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Review

Modeling the Analysis Process of a Lipid-Based, Multi-Compartment Drug Delivery System

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Abstract: Solid lipid microparticles (SLMs) are multi-compartment lipid drug carriers that can be used in various forms via many routes of administration, primarily to obtain prolonged release, protect the drug substance or mask its taste. It is practically impossible to theoretically predict the effectiveness of the incorporation and distribution of active pharmaceutical ingredients (APIs) in SLMs, and these are fundamental features that determine the key properties of the dosage form. The possibility of an effective assessment of these features by selecting or developing sensitive, universal methods, therefore, conditions further development and practical use of this carrier. Therefore, unlike the already available review papers on SLMs, the aim of this mini-review is to focus solely on the issues of API distribution in SLMs and their release. For this purpose, the most important observations and results of our own research were collected and summarized, and then an attempt was made to confront them with the available literature data. Among the methods describing the critical attributes of SLMs, instrumental methods (DSC, AFM, Raman spectroscopy and NMR), quantitative studies for assessing API distribution in SLMs (including entrapment efficiency and drug-loading parameters) as well as different release techniques (without a membrane, in a dialysis bag and in horizontal chambers, taking into account physiological factors) were characterized and compared. The aim of this review is to facilitate the understanding of the SLM properties and to assess their ability to achieve the intended effect *in vivo*, as well as to standardize studies of such carriers, facilitating a comparison of the results between centers.

Keywords: solid lipid microparticles; microspheres; drug distribution; release study; DSC; AFM

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1. Introduction

An appropriate composition of excipients forming a drug delivery system (DDS) is essential for the administration of an active pharmaceutical ingredient (API). It is the composition and properties of the drug carrier and its behavior at the site of application that enable the proper administration and action of the drug substance, i.e., the intended therapeutic effect. The drug carrier is the one that provides prolonged release and protects the sensitive drug substance. It is, therefore, not surprising that ongoing efforts in the search for modern active substances are accompanied by continuous work on the selection and improvement of drug carriers most suitable for their administration.

The recent DDSs are often multi-compartment carriers of various natures, which offer greater advantages over conventional drug delivery systems due to increased efficiency, precision of administration and therapeutic efficacy [1,2]. Among multi-compartment structures, lipid systems are becoming increasingly popular because they facilitate the use

of poorly water-soluble APIs and are biocompatible [3,4]. The use of a multi-compartment DDS allows for flexibility and the introduction of various modifications to the final composition of the formulation. As a consequence, the efficiency of drug delivery might be significantly improved. Excellent examples of such carriers are solid lipid microparticles (SLMs), combining the advantages of different dosage forms. SLMs, as a multi-compartment and biocompatible system, are suitable for application in various routes in liquid or solid form. As a lipid carrier, it is dedicated primarily to the administration of APIs that are difficult to dissolve in water. Solid particles that do not dissolve or melt at the temperature of the human body are an excellent guarantor of prolonged release, taste masking or protection of the drug substance, of course, provided that the active molecules are effectively enclosed in the lipid matrix.

Designing such a carrier for the administration of a specific API requires extensive research and development work that is aimed at optimizing the DDS in terms of the selection of the concentration/dose, the method of combining the API with the drug carrier and finally the release rate of the drug substance. The main difficulty of this stage is the *in vitro* testing of the dosage form, assessing its properties and attempting to correlate them with the expected *in vivo* effect. Not only the selection of instrumental, analytical methods, etc., as well as their sensitivity and suitability for testing the assumed feature of the medicinal product are important, but also the selection of test parameters, taking into account the *in vivo* conditions at the site of administration/action. These are key studies because it is at this stage that the following are assessed and considered: compatibility of ingredients, carrier effectiveness and finally future patient safety. Pharmaceutical progress in this field and the development of subsequent drug forms with new APIs are supported by useful and universally recognized pharmacopoeial tests of dosage forms or various types of guidelines and recommendations of registration agencies (EMA, FDA). Unfortunately, in the case of modern carriers not yet described in scientific compendia, the search for the most appropriate methods and the use of subsequent modifications of studies adapted from other DDSs slows down technological progress, as it makes it difficult to compare the results between research centers. However, some difficulties cannot be solved without the use of an unusual procedure or procedure developed specifically for the needs of a new carrier.

The presented work is a mini-review based primarily on the results of our own research and over 10 years of experience in working with SLMs, compared and confronted with the available literature data. The focus was exclusively on studies that allowed for the qualitative and quantitative assessment of the distribution of APIs in SLMs and the rate of its release because these are key features determining the use of lipid microparticles as a drug carrier. The effective binding to lipids and the distribution of the drug substance in the lipid matrix is the basis for the use of SLMs as a carrier, providing prolonged release or guaranteeing drug protection or masking its taste. Indirectly, it is possible to assess this, both qualitatively and quantitatively, in studies of the location of the active substance using instrumental and analytical methods (distribution in individual phases). Directly, however, this state is best reflected by the study of the release of the drug substance, which is crucial from the point of view of bioavailability and therapeutic efficacy [5] because, if properly designed, it enables the assessment of the quality and stability of the formulation, as well as providing a chance to predict the properties of the DDS *in vivo*.

Since there are already two classic reviews on SLMs [6,7], this paper has been prepared in a different way, focusing only on the practical aspects of SLM analysis, limited to the key features—API distribution and release. The studies described in detail in previous reports are now presented in a cross-sectional way, comparing the advantages and limitations, discussing the essence of the issues and comparing other proposed approaches

described in the literature. The experience in SLM analysis that we have tried to share in the prepared document, together with critical commentary, has been gained over the years while conducting all the described research.

The research conducted by our team focused mainly on SLMs with a size limited from a few to a dozen micrometers in a liquid or powder state. Among the numerous possible applications of SLMs, the topical application of a SLM dispersion to the eye in the form of drops was quite often referred to [8]. Due to the size of lipid microparticles, the possibility of thermal sterilization and the form of the dispersion, which can be applied in the form of sterile eye drops, this carrier meets all the requirements for such a route of application. Moreover, earlier *in vivo* studies conducted on rabbits have already demonstrated the efficacy and good tolerance of this carrier after administration to the conjunctival sac [8]. For experimental purposes, the following aspects were selected as factors differentiating the tested formulations: lipid, drug substance, active substance concentration and carrier form (dispersion or powder). SLMs with model drug substances such as cyclosporine, indomethacin, hydrocortisone, diclofenac sodium or clotrimazole were analyzed, and for comparative purposes, SLM *placebo* were tested. In selected experiments, SLM in the form of powder, which was obtained in the spray-drying process, were used for the studies. The lipid matrix of the microspheres was formed from the solid lipid Compritol 888 ATO (glycerol dibehenate), selected as the most beneficial, as well as other lipids, e.g., Precirol ATO 5 (glycerol palmitostearate) or stearic acid.

