorganic salts (for osmotic balance), trace elements, serum, phenol red dye (pH indicator) and a bicarbonate or HEPES buffer system (pH regulation).[59] Furthermore, antibiotics (against contaminations) and other media supplements can be added if required. The selection of cell culture medium is extremely important, as it significantly affects cell culture experiments. The choice strongly depends on the cell type as their growth requirements differ widely.[59] Regarding *in vitro* cell tests, human cell lines like CaCo-2 and HEK-293 show several advantages over other mammalian cell lines, as their protein expression resembles more the human *in vivo* conditions.

2.5.2 CaCo-2 cells

The well-characterised human cell line CaCo-2 is derived from human colon adenocarcinoma, which serves as an *in vitro* model of the intestinal epithelial barrier.[60,61] One of the most advantageous properties is their ability to differentiate spontaneously after reaching confluence (14 - 21 days). The cell monolayers show many typical properties of enterocytes. Often, the reason to choose the colon carcinoma cell line is based on their high similarity to normal intestinal cells with respect to morphology and physiology.[60] As they are heterogeneous cell properties slightly differ, making it difficult to directly compare cell cultures of distinct laboratories. CaCo-2 cells feature tight junctions between cells and expression of typical enterocytic enzymes.[62] This cell model still cannot be put in direct relation to the *in vivo* situation, as normal intestinal epithelium is made up of several different cell types which are protected by mucus layer and covered by an unstirred water layer. However, absence of protective mucus layer and lack of ability to recover from trauma over time are some of the limitations of the CaCo-2 cell model.[63] Still, these cells are in many instances a good choice for several studies, and are frequently used for high-throughput screening for drug delivery programs, especially drug permeability investigations.[61]

2.5.3 HEK-293 cells

Another cell line frequently used in research as an *in vitro* model are HEK-293 cells, retrieved from human embryonic kidney cultures. They are easily grown in cultures using serum-free medium, other benefits are that the cells reproduce rapidly and are relatively easy to transfect.[64–66] Moreover, HEK-293 cells have been widely used to efficiently produce proteins.[65] These advantageous properties recently made this cell type increasingly attractive for cell tests.

2.6 Cellular Uptake Mechanisms and Impacts

2.6.1 Mechanisms of cellular uptake

Passive Transport

Passive transport can be described as a process where particles cross the cell membrane without any energy needs. The driving force is a concentration gradient that leads to particle movement from higher to lower concentration areas. Simple passive diffusion is the most common route, which is strongly depending on lipid-solubility. Hence it is affected by polarity, as the lipophilic molecules diffuse more easily through the phospholipid bilayer of cell membranes. The process involves three steps: the permeants firstly attach to the membrane, then diffuse across and finally are released into the cytosol as free particles or molecules. To facilitate the entry of molecules or particles, membrane-bound carrier proteins induce facilitated diffusion by forming a molecule-protein complex to cross the membrane. It is still a passive way of transportation down the concentration gradient, but it is faster than simple diffusion. In general, small molecules with low molecular weights diffuse much more readily than large ones. Furthermore, the rate of diffusion depends on the concentration gradient. Thus, a higher gradient leads to faster diffusion. Many drugs can exist in the unionized or ionized form, because they are weak acids or weak bases. Therefore, the transfer rate is also dependent on the pK_a value of the molecules and the pH of the solution, as only the unionized form can passively migrate through the membrane.[18,67]

Active Transport

To transport molecules against their concentration gradient, energy is required and thus this process is called active transport. It is performed by molecular pumps and therefore is subject to antagonism and blockades. Additionally, competition or saturation may occur, as there is a fixed number of active transport binding sites.[18] There are several specific or non-specific pathways by which particles and molecules can enter the cells.Firstly, the particle is attached to the cell membrane, then, an area of the cell membrane invaginates around the target and moves into the cell as membrane-bounded vesicles.

There, it can stay inside the created vacuole or be released into the cytoplasm. The unspecific internalisation of large particles of around 1 µm is conducted primarily via phagocytosis by specialized cells (phagocytes) which are macrophages, monocytes or neutrophils.[68] Pinocytosis on the other hand, can be realized by all mammalian cells. It subdivides into macropinocytosis (for particles around 1 µm) and clathrin-mediated, caveolin-mediated, or clathrin- and caveolin-independent endocytosis. The three latter mentioned are the more specific means of receptor mediated cellular entry for particles in the size range of around 200 nm or smaller.[22,69] Which of these pathways is predominant in a particular situation depends on several factors like size and surface characteristics of particles, cell type and ambient physiological conditions.[22,68]

An overview on different pathways of cellular uptake by means of active transport including corresponding particle sizes as suggested is illustrated in *Figure 5,* reprinted from Neoh K. and Kang E. (2012).[70]

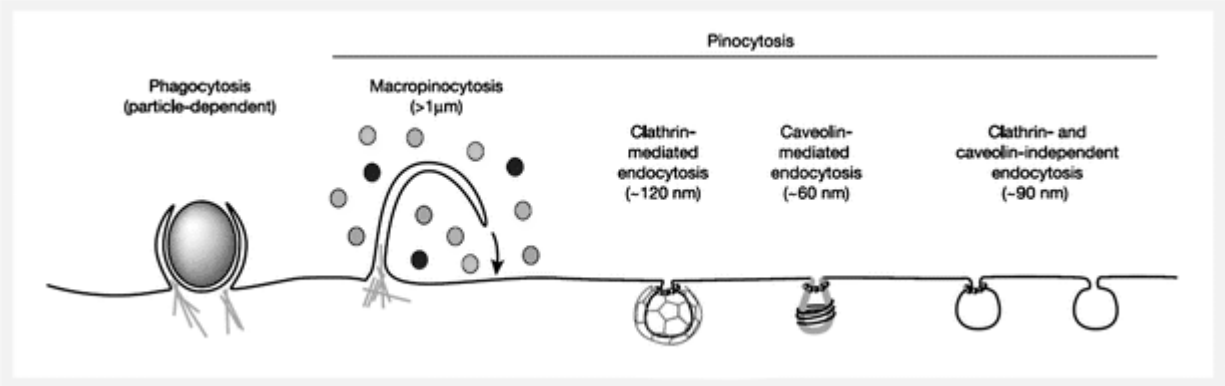


Figure 5: Active transport mechanisms of cellular uptake.[70]

As particles or molecules have entered the cells as endosomes, pinosomes or phagosomes, they can be transported to different cell organelles. Biodegradable particles are broken down by cell lysosomes, while non-degradable particles might be accumulated in the cells. Therefore, long-term cytotoxicity has to be evaluated for the biological use of nanocarriers.[68,71] Other than that, exocytosis or the escape from the cells by metabolism may occur.

2.6.2 Influences of properties of nanoparticles on cellular uptake

Physicochemical properties of particles including size, shape, charge, mechanical properties, chemical composition and hydrophilicity among others, were described to have a crucial impact on the interaction with cells.[68,72] Internalization amount and rate of carriers into cells are of great importance for designing the materials with a most desirable biological performance. Furthermore, cell physiology has an effect on particle internalization, which makes cellular uptake conditional on the cell-type.[66,73]

Size

Cellular uptake can take place for colloidal particles with sizes ranging from several nanometres to over a micrometer. There are several different pathways of cellular uptake, strongly determined by the size of particles. Even though the processes have not been fully understood yet, some rules regarding scales already exist. It is known that amount and rate, thus efficiency, of uptake, are dependent on the membrane wrapping time, which leads to an inefficient uptake of extremely large or small particles.[10]

Many investigations on size-dependence have been carried out for a great number of nanocarriers under varying conditions. Smaller particle sizes were often correlated to a greater extent of uptake,[74] though there might a limit at the bottom end. 100 nm sized nanoparticles showed a many times higher uptake by intestinal tissues compared to larger particles.[12] The same size dependence was found for CaCo-2 cells for microparticles, showing that the smallest among them (100 nm) achieved the highest uptake.[74] Furthermore, it was demonstrated that several polymeric nanoparticles reached best uptake efficiency if size was between 100 and 200 nm.[25] In regard of lipid based nanocarriers, a strong size-dependence cellular uptake was found for liposomes on CaCo-2 cells.[11] Particle sizes between 40 and 300 nm were tested, among which the smallest showed almost a 12-fold increase of uptake in comparison to the largest (> 160 nm).

Additionally, it was found by pharmacological inhibitor block method that uptake mechanisms of liposomes were size-dependent. In an *in vitro* and *in vivo* study about the influence of particle size on the bioactivity of curcumin lipid nanoemulsion,[29] it was shown that among various particle sizes (50, 100 and 200 nm), the lipid nanoemulsion with a droplet size of 100 nm had the best bioactivity and thus highest potential for enhancing bioavailability. Highest cellular uptake efficacy for particles sized around 100 nm could be explained by the internalization via receptor-mediated endocytosis. This can only take place for particles smaller than 100-200 nm, while larger particles have to be taken up by phagocytosis or macropinocytosis.[75]

However, experimental observations are not always concurrent and may generate controversial results, as the uptake of particles follows different mechanisms depending on a number of parameters. Moreover, sedimentation and agglomeration of the nanosized colloids may become a significant problem, which is rarely taken into consideration in most investigations. Not to forget that the cell type determines the endocytic pathways.

Other impacts

Apart from particle size, there are many other factors that have an effect on cellular uptake. The shape of colloidal particles shows a more complicated dependency and has therefore been less investigated so far. Among the various shapes, the more complex ones show higher membrane wrapping time, which is due to a higher surface area in comparison to the simpler spherical particles. This is a problem to clarity of results, as shape and size depend on each other. Thus, only the overall use of spherical particles eliminates the shape factor.

Surface chemistry of colloidal particles is an important factor affecting cellular uptake, as the particle-cell surface interactions determine the pathway and the extent of interaction to receptors. The driving forces are electrostratic, van der Waals and hydrogen bonding interactions. The most relevant surface characteristics are hydrophilicity, charge and presence of ligands.[68] Concerning surface charge, it was found out that positively and negatively charged particles utilize different endocytosis pathways.[76] In addition, uptake efficiency was reported to be highest if the zeta potential of nanoparticles was positive.[49] Since endocytosis involves several mechanical steps which induce deformation of particles, their mechanical properties are suggested to have an influence on cellular uptake. Therefore, the stiffness of particles is supposed to affect uptake efficiency and pathway, as wrapping might be easier for stiffer particles.[77] Moreover, surface roughness of the substrate might influence the cellular uptake.

3 Experimental Part

3.1 Materials

Dermofeel MCT and Captex 200 were gifts from Abitec and propylene glycol was purchased from Gatt-Koller. All other reagents were purchased from Sigma–Aldrich, Vienna, Austria and were of analytical grade. Semisolid lipids were melted and homogenized before use and divided in small portions to avoid heating cycle stress.

3.2 Preparation of SNEDDS Formulations

Formulations were screened by homogenising lipids, surfactants and solubilizer with a vortex mixer and analysed visually regarding the stability immediately and after 24 h incubation time at room temperature. Formulations not presenting any phase separation were considered as stable. Thus, those with cloudy appearance were discarded due to incompatibilities between the excipients, whereas clear and stable formulations were selected for further studies. The chosen components for this study are listed in *Table 3* with corresponding HLB values[33,78] and respective function.

Table 3: Components used for the preparation of SNEDDS formulations.

Trade name	Chemical name	HLB	Function
Propylene glycol	1,2-Propandiol	-	solubilizer
Cremophor RH40	PEG-40 hydrogenated castor oil	14-16	surfactant
Cremophor EL	PEG-35 castor oil	12-14	surfactant
Tween 80	Polysorbate 80	15	surfactant
Capryol 90	Propylene glycol monocaprylate	5	surfactant
Captex 200	Propylene glycol dicaprylocaprate	< 1	oil
Dermofeel MCT	Glyceryl tricaprylate	< 1	oil

3.3 Characterisation of SNEDDS

The SNEDDS formulations were emulsified at a concentration of 0.1 % in different media: phosphate buffer pH 6.8, distilled water and OptiMEM at room temperature and subsequently characterized regarding size, polydispersity index (PDI) and zeta potential. Formulations were selected according to size: those with a size below 100 nm had a bluish appearance, whereas formulations with size above 100 nm tended to progressively display more white emulsions. Cloudy and instable emulsions were discarded.