All analyses presented in this paper can be used in SLM studies, regardless of the proposed route of administration. The only limitation is the type of parameters prevailing *in vivo* in the conjunctival sac proposed for the release study (enzyme lysozyme, artificial tear fluid), which require appropriate modification, depending on the method of application. Similarly, the model active substances used do not limit in any way the broader usefulness and interpretation of the presented results.

2. Types of Solid Lipid Particles

Solid lipid particles, also called lipospheres, are an example of a versatile carrier with many potential applications [9–11]. They can be divided into three categories: the first one, developed in the 1990s, is called SLNs (solid lipid nanoparticles), and the next ones are NLCs (nanostructured lipid carriers) and SLMs (solid lipid microparticles). SLNs were obtained by replacing the oil in an o/w emulsion for parenteral administration with a matrix of lipids solid at human body temperature [12] and were proposed as an alternative drug carrier to emulsions or liposomes [13]. Thus far, SLNs have been the most often studied among solid lipid particles.

SLMs were created based on SLNs, but as larger particles, combining the advantages of lipid carriers (such as SLNs) with microparticle-scale formulations (such as polymer microspheres) while eliminating the risk associated with the “nano” size or the potential toxicity of polymers [14]. The lack of accumulation in the body and toxicity of polymer decomposition products is a necessary condition for exceeding the regulatory requirements and their practical application [15]. Even in the case of the already commonly used PLGA (poly(lactide-co-glycolic acid)) polymers, it was found that their local toxicity may depend on other formulation components (e.g., chitosan, polyvinyl alcohol or poloxamer 188) [16]. The advantage of microcarriers over nanoparticles is also the fact that they do not penetrate the interstitial tissue and are not transported by the lymph [17]. Lipid dosage forms are currently of great interest because they provide good *in vivo* tolerance and biodegradability, as they are composed mainly of physiological and biocompatible components.

In justifying the purpose of the presented data, enriched with commentary resulting from our own experience and research, it should be noted first of all that SLMs have a

much shorter research history and a much smaller amount of available data than their "nano" counterparts, i.e., SLNs [18]. Although SLMs and SLNs are similar in terms of their compositions, the discrepancy in their size of at least one order (200 nm vs. 2 μm) results in undeniable differences, both in terms of the manufacturing methods, methods and routes of administration and finally the properties and morphology of particles. For this reason, a much larger experimental database on SLNs cannot be an exhaustive source of information for the development of SLM formulations. Of course, due to the aforementioned similarities and the much poorer scope of literature data relating to SLMs, the SLN test results often become a starting or reference point, but they cannot replace the analyses of lipid microparticles, which significantly differ in properties and require an appropriate research methodology, adapted primarily to the size of the tested particles.

3. Nomenclature

Microparticle systems other than SLMs, such as polymer microspheres, microcapsules or micropellets, are already widely used as DDSs because they provide significant therapeutic and diagnostic efficacy compared to traditional dosage forms [19]. These carriers are produced from different materials, especially polymers, which form the carrier and determine its properties. Unfortunately, due to the variety of forms of different sizes (often measured even in mm) and excipients that make up these formulations, the classification of microparticles is not obvious, and the nomenclature used may be ambiguous or even misleading.

In general, microspheres are defined as spherical particles with dimensions in the microscale, i.e., in the range from 1 μm to 1000 μm [20]. The term microparticle refers only to the size but not necessarily to the shape (they may have, for example, a spheroidal shape). According to this criterion, microspheres should, therefore, be considered a special—spherical type of microparticles [20]. In the same way, nanospheres are usually defined as particles with a diameter of less than 1 micron (1 μm) or 1000 nm, although some scientists narrow this definition, classifying nanospheres as particles with a diameter from 10 to 200 nm [20]. Generally, however, in the latest literature, the terms "microparticles" and "nanoparticles" refer to particles whose dimensions are measured in micrometers and nanometers, respectively [21].

SLNs and SLMs can also be referred to as lipospheres, which in turn refers to water-dispersible lipid-based solid particles with a diameter ranging from 0.01 to 100 μm, consisting of a solid hydrophobic lipid core surrounded by a stabilizer (surface-active compound) facilitating the dispersion of the lipid phase in the aqueous phase [22].

Microparticle dispersions, like microspheres, are multi-compartment DDSs with specified physiological and pharmacokinetic advantages, offering both therapeutic and technological benefits [17]. The basic characteristic of such a carrier should, therefore, be considered to be its multi-reservoir nature, thanks to which the dose of an API is distributed over many separate "portions" from which it is released [17]. However, unlike microcapsules, which have a typical reservoir character, microparticles have a matrix structure, so they are carriers in which the active substance is dissolved or dispersed homogeneously or inhomogeneously.

4. Advantages and Routes of Administration of SLMs

SLMs combine the advantages of various DDSs, and the basic advantages of their selection as a drug carrier include the following facts [6,7,22,23]:

- Particles formed from a solid lipid at body temperature can provide not only a prolonged release effect but also protection of the incorporated drug substance or taste masking;
- Due to the size of the particles, compared to SLNs, they provide a higher level of incorporation of the drug substance in the lipid matrix and a more visible prolonged release effect;
- As a multi-compartment carrier, they offer uniform dispersion at the site of administration, which ensures more even absorption and better protection of the applied drug substance;
- Cost-effective production on a large scale;
- Possibility of production using various techniques, depending on the desired size of the microparticles or form (powder or dispersion);
- Due to the composition of the matrix consisting of GRAS (*generally recognized as safe*) substances, the carrier is well tolerated, considered non-toxic, biocompatible and biodegradable;
- Unlike SLNs, they do not require a high-pressure homogenization process at the production stage;
- SLMs in a powder form can be obtained without the use of organic solvents;
- Possibility of thermal sterilization of dispersion and application of the sterile formulation;
- Possibility of application in both solid and liquid forms;
- Possibility of administration by the most common routes, such as oral or topical.

Despite the many advantages mentioned, the basic purpose of using SLMs is to obtain the effect of a prolonged release of an API and mask the taste of the active substance or protect it, both *in vitro* (e.g., against hydrolysis or oxidation), in order to increase stability, and *in vivo* (e.g., in the gastrointestinal tract against the action of hydrochloric acid or enzymes) [24–27]. These advantages will become possible to use as a result of the development and implementation of SLM technology.

It is expected that the use of lipid carriers in the form of SLMs will contribute to increased therapeutic efficacy of the drug substance administered in this carrier not only due to their favorable release profiles but also due to their optimal organoleptic properties. SLMs, as multi-compartment drug delivery systems, create unique opportunities related to the adaptation of the dosage form to the planned route of drug administration [6,28,29]. SLMs can be used in various pharmaceutical forms: solid (in the form of powder, capsules, tablets), liquid (as dispersions/suspensions) or semi-solid (concentrates); therefore, they can be administered by various routes (Figure 1): orally [25,30], parenterally [31] (intramuscularly, subcutaneously), topically on the skin [32–34], nasally [35] or into the eye [8] and even by inhalation [36–38].