Mean droplet size and polydispersity index were determined by dynamic light scattering using a Zetasizer Nano Series (Malvern Instruments). The measurements were performed at a concentration of 0.1 % SNEDDS, directly after preparation of nanoemulsions and after 4 h of incubation at 37 °C. The zeta potential was measured at a concentration of 1 % SNEDDS in distilled water at pH 7. The automatic optimization method was used for both kinds of measurements.

3.4 Cell Culture

HEK-293 and CaCo-2 cells were cultivated in minimum essential medium (red MEM) containing phenol red, Earls salts, 10 % fetal bovine serum and 1 % penicillin and streptomycin. The cells were split twice a week and the culture medium was replaced every 48 hours. The cells were maintained in an incubator at 37 °C under 5 % CO_2 and 90 % relative humidity.

OptiMEM Reduced-Serum Medium, which was used to prepare SNEDDS emulsions, contains insulin, transferrin, hypoxanthine, thymidine and trace elements. These additional components allow for the reduction of the serum supplementation by at least 50 %. This medium contains a sodium bicarbonate buffer system (2.4 g/L), and therefore requires a 5-10 % CO_2 environment to maintain physiological pH.

3.5 Cytotoxicity Study

The potential cytotoxic effect of chosen formulations was tested via resazurin assay on HEK-293 and CaCo-2 cells monolayers.[79] Cells were seeded at a concentration of 4×10^5 cells / mL MEM and cultured on 24 well plates, using 500 µL per well in total. The cells were incubated at 37 °C under 5 % CO_2 until reaching nearly confluence in monolayers. Firstly, cells were carefully washed with pre-warmed (37 °C) DPBS (phosphate buffer containing Ca^{2+} and Mg^{2+}) which was chosen because HEK-293 cells easily detached within the washing steps with PBS not containing Ca^{2+} and Mg^{2+}. Then, the cells were incubated with 500 µL per well of SNEDDS emulsified in OptiMEM at different concentrations (0.5 %, 0.25 %, 0.1 % and 0.05 % m/v) in triplicates. Untreated cells (only OptiMEM) were used as positive control whereas a Triton-X-100 solution (0.2 % in water) served as negative control.

After 4 h of incubation, the samples were removed and the supernatant was discarded. Subsequently, the cells were washed with pre-warmed DPBS and replaced with 500 µL of resazurin solution previously prepared in a concentration of 0.15 mg / mL in white MEM. After 90 min of incubation, 10 µL of each well were diluted to 100 µL with white MEM into a 96 black well plate (Greiner 96 Flat Bottom Black Polystyrol). The fluorescence of converted resazurin was measured with a 540 nm $_{excitation}$ / 590 nm $_{emission}$ filter set with a microplate reader (Tecan infinite, M200 spectrometer, Grödig Austria).[45,79]

The viability of cells was calculated as a fraction of untreated cells control according to equation (1):

$$cell\ viability\ [\%] = \frac{Fs - Fx}{Fu - Fx} \times 100 \qquad (1)$$

where *Fs* represents the fluorescence of the samples, *Fx* is the fluorescence of the Triton-X-100 treated cells (blank) and *Fu* is the fluorescence of the untreated cells (reference value).

3.6 In vitro Cellular Uptake Study

SNEDDS formulations were labelled with 0.1 % (v/w) fluorescein diacetate (FDA) previously dissolved in ACN (25 mg / mL).[46] Then, the loaded formulations were emulsified in OptiMEM at a concentration of 0.05 % (w/v). Cells were cultured in 24 well plates as described previously. Firstly, the cells were washed with pre-warmed DPBS, afterwards the samples (500 µL per well) were added in triplicates to the cells. Untreated cells served as blank (0 % control), whereas the unwashed cells previously incubated with the sample served as 100% reference.

After 4 h of incubation time at 37 °C, the nanoemulsions were removed and the cells were washed twice with pre-warmed DPBS. Then, 500 µL of an aqueous solution of 5 M NaOH, 2 % Triton-X-100 was added to the content of wells for cell disruption and activation of FDA. After 30 min, the complete content of each well was transferred in Eppendorf tubes and centrifuged at 13 400 rpm for 5 min to remove the solid cell components. 100 µL of the supernatant were transferred in a 96 black well plate (Greiner 96 Flat Bottom Black Polystyrol) for analysis. The fluorescence was measured at 485 nm $_{excitation}$ / 515 nm $_{emission}$ with a microplate reader (Tecan infinite, M200 spectrometer, Grödig Austria).[46]

The uptake efficiency was calculated in percent according to equation (2):

$$uptake\ efficiency\ [\%] = \frac{Fa - Fx}{Fb - Fx} \times 100 \qquad (2)$$

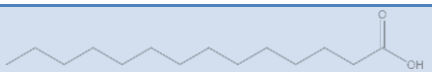
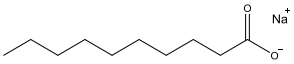
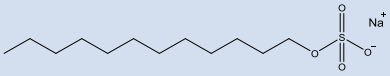
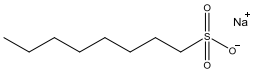
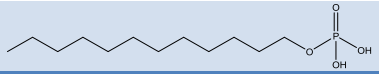
where *Fa* and *Fb* represent the fluorescence of the samples with and without removal of the formulation, respectively. *Fx* represents the fluorescence of the untreated cells (blank value).

3.7 Functional Excipients: Anionic Surfactants

Anionic surfactants were dissolved into the pre-mixed formulation via vortexing, heating to 70 °C and sonication. The maximum solubility of each excipient in the chosen formulation was evaluated. The selected anionic surfactants are listed in *Table 4.*

The loaded SNEDDS were characterized regarding size, zeta-potential, cell toxicity and uptake efficiency as reported previously. Thereafter, the potential cytotoxic effect and uptake of SNEDDS loaded with negatively charged surfactants was tested on CaCo-2 cells, which were grown on 24 well plates for 10 days. Therefore cells were incubated with 0.05 % SNEDDS containing 1 % of anionic surfactants in OptiMEM for 4 h.

Table 4: Selected anionic surfactants used as functional excipients.

Name	Chemical formula		Abbreviation
Myristic acid	$C_{13}H_{27}COOH$		COOH
Sodium decanoate	$C_9H_{19}COONa$		COONa
Sodium dodecyl sulfate	$C_{12}H_{25}SO_4Na$		SO4
Sodium heptanesulfonate	$C_7H_{15}SO_3Na$		SO3
Mono-N-dodecyl phosphate	$C_{12}H_{25}PO_4H_2$		PO4

4 Results and Discussion

4.1 Preparation of SNEDDS Formulations

The components used in SNEDDS formulations can be classified into non-polar lipids, w/o surfactants (HLB < 12), o/w surfactants (HLB > 12) and solubilizers, where the water immiscible w/o surfactants can also be used as oils.[39] According to this classification, selection criteria of excipients for SNEDDS formulations were developed. SNEDDS are typically composed of 10 % solubilizers, 30-35 % o/w surfactants, 20-30 % w/o surfactants and 20-30 % oils. It is known that the surfactants Cremophor EL and Cremophor RH40 are biocompatible and provide great bioavailability in combination with propylene glycol.[80] Furthermore, Tween 80 already demonstrated the ability to reduce particle size effectively.[29] To develop formulations that meet the requirements of this study, propylene glycol was used as solubilizer and one of the previously mentioned Cremophors as surfactant. They were screened in different combinations and ratios with co-surfactants and lipids.

As illustrated in *Table 5*, lipid formulations can be classified after *C.W Pouton* into type I, II, III A, III B and IV, where the most hydrophilic formulations belong to type I and the most hydrophobic to type IV. This classification was done according to the composition of lipid-based formulations and the effect of dilution and digestion.[81] Class I formulations are only composed of oils (or added very low amount of surfactant). As class IV formulations are oil-free, they are fairly polar systems of surfactants and solubilizers. Self-emulsifying drug delivery systems are represented by classes II and III,[45] showing a rather balanced composition. It should further be mentioned that the presence of organic solubilizers has a negative impact on shelf-life stability of preconcentrates.[33]

Table 5: Lipid formulation classification scheme (LFCS) according to Pouton.[81]

	content of formulation [%, w/w]				
Class	**I**	**II**	**III A**	**III B**	**IV**
Oils (HLB < 1)	100	40-80	40-80	<20	-
Water-insoluble surfactants (HLB < 12)	-	20-60	-	-	0-20
Water-soluble surfactants (HLB > 12)	-	-	20-40	20-50	30-80
Hydrophilic solubilizers	-	-	0-40	20-50	0-50
Particle size of dispersion	coarse	100-250	100-250	50-100	<100
Lipophilicity					
Dispersibility					
Digestibility					
Effect of dilution					

The aim of this study was to compare different droplet sizes of formulations composed of same excipients but at different ratios. As displayed in *Table 6* and *Table 7*, two main compositions were developed (termed C and E lines). Thereby, the size of the formulations was tuned by varying the ratio between the oil and the surfactants. It was noticed that the size increased by decreasing the amount of surfactants. This is due to the fact that surfactants, being surface active agents, lower the interfacial tension between oil and water phases. In order to obtain stable formulations, it was found out that the oil share needed to be between 35 and 55 %. Furthermore, many of the developed formulations had to be excluded from further studies, because the phase separation of the preconcentrate after some time implies instability of the mixture.

4.2 Characterisation of SNEDDS

Droplet size (by intensity), polydispersity index (PDI) and zeta potential of the selected formulations are reported in *Table 6* and *Table* 7. Droplet sizes are ranging from 30 to 150 nm with a PDI below 0.25, which is the suggested highest acceptable PDI value.[82] Two kinds of compositions (C and E) were selected, as stable preconcentrates and nanoemulsions with variable droplet size and narrow PDI were obtained. For each composition, three formulations each were chosen showing small (~ 40 nm), medium (60-90 nm) and large (> 100 nm) droplet sizes. Formulations with a PDI above 0.3 were ruled out, because these nanoemulsions were too polydisperse for this purpose. Indeed, to evaluate the effect of the droplet size of SNEDDS on the cellular uptake efficiency, a PDI as narrow as possible is required to avoid overlapping populations. Moreover, no significant change in size distribution could be observed after 4 and 24 h at 37 °C, which demonstrates the stability of the developed formulations over time.

SNEDDS dispersed in different media, such as 100 mM phosphate buffer (pH 6.8), distilled water or culture medium, did not vary in droplet size. Furthermore, it was found out that the concentration of nanoemulsions had no significant influence on size distribution. The incorporation of FDA into SNEDDS had no negative impact on their droplet size and stability (*Table 8*).

The zeta potential could be measured solely in distilled water due to conductivity issues. At concentrations below 1 % (m/v) the signal-to-noise ratio was too high for retrieving an acceptable signal. The formulations displayed not significantly different zeta potential, which was slightly negative (~ -5 mV) for all formulations except for formulation E3 which displayed a slightly positive zeta potential. Although several optimisation efforts were made, standard deviation of zeta potential remained quite high.

Table 6: Composition, droplet size, PDI and zeta potential of chosen formulations (C 1-3).

	Composition [m%]								
	PG	TW80	RH	C200	Droplet size [nm]		PDI	Zeta potential [mV]	
C1	10	30	30	30	35,7	± 11,6	0,096	- 4,5	± 4,6
C2	10	20	30	40	60,1	± 21,0	0,103	- 5,2	± 8,9
C3	10	25	15	50	100,2	± 46,3	0,177	- 6,8	± 4,9

PG: Propylene glycol, TW80: Tween 80, RH: Cremophor RH40 and C200: Captex 200.

Table 7: Composition, droplet size, PDI and zeta potential of chosen formulations (E 1-3).

	Composition [m%]								
	PG	Cp90	EL	MCT	Droplet size [nm]		PDI	Zeta potential [mV]	
E1	10	25	35	30	46,0	± 10,0	0,026	- 4,4	± 18,0
E2	10	10	40	40	89,9	± 34,4	0,131	- 3,7	± 5,6
E3	10	20	20	50	144,0	± 45,0	0,086	+ 2,5	± 4,7

PG: Propylene glycol, EL: Cremophor EL, Cp90: Capryol 90 and MCT: Dermofeel MCT.