The production of SLMs in powder form is also justified due to the increased long-term stability of the formulation (physical, chemical and microbiological) [39,40]. If necessary, the obtained dry preparations can be used not only in powder form but also after reconstitution of the powder ex tempore to the form of a liquid dispersion. Although stability studies have confirmed that both SLM and SLN preparations in the form of a liquid dispersion remain stable, even for two years [41,42], depending on the composition and properties of the incorporated API, in some aqueous SLM dispersions, there is a greater risk of unwanted changes than in the form of powder. Unfavorable physicochemical changes may concern both the lipid matrix or excipients, as well as the active substance, which may undergo chemical degradation (e.g., hydrolysis) or premature release. Moreover, changes, such as the degradation of the particle matrix or aggregation/fusion of lipospheres, have been observed in some SLM or SLN dispersions, resulting in an undesirable increase in particle size [6,39]. Once the microparticles are converted into a fine, dry powder, SLMs can be stored for long periods of time without the risk of the physicochemical changes observed in liquid dispersions.

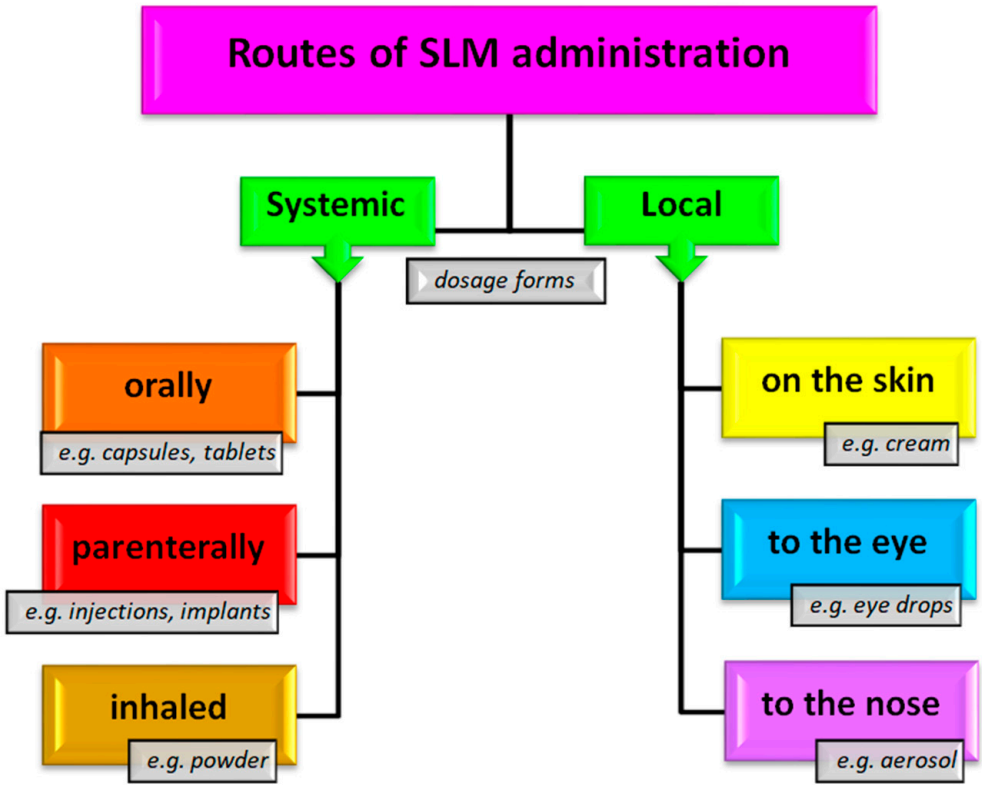


Figure 1. Potential routes of administration of SLMs in different dosage forms.

There are several methods for manufacturing SLMs in solid form, such as spray-congealing/spray-chilling, spray-drying from an organic solution, freeze-drying, cryogenic micronization or using the supercritical micronization technique (particles from gas-saturated solutions) [43]. The main problems with using these methods are the large sizes of produced microspheres (up to 2000 µm), the time-consuming complex process and often the need to use organic solvents (ethanol, chloroform, dichloromethane) [43,44]. However, it is also possible to spray-dry an aqueous dispersion of SLMs [45]. In this way, it is possible to obtain a SLM powder from a few to a dozen micrometers in size without using organic solvents. In the drying process, the aqueous dispersion is sprayed through a nozzle into small droplets, which, in contact with a hot drying agent (usually air), are dried by evaporation of water [46]. Although the sprayed liquid is in contact with hot gas, this contact is very short, and the cooling effect of the evaporating solvent maintains the temperature of the droplets at a relatively low level. In this way, it is possible to dry lipid systems even at temperatures above 100 °C [28,29]. However, since the melting of the microspheres' lipid occurs at a temperature lower than the drying process, in the case of improperly selected conditions, melting of the lipid matrix should be expected. This, in turn, may result in a significant deterioration of the initial properties of the SLM (mainly the distribution or release rate of the API), as well as negatively affect the drying efficiency and the smooth course of the process. As a result, process optimization is necessary to preserve the attributes of dried SLMs, which can, however, be successfully performed on a *placebo* SLM formulation (the established conditions can then be used to dry SLMs with the selected API).

5. Assessment of Drug Substance Distribution in SLMs

Due to the solid nature of the lipid forming the microsphere matrix, the main purpose of SLM utilization as a DDS is to provide prolonged release, protect the API or mask its taste [6,7,47]. In each of the above cases, effective incorporation of API molecules into the

lipid matrix is necessary to achieve the desired effect. The distribution of the drug in the carrier also affects other properties, including the stability of the formulation [7,47–50]. For the above reasons, an assessment of the API distribution within the solid lipid microparticles is a key aspect of the assessment of SLMs as a dosage form. Various techniques and research methods have been attempted for this purpose. There are different descriptions of the expected or predicted drug location in SLMs or SLNs [12,51], which result from the adopted concepts of a multi-compartment carrier based on the results of instrumental analyses, spatial modeling or even the effect of rapid release. The models considered are presented in Figure 2.