Table 8: Droplet size and PDI of FDA loaded SNEDDS. Immediate measurement (0 h) and stability test after 4 h of incubation at 37 °C.

	0 h			after 4 h		
	Droplet size [nm]		PDI	Droplet size [nm]		PDI
C1	43,5	± 18,3	0,138	34,5	± 11,8	0,091
C2	60,3	± 25,1	0,186	73,3	± 30,4	0,204
C3	135,8	± 83,8	0,213	116,7	± 52,2	0,185
E1	45,0	± 10,3	0,042	49,6	± 15,2	0,075
E2	83,2	± 35,0	0,133	72,2	± 26,6	0,104
E3	147,0	± 40,1	0,061	149,3	± 46,0	0,076

4.3 Cytotoxicity Study

Among several cell viability assays, the resazurin method is frequently used to monitor viable cell numbers.[79] Resazurin is a cell permeable redox-indicator that can be dissolved in a physiological buffer or culture medium, resulting in a deep blue solution that can be directly added to cells. As illustrated in *Figure 6*, resazurin molecules are reduced by the coenzyme NADH of living cells to form pink fluorescent resorufin. The number of viable cells is proportional to the quantity of resorufin produced which is measured by fluorescence using a microplate reader with a 560 nm / 590 nm filter set. Major advantages over other cell viability assays are higher sensitivity, homogeneous format and relatively inexpensiveness. However, resazurin itself causes moderate cytotoxicity, thus the incubation period is usually restricted to 1 to 4 hours.

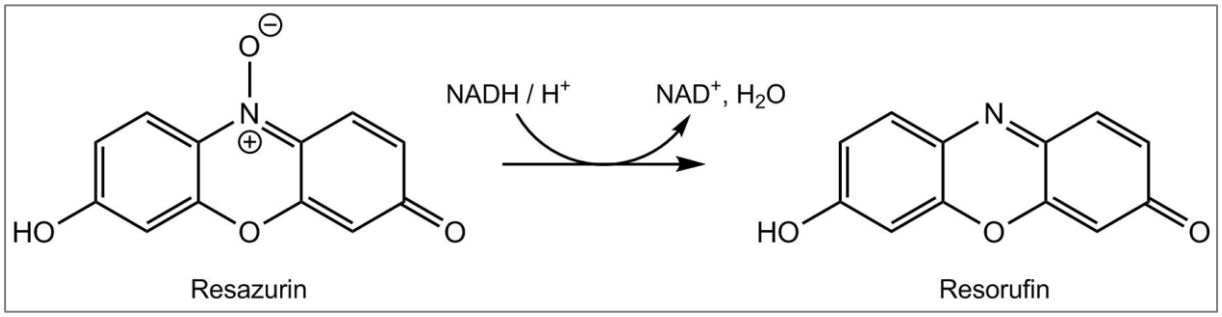


Figure 6: Reduction of Resazurin to Resorufin by NADH/H$^+$ in viable cells.

The cytotoxic effect of the chosen formulations was evaluated using the resazurin method as proposed by the *Assay Guidance Manual*.[79] Thereby, a concentration dependent toxicity was observed. As displayed in *Figure 7*, a concentration of 0.05 % of SNEDDS formulation C1 had no cytotoxic effect. Since cell viability was requested to exceed 90 %, a concentration of ≥ 0.1 % of formulation C1 was too toxic on HEK-293 cells. Subsequently, 0.05 % was selected as concentration for further studies and used with all chosen formulations (C 1-3 and E 1-3) on HEK-293 and CaCo-2 cells. As it can be seen in *Figure 8,* all formulations displayed similar toxicity on both cell lines. Values exceeding 100 % might be due to measurement errors, or caused by a more pronounced conversion of resazurin if treated with low amounts of SNEDDS formulations.

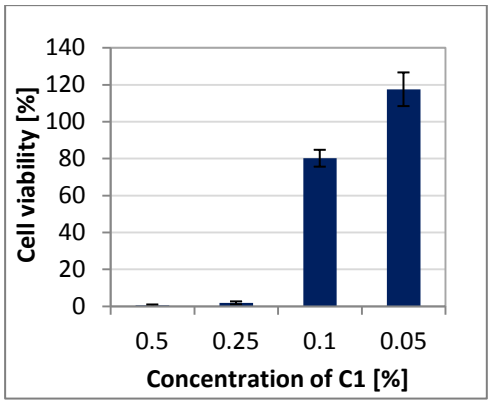


Figure 7: Viability of HEK-293 cells at different concentrations (0.5, 0.25, 0.1, 0.05 %) of formulation C1 SNEDDS in OptiMEM.

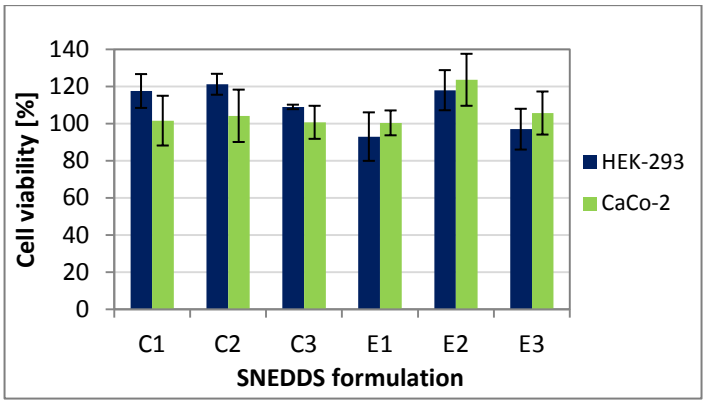


Figure 8: Viability of HEK-293 and CaCo-2 cells for chosen formulations at 0.05 % SNEDDS in OptiMEM.