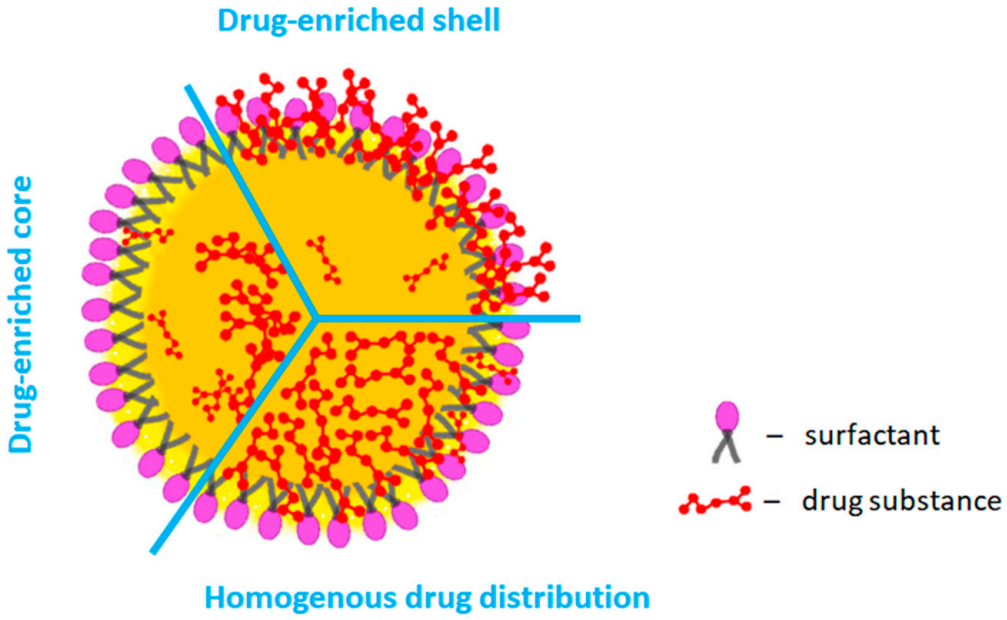


Figure 2. Models of probable drug substance distribution patterns in SLMs.

Below, both the experiences related to the use of various instrumental tests and analytical methods are summarized, allowing for a more or less effective identification of the distribution but also quantitative characterization of the API in the different phases of the tested SLMs.

5.1. Instrumental Methods for Characterizing API Distribution in SLMs

Identification of the API distribution in microparticles is possible by direct localization of the drug substance, by assessing the influence of the API on the properties of the matrix components or by detecting the interactions between these components and the drug molecules.

In our studies, four instrumental techniques were used to identify the distribution and interactions of active substances with other components of lipid microparticles: a thermal analysis based on differential scanning calorimetry (DSC) combined with thermogravimetric analysis (TG), atomic force microscopy (AFM), Raman spectroscopy and nuclear magnetic resonance spectroscopy (^{1}H NMR). The proposed methods were assessed in terms of their suitability for characterizing, directly or indirectly, the distribution of active model substances in SLMs. Not only were the aqueous dispersions of lipid microparticles studied but also spray-dried SLM powders. This allowed us to consider the suitability of the proposed methods for identifying changes in the distribution of APIs induced by drying.

5.1.1. Simultaneous Thermogravimetric Analysis (STA)

DSC is a commonly used method for studying lipospheres mainly due to the polymorphism of the lipids that make them up. This obvious fact is the basis for studies of

many other lipid forms of the drug. Detection and study of active substances incorporated in SLMs using this technique is definitely more difficult. The basis of DSC studies is the fact that different polymorphic forms are characterized by different melting temperatures and melting enthalpy, which is also influenced by modifications in the solid state. This method can also provide information on the structure of solid microparticles, i.e., in what form—crystalline/amorphous—the incorporated drug substance is in the matrix. It is known that detecting low concentrations of the substance is problematic, and such concentrations usually occur in microspheres, which is why confirmation of API incorporation in the matrix by direct analysis of its thermal changes sometimes turns out to be impossible. The studies undertaken focused on attempts to identify and characterize the thermal changes originating from APIs and the thermal effects resulting from their interaction with the ingredients of the carrier.

The simultaneous use of two methods (DSC and TG) enables the so-called simultaneous thermogravimetric analysis (STA), the advantage of which is obtaining a comprehensive thermal characteristic of the tested formulations [52]. In such an experiment, both signals are recorded at the same time, on the same equipment, and the conditions prevailing during the test (atmosphere, pressure, heating rate, the same measuring vessel, etc.) are identical for TG and DSC analyses, which is of significant importance for the quality of the obtained results. The tests were performed on liquid and solid SLMs with model APIs. Thermal characteristics of the SLM placebo formulations and physical mixtures were also performed. The dispersions were tested immediately after preparation, after processing into powder form and also during the stability tests [53].

One of the important aspects of studying SLM liquid dispersions is the sample preparation for analysis. Theoretically, DSC/TG analyses are appropriate for the direct examination of SLM liquid dispersions, but then water is removed at the initial stage. As a result, the DSC curve presents a single conversion peak related to water evaporation, within which it is not possible to separate a much smaller lipid melting peak. In turn, the TG curve can be distinguished by two steps of decomposition with mass loss, which mainly correspond to water evaporation, followed by thermal decomposition with carbonization, which is difficult to interpret from the point of view of lipid analysis. When SLM dispersions are studied after the prior removal of water, a single endothermic transition stage can be clearly distinguished on the DSC curves without mass loss on the TG curves, which corresponds to the melting process of the lipid forming the SLM matrices [53]. It is, therefore, advisable to remove water from the sample before thermal analysis, but the method becomes crucial so that it does not impact the features of the tested microparticles. Several different approaches have been proposed in the literature. One of the recommended methods of water removal is to preheat the sample in a crucible and then cool it again before analysis. However, due to the temperature being higher than the melting point of the matrix, such a procedure certainly has an impact on the properties of the preparation [54,55]. Also, the freeze-drying of liquid SLMs before DSC was utilized, but this process can also result in serious changes in the sample due to the effect of reduced temperature or unfavorable forces affecting the microparticles during freezing [56]. To avoid applying additional heating or lyophilization immediately before DSC, water was evaporated at room temperature under compressed air, without or after ultracentrifugation of the dispersion [53]. Taking into account the small amount of microparticles needed for DSC analysis, the method of evaporating the solvent from a small volume of SLM dispersion under compressed air at room temperature, without the centrifugation process generating centrifugal forces, as has been shown to affect SLMs, proved to be sufficient.

During the DSC studies, no melting peak of any model API was observed in the SLM thermograms [53], regardless of their concentration in the preparation or the distribution

of the API in the microparticles. In some formulations, the disappearance of the melting peak of the API in SLMs can be explained by the fact of low concentrations, which is also indicated by the results of other research teams [57–59], but these peaks are not visible even when the API to lipid ratio was 1:2. Therefore, it is also justified to explain this fact by drug–lipid interactions (or drug–other components of the dosage form, e.g., polysorbate) and the incorporation of the API into the matrix structure as a result of its dissolution in the melted lipid. This effect is confirmed by the conducted studies of physical mixtures. The presence of the substance dissolved in the microsphere matrix, by influencing the crystal structure, causes a decrease in the melting temperature of the lipid matrix, depending on the concentration of the incorporated API, and in the DSC spectrum, a shift of the melting peak of the matrix lipid towards a lower temperature. Thus, the presence of APIs and their impact on the SLM properties in STA analysis can only be inferred indirectly, based on the decrease in the melting temperature of lipids (DSC) or a subtle change in the decomposition temperature (TG). Importantly, the thermal behavior of liquid SLMs was very similar, irrespective of the introduced API, which allows the use of the observed changes to assess the properties of the tested formulations. The thermal decomposition visible on the TG curves is also subject to characteristic changes due to the incorporation of the API in microparticles. The decomposition temperature in SLMs with an API shifts, this time towards higher temperatures (with a slight slowdown in the degradation process), while the temperature of the placebo formulation is the same as the lipid used to obtain the carrier, which confirms the dependence of the observations on the API. A very similar effect was noticed in the SLMs with different APIs.

STA studies also confirmed the stability of SLMs in liquid and solid forms, when the carrier was made of glycerol behenate (Compritol), which was found also in other experiments [60]. The stability in STA tests of microsphere suspensions with Compritol and various APIs was confirmed after one year in a refrigerator. Spray-dried powders of the same composition but stored for two years at ambient temperature were also stable. As was shown, the lipid forming the microparticle matrix has a significant effect on the properties of the SLMs obtained after the spray-drying process. No thermal changes were observed in the microspheres with Compritol, which did not undergo adverse changes during the drying process and was also stable during storage, in contrast to the much less stable stearic acid.

5.1.2. Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) is a method that has already been used in lipid nanosphere studies, but previously, it was used only for imaging the surface topography of SLNs and for presenting the shapes and sizes of nanoparticles [61], i.e., more as a specific imaging analysis. Meanwhile, the AFM technique can also be used to measure the viscoelastic properties of the studied surfaces, which becomes a source of information about the surface properties of the formulation based on the interaction of the particle surface with the probe [62]. The introduction of AFM to the spectrum of SLM physical analyses allows for the understanding of the interaction of the lipid matrix with the active substance and the surface identification of APIs.

The AFM method is becoming a promising technique for use in this area of research because the measurement of mechanical properties, such as stiffness/elasticity, microfriction or adhesion, turns out to be a precise source of information about the properties of the tested surface [63]. The undoubted advantage of AFM is the measurement of tested surfaces with very high resolutions. The wide range of measured forces indicates the sensitivity and ease of detecting the variability of the tested structure, depending on its composition (type of lipid, type of API, amount of API localized on the surface) or processes to which the

formulation was subjected (conversion of microparticles from dispersion to powder in the spray-drying process), as well as changes occurring over time (stability studies).

The nanomechanical properties of the microparticle surface studied using AFM allowed us to distinguish between a SLM placebo and SLM with a drug substance located on the surface, as well as to observe the changes resulting from the drying process. This confirms the potential of the AFM method not only for studying the topography of particles and obtaining real, three-dimensional images but also for characterizing the surface of lipid microspheres by assessing its interaction with the probe. The advantage of the AFM method is the relatively simple and time-saving preparation of the sample for testing. Unfortunately, this technique is more difficult in terms of the proper selection of the measuring probe for testing.

5.1.3. Raman Spectroscopy

Raman spectroscopy is another advanced method of microscopic chemical analysis, proposed for the evaluation of the distribution and interaction of active substances in microspheres. As is known, Raman mapping can generate high-resolution chemical images of complex materials (e.g., tablets), which allows for the evaluation of not only differences in the composition but also the microstructure of the preparation. Creating such chemical maps requires imaging the distribution of the intensity of Raman signals characteristic of individual components in the tested plane. Obtaining two-dimensional images showing the distribution of individual chemical components in lipid microparticles using Raman spectroscopy would be an ideal confirmation of the surface localization of a significant fraction of APIs, expected on the basis of other studies. However, such studies have not been conducted so far in relation to the multi-compartment dispersions of microparticles. Perhaps this is because initial experiments did not bring the expected results. Although it was possible to identify tested APIs (e.g., cyclosporine, spironolactone) on the SLM surface, the obtained maps did not confirm the superficial location of large amounts of the drug substance, as indicated by other results (distribution or release studies) [63]. The reason may be the dominance of signals on the obtained chemical maps mainly coming from the lipid forming the microparticle matrix, as well as other excipients localizing on the surface, such as polysorbate (a surface-active compound stabilizing microspheres) or polyvinylpyrrolidone (an excipient used during drying). The factor limiting the use of this technique turned out to be the problem of significant intensity in the spectra of bands originating from lipids overlapping with low-intensity bands originating from active substances. In Raman spectroscopy, detectability depends, to a large extent, on the relative intensity and, above all, the position of the signals of the interesting component in comparison with the signals generated by the carrier matrix. Even the occurrence of signals of relatively high intensity is difficult to detect when they are superimposed on the signals of the lipid matrix. At low drug concentrations, the signals are almost indistinguishable from the baseline noise. Interfering signals, additionally significantly different in intensity, have limited the usefulness of this test method to formulations with APIs with favorable (here, in the sense of bands that allow for differentiation in comparison with the lipid spectrum) spectral properties. At the same time, it should be added that the advantage of Raman spectroscopy is the undemanding preparation of the sample, as well as the possibility of conducting observations in the liquid state. Therefore, it is recommended to conduct a preliminary analysis of the spectra to assess the justification for continuing this type of research, depending on the specific properties of the tested formulations.

5.1.4. Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) spectroscopy has also been proposed as one of the considered methods for assessing the interactions of SLM components with the drug substance and its distribution or changes in distribution due to spray drying. While the techniques of nuclear magnetic resonance spectroscopy in a solution have found wide application in pharmacy, ^{1}H NMR spectroscopy in liposphere studies is still rarely used, and its application potential as a method for SLM research is virtually unknown.

NMR spectroscopy, for the detailed study of molecular structures, uses electromagnetic radiation with frequencies in the radio range to detect atomic nuclei (1H) in molecules. Studies conducted on lipid nanospheres have shown that NMR signals for triglycerides are difficult to observe due to very short relaxation times [64,65]. Due to the relaxation phenomenon dependent on the degree of molecular mobility [61], the ^{1}H NMR method enables the distinction of a moving liquid from immobilized solids. Therefore, it could be expected that this method would enable the detection of mobility and rearrangement of active substance molecules excluded from the matrix if such an event occurred, for example, as a result of spray drying or during stability studies. This is based on the assumption that the lipid-active substance interaction and/or the change in the distribution of the API in the formulation will be reflected by the appearance of signals characteristic of the drug substance or a change in the width or amplitude of the signals generated by the tested microspheres. Unfortunately, in the conducted studies, it was not possible to identify signals originating from the APIs if they were incorporated into the solid lipid matrix [63]. The fraction of cyclosporine present in the aqueous phase of the dispersion resulted in the presence of a specific signal, but this was outside the characteristic region of the spectrum (the so-called *finger print*). Such signals were not recorded in the case of another tested substance—spironolactone. Although the ^{1}H NMR spectra of the spray-dried formulations presented characteristic changes related to the polymer added during drying (PVP), no signals related to the active substances were still visible. It should, therefore, be stated that when using ^{1}H NMR spectroscopy, it is very difficult to identify signals originating from the APIs incorporated in the solid lipid matrix, and the free fraction of the substance gives rise to non-characteristic changes in the spectrum or they are not detectable at all. In general, solid-state NMR can also be used to characterize solid surfaces as an alternative to liquid NMR, although its resolution is inferior because solid-state NMR peaks might be broader than liquid-state peaks, which may obscure peak assignment and integration [62]. Solid-state NMR has been used, for example, in the study of lipodisq nanoparticles for their structural characterization depending on the lipid and polymer compositions [66].