4.4 In vitro Cellular Uptake Study

For the quantification of the cellular uptake of SNEDDS, fluorescein diacetate (FDA) was chosen as fluorescence marker due to its simple application and its lipophilicity, which leads to good encapsulation into the oil phase.[8,83] As FDA was not completely soluble in any components of the formulation, it was added as saturated acetonitrile solution to the preconcentrates. After accomplishing incubation time with FDA-loaded SNEDDS, the acetate groups of the fluorescence marker were cleaved from the molecule by addition of NaOH solution. *Figure 9* displays the reaction equation of the FDA activation. Triton-X-100 was an additional adjuvant in the sodium hydroxide solution to lyse the cells in order to release the agent from the cytoplasm. As measurements should be done in a homogeneous medium,[83] cell components are centrifuged off to minimize fluorescence interferences.

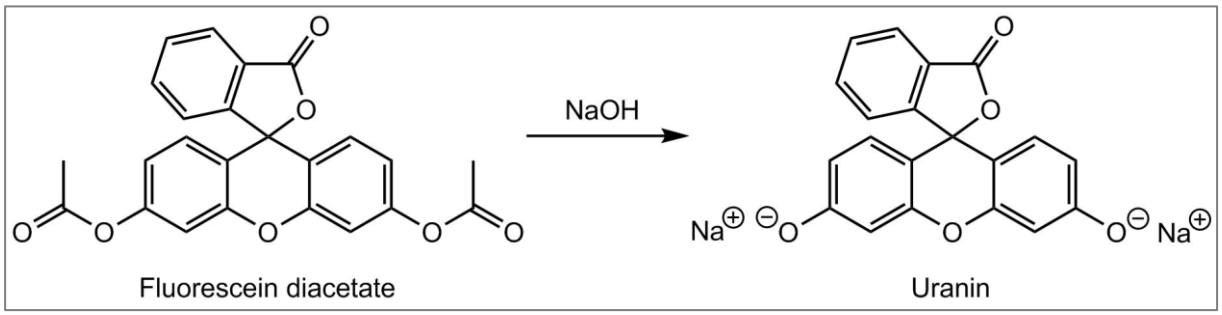


Figure 9: Conversion of fluorescein diacetate (FDA) to green fluorescent uranin by NaOH.

In a preliminary study, no significant uptake of naked FDA could be observed under the same conditions when using the same amount of FDA per well. Furthermore, it could be assumed that the lipophilic, water insoluble molecules remained incorporated in the SNEDDS.[8]

Uptake efficiencies are reported in *Figure 10* and *Figure* 11, where, regarding formulations E 1-3, no size dependent uptake efficiency could be observed for neither cell type. However, formulations C 1-3 showed a size dependence, but not tendency to higher uptake efficiencies for smaller particles. The differing efficacies for formulations of the C-line are not necessarily due to changes is particle size, but are more likely due to the particular ratio between excipients of formulations. Moreover, as the C-line showed a higher cellular uptake than the E-line on both cell types, it can be concluded that the cellular uptake is influenced to a higher degree by the composition than by the size of SNEDDS.

Research has shown a concentration dependent effect of surfactants on permeability on cell membrane.[84–86] Therefore a conflict occurs, as a change in droplet size of SNEDDS is achieved by modifying the composition, mainly by varying the amount of surfactant. Keeping the ratios constant does not lead to satisfying conditions either, as a change in droplet size is then achieved by using different compounds, which was already shown to influence cellular uptake. Furthermore, it is possible that SNEDDS are taken up by the cells via multiple endocytosis pathways, as it was suggested for the internalisation of nanoemulsions.[46] The different uptake efficiencies on HEK-293 and CaCo-2 cells are consistent with the investigated cell line dependence of internalization pathways.[66]

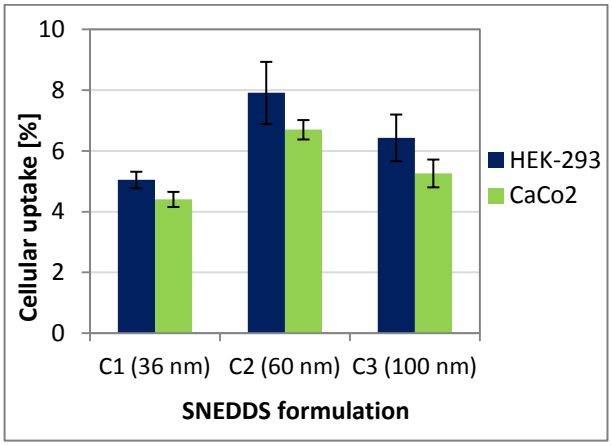


Figure 10: Uptake efficiencies of FDA loaded SNEDDS formulations C 1-3 at 0.05 % in OptiMEM on HEK-293 compared with CaCo-2 cells after incubation of 4 h.

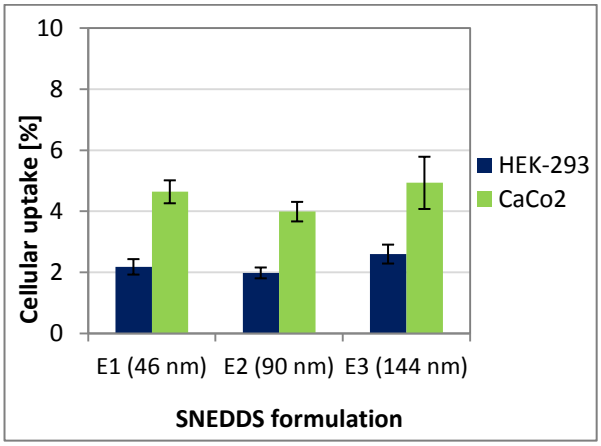


Figure 11: Uptake efficiencies of FDA loaded SNEDDS formulations E 1-3 at 0.05 % in OptiMEM on HEK-293 compared with CaCo-2 cells after incubation of 4 h.

4.5 Functional Excipients: Anionic Surfactants

4.5.1 Preparation and Characterisation

The tested anionic surfactants were incorporated in formulation C2, which was chosen because it exhibited the highest cellular uptake efficiency. Different concentrations of the anionic excipients were investigated regarding solubility and possible change in droplet size of loaded SNEDDS (*Table 9*). The solid additives showed very dissimilar solubility behaviour in the formulation C2. Myristic acid dissolved quickly up to high concentrations (≥ 10 % at 70 °C), whereas sodium decanoate was not soluble at 2 % through heating at 70 °C and sonication for 2 h. Sodium dodecyl sulfate and heptanesulfonate were soluble at up to 3 % in formulation C2. It was possible to dissolve dodecyl phosphate at 2 % via heating at 70 °C, but the mixture crystallised after reaching room temperature. Since all the surfactants could be loaded by at least 1%, this concentration was used for further studies.