In the presented approach to the assessment of API distribution in SLMs using instrumental methods, traditional microscopic methods were not described because they did not bring the expected results in recognizing and identifying APIs on the surface of lipospheres. However, in the literature, one can find attempts to more precisely localize the drug in SLMs using imaging analyses like SEM (scanning electron microscopy), confocal scanning microscopy, fluorescence spectroscopy or XPS (X-ray photoelectron spectroscopy) [6,50,51,62,67]. However, in most cases, these methods also do not bring the expected results; some of them are much more demanding and complicated, or they can be applied only selectively to some APIs (for example, those presenting fluorescent properties, which many substances do not have). HR TEM (high-resolution transmission electron microscopy) has also been used in studies of solid lipid particles. This technique is considered a good imaging tool not only to analyze the size, shape and homogeneity but also to characterize the crystallinity and lattice structures of materials at an atomic resolution [62]. TEM microscopy was used, among others, to confirm the sizes of SLNs [68,69], to show the topologies of the spongosome lipid nanoparticles loaded with bioactive ingredients

and to reveal the domains' inner organization or a phase separation resulting from the encapsulation of oil in the lipid particles [70], as well as to examine the morphology [71] and distribution of SLNs and to determine the core–shell structure of these particles [72]. The microstructure of particles with different lipid compositions (the lamellar layers or terrace-like layers) was also compared [73]. Moreover, such methods still do not provide knowledge about the quantitative partitioning of the drug substance between the particle core and its surface.

Summarizing the data presented in the above chapter, it can be stated that STA analysis should be considered useful for the analysis of lipid interactions with active molecules and modification of the SLM matrix structure, although these processes can only be assessed indirectly based on the changes in the lipid melting temperature (DSC) or subtle changes in the decomposition temperature (TG). SLM, in the form of dispersion, turned out to be much more difficult to study compared to the powder form. In order to clearly distinguish lipid melting events, which are key changes in the DSC spectra and the main source of information about lipid microparticle properties, the SLM analysis should be carried out in solid (dry) form after appropriate sample preparation. Assessment of lipid microparticle properties by thermal analysis can be used not only at the stage of development studies and for stability assessment but also in quality control. Also, AFM can be a useful tool in SLM analysis, not only to obtain real topographic images but also to analyze the presence and location of drug substances on lipid microparticle surfaces by assessing the viscoelastic interactions of the surface with the probe. Both in the AFM and DSC/TG methods, the selection of measurement parameters and the research procedure are crucial to obtain reliable and repeatable results.

Raman spectroscopy, as a method of API mapping in lipid microparticles, has significant limitations in routine use, mainly due to the spectral properties of active substances, which, combined with their small percentage share in the formulation, make it difficult or impossible to develop and further process spectra and Raman maps. Studies using nuclear magnetic resonance spectroscopy (^{1}H NMR) have proven to be the most demanding from an analytical point of view; therefore, in comparison with the number and value of the obtained results, this technique has been considered definitely unsuitable for in-depth SLM analysis.

5.2. Quantitative Method for Assessing API Distribution in SLMs

According to studies in various scientific centers, the amount of drug contained in lipospheres is usually characterized by two parameters: entrapment efficiency (EE) and drug loading (DL) [32,47,50,74–76]. Entrapment efficiency is the amount of drug introduced into lipid particles (successfully encapsulated) divided by its total amount added to the formulation, and the drug loading is the amount of drug incorporated per unit mass of the entire system or lipid. Determination of these parameters in SLM dispersions consists of separating the aqueous phase (by dialysis, ultracentrifugation or membrane filtration) and determining the concentration of the dissolved substance—the dissolved fraction is considered as free drug, and the remainder of the drug as the amount incorporated into the microparticles [6,47,75]. When microparticles are obtained in a dry form (SLM powder), the content of API is determined directly in the lipid particles after their melting and extraction [6,23,75]. The parameters EE and DL, determined in this way, are usually considered a measure of the expected therapeutic potential of the formulation. However, this ignores the important fact that the drug does not have to be incorporated in the microsphere matrix but can be located in different areas of the lipid particles, which are not distinguished by the given parameters EE and DL and which are crucial for the properties of SLMs.

Available are the various characteristics of the drug distribution in SLMs or SLNs [12,51], taking into account a multi-compartment form of DDS based on the results of instrumental analyses, spatial modeling or even the effect of release study, which assume different localizations of the API within the lipospheres. At the same time, no experiments were conducted to quantitatively present the distribution of APIs in the SLM suspension. This fact is usually completely omitted by the authors of the publications for totally incomprehensible reasons. The described attempts to localize the drug in SLMs using instrumental techniques have already been commented on above; besides, such methods do not provide knowledge on the quantitative distribution of the active substance between the core of the microparticle and the surface of the SLM.

The distribution of APIs between the individual SLM dispersion phases depends on many factors, such as the hydrophobicity of the drug, the properties of the lipid, the type and concentration of the surfactant or the method of obtaining the SLM [6,7,47]. As a result, this process is so complex that it is impossible to predict the distribution of the drug and its incorporation into the lipid matrix based only on the properties of the formulation components or even solubility studies. There is no direct relationship between the solubility of the API determined in the lipid and its ability to be incorporated into SLMs containing this lipid.

SLMs are stabilized with surfactants, which, during the production process, are localized in the interphase associated with the surface of microparticles [7]. Active substances can be localized in different areas of the SLM dispersion: in the inner part of the microparticles (the so-called lipid core), on the surface of the SLM (the so-called interphase) or, in the case of dispersion, also in the aqueous phase (outside the microparticles). The location of the API obtained directly after the preparation of microspheres may change during further processing (e.g., in the process of thermal sterilization, leading to the re-melting of microparticles) or storage (e.g., as a result of polymorphic transformations of the matrix lipids, resulting in the reorganization of the spatial arrangement of lipid molecules and the spaces created by them for the incorporated substance). As a result of such changes, not only the redistribution of the substance in the system between the individual phases is possible, but also the precipitation of the substance present in the aqueous phase due to separation from the lipid matrix outside the SLM and precipitation in the aqueous phase as a result of exceeding the solubility.

The presence of a significant fraction of the drug substance on the surface of microparticles or nanoparticles has already been reported in numerous publications [47,67]. These reports were most often based on the obtained rapid-release profiles of a significant fraction of the drug in the initial phase (*burst effect*), as well as on the above-mentioned imaging techniques. However, there is still a lack of quantitative studies that would allow for a detailed determination of the drug distribution when taking into account the structural domains in lipospheres. In SLMs, the API located on the surface of lipospheres is anchored in the interphase formed by the surfactant molecules stabilizing the system. This fraction is relatively easily accessible, so it can result in a rapid initial release, which, depending on the formulation, can also be an advantageous feature (the stable dispersion of SLMs prevents precipitation of the drug located on the surface of lipid particles, and the rapid release of the surface fraction after application provides an easily accessible initial dose at the site of administration). However, the surface location of the predominant amount of the drug in SLMs will not effectively mask the taste or prevent chemical degradation of the drug if the dosage form is used for this purpose. To sum up, the EE and DL parameters do not take into account the distribution of the drug within the lipid matrix of the microparticles or the phenomena occurring on the surface of the particles, and therefore, they are insufficient to determine the efficiency of drug incorporation in SLMs, and thus the suitability of this

carrier for the selected drug substance and the purpose of its use. Only studies of the distribution of different APIs in the various phases of the SLM dispersions allow for insight into the distribution of APIs within lipid microspheres. The method of shaking the SLM suspension with an organic solvent (methanol) allows for the extraction of the tested drug substance from the interface without dissolving and damaging the lipid matrix of the microparticles [77]. This procedure is simple and allows for the quantitative determination of the fraction of the drug located in the interface. (It has been proven that mixing the microsphere dispersion with methanol does not destroy the carrier while dissolving the drug substance from the microparticle surface.) Thus, the distribution study involves three parallel steps: complete extraction of the API with methanol after melting the lipospheres (analysis of the total drug content in the SLM dispersion), ultrafiltration of the undiluted SLM dispersion (determination of the fraction of the API dissolved in the aqueous phase) and extraction of the drug substance from the interface with methanol and separation of the water–methanol phase by centrifugation (the sum of the drug content in the aqueous phase and the interface is then determined). This procedure allows for the quantitative determination of three drug fractions in the SLM, i.e., the fraction incorporated into the lipid matrix; the fraction associated with the microspheres but located on the surface of the lipospheres (in the interphase), this fraction does not include the drug from the SLM core; and the fraction dissolved in the aqueous phase of the dispersion (not incorporated in the SLM).

Comparing the results of the studies obtained in this way with the EE and DL values (using the example of four model drug substances at different concentrations), fundamentally, different conclusions were obtained [77]. Since the EE value does not include only the fraction of the active substance from the aqueous phase, the results suggest excellent efficiency of API incorporation in lipid microparticles. Meanwhile, the results of the distribution study clearly indicate that the aforementioned affinity is definitely weaker, and the largest part of the model substances is located on the surface of the microparticles and not in the lipid matrix, which could be indicated by high EE values. In fact, the fraction contained in the lipid core in most formulations does not exceed 30%, even at low API concentrations. Although the fractions of the APIs found in the aqueous phase were insignificant, it was difficult to obtain a high share of these drug substances in the lipid matrix, even if the results of the solubility studies suggested such a possibility. On this basis, it should be concluded that the effective and permanent incorporation of the drug substance into the SLM lipid core in practice seems to be much more difficult and limited than it would result from the analysis of the EE and DL parameters. In general, the fraction of the drug present in the aqueous phase was most often negligible, but the remaining part of the API associated with lipid microparticles was mainly localized on the surface and only partially incorporated in the lipid core. Such a distribution was independent of the solid lipid forming the matrix, but it was significantly reduced after introducing liquid lipid into the matrix. The nature of the drug substance itself had a greater impact on the manner of API distribution. Increasing the concentration of the lipid phase from 10% to 30% in the dispersion also did not cause a significant change in the distribution of the substance in the individual phases, which may suggest a stable affinity of the tested API to the SLM lipid. Although, in microparticles composed of the same auxiliary substances, the increase in the solubility of the API in lipids is consistent with the increasing possibility of incorporating drug molecules into the lipid matrix; however, this correlation is not analogous, and the amount of the drug in lipospheres may even exceed the amount determined during the solubility study, which was confirmed in the SLMs with cyclosporine.

Considering the API distribution with its release rate from SLMs, one can find clear dependencies related to the initial *burst effect*, during which the released amount of the drug

is not greater than that marked in the interphase. The total release of the drug substance, including the fraction incorporated in the lipid matrix, is slow and, in the conditions of the study (if the acceptor fluid does not contain enzymes), is most often incomplete.

Due to the changes occurring over time and related to lipid transformation (transition from unstable α and β′ polymorphic forms with higher energy to more stable and ordered β), distribution studies should also be an element of stability studies. As a result of redistribution, active substance molecules removed from the lipid matrix can be excluded from the SLMs to the aqueous phase. The phenomenon of exclusion from the lipid matrix (drug expulsion) also applies to SLNs, for which it has been described many times [12,13,48,49]. In addition, the API can be moved from the core to the interphase without the adverse effect of precipitation outside the microspheres. Moreover, the beneficial effect of autoclaving (important in drugs intended for parenteral or ocular administration) is worth noting, observed in some formulations, and manifested even by a quite significant reduction in the interphase fraction and an increase in the amount of the drug in the lipid matrix. The detection of this phenomenon was possible only on the basis of distribution studies distinguishing the amounts of drug in individual phases of SLM suspensions, while it was not visible on the basis of the analysis of the EE and DL parameters, which do not change after thermal sterilization [77].

In summary, the evaluation of multi-compartment DDSs, such as SLM suspensions, based solely on the EE and DL parameters is insufficient, even misleading and incorrect because the drug substance located on the surface of the microparticles and the substance contained in the lipid core of SLMs cannot be considered in the same way. The fraction of the drug located on the SLM surface will be released more easily/faster, and it will not be protected or masked to the same extent as the fraction incorporated in the lipid core of the particles. The EE and DL parameters do not allow for distinguishing the amount of drug substance attached to the surface of microparticles from the amount actually contained in the lipid cores of microparticles. They only indicate the partitioning of the drug substance between the aqueous and lipid phases of the dispersion and the efficiency of the process, but only in terms of the attachment of the API to the lipid, not its incorporation in it. Only the results of distribution studies allow for a critical assessment of the actual degree of drug incorporation in SLMs and the manner of their distribution in the lipid matrix, also quantitatively. In this way, the API fraction that is generally considered to be incorporated into SLMs can be divided into two: bound in the interphase and incorporated into the lipid matrix core. Such a characterization of SLMs is an important tool and helpful in understanding the effects of SLMs *in vitro* and *in vivo*. Its use in development studies, stability studies or batch control is therefore justified.