Table 9: Characterisation of SNEDDS formulation C2 loaded with different concentrations of anionic surfactants.

		A			B			C	
	Loading	**Droplet size [nm]**		**PDI**	**Droplet size [nm]**		**PDI**	**Zeta potential [mV]**	
Myristic acid	0,5 %	55,7	± 18,1	0,089	-	-	-	-	-
	1 %	53,7	± 17,7	0,096	47,3	± 15,3	0,121	-6,22	± 11,3
	2 %	51,5	± 16,9	0,121	-	-	-	-	-
Sodium decanoate	0,5 %	57,1	± 19,9	0,102	-	-	-	-	-
	1 %	53,0	± 21,5	0,142	59,3	± 21,6	0,113	-13,1	± 5,8
	2 %	-	-	-	-	-	-	-	-
Sodium dodecyl sulfate	0,5 %	60,7	± 21,9	0,128	-	-	-	-	-
	1 %	56,5	± 18,8	0,155	51,7	± 20,0	0,134	-28,2	± 28,5
	2 %	59,1	± 25,8	0,183	-	-	-	-	-
Heptane sulfonate	0,5 %	55,4	± 19,3	0,108	-	-	-	-	-
	1 %	57,0	± 18,2	0,160	57,1	± 20,0	0,103	-4,07	± 9,9
	2 %	64,5	± 27,9	0,147	-	-	-	-	-
Dodecyl phosphate	0,5 %	62,8	± 27,8	0,234	-	-	-	-	-
	1 %	53,3	± 17,2	0,232	50,8	± 19,4	0,123	-19,9	± 27,6
	2 %	55,7	± 20,0	0,132	-	-	-	-	-

A: Droplet size and PDI of loaded nanoemulsions (0.1 % in PBS), B: droplet size and PDI with additional 0.1% FDA loading (0.1 % in PBS), and C: zeta potential of nanoemulsions without FDA using 1 % SNEDDS in deionized water.

As can be seen in *Table 9*, droplet size of nanoemulsions depends on the amount of loaded anionic surfactants. A tendency to smaller sizes at increased loading concentrations could be observed when compared to the empty formulation C2. One exception is the addition of heptane sulfonate, where the droplet size seems to increase with higher amounts of loading. Though, it has to be noted that standard deviation and PDI of loaded SNEDDS is increased compared to the empty formulation. Moreover, the addition of myristic acid and heptane sulfonate did not significantly alter the zeta potential of the loaded formulations, whereas sodium decanoate, sodium dodecyl sulfate and dodecyl phosphate showed an increase in negative zeta potential in respect to formulation C2 (*Table 9*).

4.5.2 Cytotoxicity Study on CaCo-2 Cells

The potential cytotoxic effect of SNEDDS loaded with negatively charged surfactants was tested on CaCo-2 cells. The results are displayed in *Figure 12*. Incubation of 0.05 % SNEDDS exhibiting 1 % loading of anionic excipients on CaCo-2 cells showed no cytotoxic effect (viability > 90 %) after 4 h. This concentration of emulsion was used for comparability reasons with previous experiments.

4.5.3 Cellular Uptake Study on CaCo-2 Cells

All anionic surfactants revealed an increased cellular uptake efficiency in comparison to the empty C2 as illustrated in *Figure 13*. Myristic acid and heptane sulfonate led to an approximately 1.6-fold increase in uptake efficiency, whereas sodium decanoate and sodium dodecyl sulfate showed a roughly 30 % higher uptake compared to the empty formulation. On the contrary, dodecyl phosphate did not cause a significantly higher cellular uptake in CaCo-2 cells after 4 h. Thus, it can be assumed that SNEDDS containing anionic surfactants in general enhance uptake, which is also dependent on the exact type of functional group. This finding is coherent with reports of increased permeability in the presence of sodium dodecyl sulfate (SDS).[87,88]

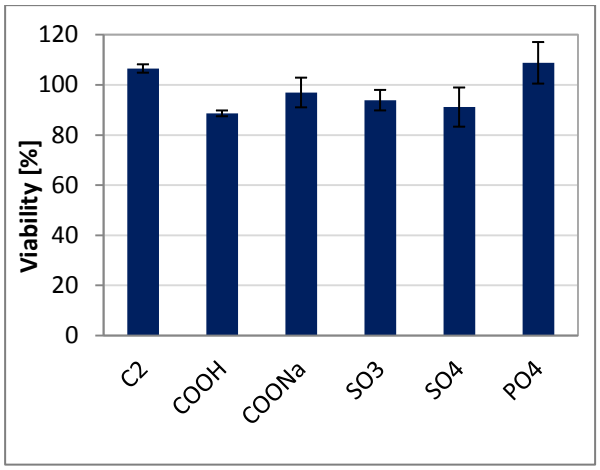


Figure 12: Viability of CaCo-2 cells after 4 h of incubation with 0.05 % SNEDDS loaded at 1 % of anionic surfactants.

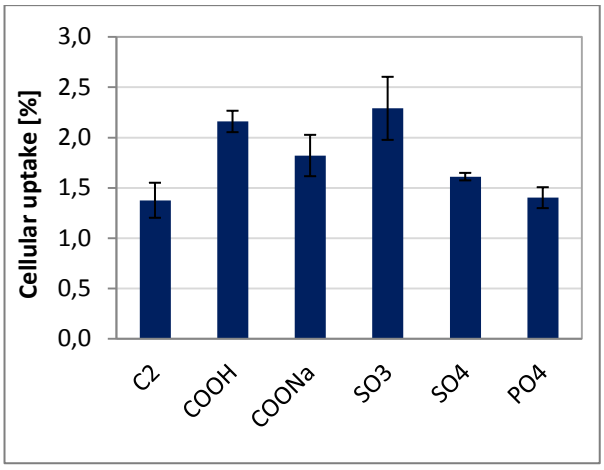


Figure 13: Cellular uptake efficiency in CaCo-2 cells after 4 h of incubation with 0.05 % SNEDDS loaded at 1 % of anionic surfactants.

5 Conclusion and Outlook

Contrary to prior expectations, no tendency in uptake efficiency was observed with regard to droplet size. As particle sizes around 100 nm showed highest uptake efficiencies for nanoparticles,[25] liposomes[11] and microemulsions,[38] similar results were suspected for SNEDDS. But, according to these experiments, droplet sizes of self-nanoemulsifying systems showed no direct impact on the cellular uptake efficiency on either CaCo-2 or HEK-293 cells. It can be ruled out that overlapping size populations eliminate a size-dependent effect, as the PDI was kept low for chosen formulations. Still, *in vitro* aggregation on the cells cannot be excluded. Furthermore, the zeta potential was much the same for the formulations (those without the anionic surfactants), hence an interfering influence can be excluded. What has been taken into account though, is the concentration-dependent effect of surfactants on cell membrane permeability. Hence a higher amount of surfactant increases the permeation of the cells and therefore leads to a higher cellular uptake. Additionally, it can be assumed that uptake efficiency is dependent on the SNEDDS composition, as different excipients lead to varying efficacies.

To conclude, many factors play a role in cellular uptake and in this way mechanisms and efficiency, which affect each other, as described previously. Therefore, all except one factor need to be kept constant to ensure elimination of other impacts. This was achieved for polymeric nanoparticles, liposomes and microemulsions, where the particle size depends on their preparation, and the composition stays the same. In the case of SNEDDS, this requirement cannot be fulfilled, as droplet size is determined only by the composition of formulation.

It was shown that many anionic surfactants improve cellular uptake efficiency up to 60 % if incorporated into SNEDDS. Though, there is an important distinction between the functional groups, their enhancement is ranked as follows: SO3 > COOH > COONa > SO4 > PO4, where the latter showed no significant different uptake efficiency compared to the empty SNEDDS formulation. These findings prove that surface charge has a crucial impact to cellular uptake, as zeta potentials of SNEDDS containing anionic excipients differ from another. Interestingly, the zeta potentials of loaded SNEDDS are in the same order as previously mentioned: SO3 as the closest to neutral and SO4 and PO4 exhibit the most negative values.

These findings indicate that incorporation of surfactants with functional groups enhance cellular uptake efficiency, but zeta potential should preferably be close to zero, as a highly negative surface charge is suggested to decrease permeability of cell membranes.

Further investigations about the effects of anionic excipients would include studies with different loading-ratios in SNEDDS. Still, there are limitations to solubility for most excipients, making direct comparison to one another more difficult. Additionally, the zeta potential of the different loaded SNEDDS need to be taken into account when applying on cell tests.

In order to get a more profound understanding about the cellular uptake of SNEDDS, it has to be investigated which internalisation mechanisms take place. This can be done by specific inhibitor block method to examine which pathways are operating for the uptake of SNEDDS of different sizes.

SNEDDS were applied in a concentration as low as 0.05 %, but cellular uptake was found to be concentration dependent. Thus, formulations with lower cytotoxicity should be evolved to achieve higher overall uptake efficiency. Still, a compromise has to be found between toxicity and self-emulsifying properties.

Apart from the HEK-293 and CaCo-2 cells which were used in this study, also other cell lines have to be tested regarding their SNEDDS uptake efficiency. Additionally, comparisons in uptake efficiency between SNEDDS and liposomes under the same conditions could be carried out to be able to directly compare the two lipid-based nanocarriers. Finally, *in vivo* studies will need to be done in order to analyse the overall bioavailability, which might still be size-dependent for SNEDDS as it is for other nanocarriers. It was reported that polymeric nanoparticles sized larger than 200 nm are cleared more rapidly from the body, thus their circulation time is limited by their size. Still, particle size reduction might not be desirable in all situations.

6 Appendix

Table 10: Composition, droplet size, PDI and zeta potential of all developed formulations C.

	Composition [m%]						
	PG	TW80	RH	Cap200	Droplet size [nm]		PDI
C1	10	30	30	30	35,7	± 11,6	0,096
C2	10	20	30	40	60,1	± 21,0	0,103
C3	10	25	15	50	100,2	± 46,3	0,177
C4	10	40	10	40	54,5	± 21,4	0,128
C5	10	30	25	35	52,8	± 25,1	0,220
C6	10	20	20	50	98,5	± 38,3	0,128
C7	10	30	10	50	89,3	± 38,3	0,136
C8	10	25	25	40	49,1	± 17,1	0,106

PG: propylene glycol, TW80: Tween 80, RH: Cremophor RH40 and C200: Captex 200.

Table 11: Composition, droplet size, PDI and zeta potential of all developed formulations E.

	Composition [m%]						
	PG	Cp90	EL	MCT	Droplet size [nm]		PDI
E1	10	25	35	30	46,0	± 10,0	0,026
E2	10	10	40	40	89,9	± 34,4	0,131
E3	10	20	20	50	144,0	± 45,0	0,123
E4	10	5	45	40	80,2	± 10,8	0,176
E5	10	25	15	50	248,2	± 65,4	0,428
E6	10	30	20	40	110,4	± 23,2	0,107
E7	10	20	35	35	53,5	± 13,1	0,062
E8	10	15	40	35	49,8	± 11,3	0,055
E9	10	25	25	40	110,0	± 17,8	0,137
E10	10	35	25	30	87,0	± 16,6	0,102
E11	10	25	30	35	64,3	± 13,1	0,070
E12	10	25	20	45	182,8	± 17,4	0,195

PG: propylene glycol, EL: Cremophor EL, Cp90: Capryol 90 and MCT: Dermofeel MCT.

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8 List of Figures

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10 Index of Abbreviations

ACN	acetonitrile
DLS	dynamic light scattering
FDA	fluorescein diacetate
GI	gastrointestinal
h	hour
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure
HLB	hydrophilic lipophilic balance
min	minute
mM	millimolar
nm	nanometer
o/w	oil-in-water
PBS	phosphate buffered saline
PDI	polydispersity index
PEG	Poly(ethylene glycol)
PG	propylene glycol
pK_a	negative decadic logarithm of acid dissociation constant
SD	standard deviation
SDS	sodium dodecyl sulfate
w/o	water-in-oil