6. Models, Conditions and Limitations in Drug Substance Release Studies from SLMs

The release profile of the drug substance incorporated in microparticles is the main attribute of the dosage form because obtaining a prolonged release is one of the basic goals of using SLMs as a drug carrier [6]. The size of lipid particles, their morphology and drug loading, as well as the method of incorporation, are the basic qualitative and quantitative features of the SLMs assessed at the stage of development studies. A common feature, dependent on all these factors, is the drug release profile from the carrier, which thus becomes the most important study of the DDS and, at the same time, the most critical from the point of view of the expected *in vivo* effects. In vitro release testing is commonly used in various DDSs, not only at the stage of their development but also during the quality control of production series, due to the influence of individual process factors on the obtained release profiles, and thus the possibility of monitoring the variability

of the product batches [78,79]. Finally, drug release is crucial for its bioavailability and therapeutic efficacy [5]. Properly designed, it provides a basis for predicting the behavior of the drug carrier *in vivo*. In addition, conducting the study in accordance with pharmacopeia guidelines and recommendations of registration organizations (if any are available) allows for a comparison of the study results between centers. Unfortunately, at present, due to the innovative nature of the tested DDS, there is no harmonized regulatory standard (FDA/EMA) regarding the release study method for the evaluation of multi-compartment lipid carriers, such as SLMs.

Standard release testing methods, according to the pharmacopeial guidelines, are mainly limited to classical dosage forms. Both a paddle apparatus and a flow chamber can be used to test the release of lipid microparticles; however, they are not an optimal solution, as they require modification, e.g., the use of appropriate adapters to prevent particles from being washed out, their collection during sampling, filtration of collected portions of the acceptor fluid, etc. [79,80]. Therefore, in the case of non-standard drug carriers, modifications to traditional methods are considered, and non-pharmacopeial methods are developed and used, taking into account the specificity of the tested product. Unfortunately, this leads to the multiplication of different techniques and definitely makes it difficult to compare the results obtained by different researchers. It should be remembered that different experimental configurations will also have a significant impact on the course of the release process and, consequently, on the obtained results. Therefore, it is important to know and indicate the factors that have the greatest impact on the process in order to best modify and improve the methodology used while striving for its standardization. Being aware of the reasons for differences and the lack of uniformity in the techniques used can also help avoid misinterpretation of data, for example, when comparing the results obtained using different methods.

Generally, when considering the methodology of conducting a release study from lipid microparticles, three groups of techniques can be distinguished: dialysis methods, flow methods and direct mixing with the acceptor fluid in various volumes [80]. As shown in SLM studies, the release profile best reflects the distribution of the API in microparticles, also allowing for the detection of subtle differences in incorporation, which are difficult to detect even in a distribution study. Due to the impact of release studies at different steps of SLM development and manufacturing, experiments were conducted to compare the use of different methods, with particular emphasis on the differences in dialysis membranes and acceptor fluids, in order to indicate the factors that have the greatest impact on the variability of the obtained data.

In the studies conducted in the model without a dialysis membrane, the dominant effect was the rapid initial release of a significant fraction of the drug (*burst effect*), the amount of which is similar to the amount determined in the interphase in distribution studies [81]. The effect of prolonged release, preceded by a rapid, initial release stage, has already been described in the literature for both SLNs and SLMs [82–84]. It is difficult to state unequivocally, but most likely, the rapid initial release effect is the result not only of the surface location of a large fraction of the drug substance but, above all, the result of the direct dilution and mixing of lipospheres with a large amount of acceptor fluid, which, taking into account the route and method of application, would not take place *in vivo*. The prolonged release effect results from the fraction of the drug incorporated in the lipid matrix, which may not be completely released due to the composition of the acceptor fluids, which do not cause enzymatic degradation of the lipid matrix of the microparticles.

In the release study conducted in a diffusion apparatus or dialysis bag, the fundamental difference is the limitation of the penetration of drug molecules into the acceptor compartment not only by the release rate from the carrier or solubility in the acceptor fluid

but also by the diffusion of the substance through the membrane. The most important advantages of such methods include the separation of microparticles from the acceptor fluid (the membrane retains SLMs in the donor compartment, allowing only drug molecules to pass into the acceptor compartment), with no need to filter the collected volume and maintaining the sample in the system. The capacity of the dialysis bag and the volume of the acceptor fluid can be flexibly adjusted to ensure *sink* conditions. The characteristics of the membrane selected for testing are also crucial.

The main difference in the results observed between the membrane and membrane-less models was the lack of the *burst effect* [81]. Moreover, in the studies conducted in the membrane model, significant differences were visible between SLMs with various tested APIs, while in the method without a membrane, the release profiles were very similar. These effects confirm the significant influence of the presence of the membrane and the selected model on the obtained results. The results indicate a higher sensitivity of the two-compartment method, allowing for the detection of subtle differences between SLM formulations based on the observed release profiles. The main property of the membrane used in the release study, which determines the course of the process, is the so-called molecular weight cut-off (MWCO), which, according to the suggestions of the manufacturer, means the molecular weight below which molecules can freely move through the membrane [85]. There are no clear guidelines for the selection of this parameter depending on the molecular weight of the tested API, which has already been discussed earlier [81]. Although there are some tips [86] for the cut-off point to be approximately 100 times the molecular weight of the drug, an analysis of the literature data indicates that the commonly used MWCO is usually only 12 to a maximum of 70 times the molecular weight of the substance being tested [29,86–88].

Among the two two-compartment methods using a dialysis membrane, the model consisting of glass chambers was characterized by some significant limitations. First of all, the movement of the membrane with seals in relation to the donor and acceptor chambers posed a risk of reducing the already small permeation surface, and problems with the tightness of the system were observed [81]. Moreover, in the case of testing SLM dispersions, which were suspensions, after the end of the test, the inhomogeneity of the system was observed in the donor compartment, where lipid microparticles accumulated on the membrane side (at this point, the chamber is narrowed), despite the use of magnetic stirring. This effect did not occur when the microparticles in the donor chamber were diluted before the test. Since different membranes were used in the studies (Cuprophan MWCO 10 kDa, cellulose membrane MWCO 14 kDa or 1000 kDa), also not in the form of a dialysis bag (CA 0.2 µm, PTFE 0.2 µm), subsequent tests were nevertheless conducted in the horizontal cell model. The key factors influencing the obtained results were identified primarily as the size of the membrane pores through which the API penetrates. The hydrophilic/hydrophobic properties of the membrane were also not without significance, which is in favor of hydrophilic membranes. At the same time, it should be emphasized that the comparison of the release profiles obtained from the SLM dispersion and the API suspension proves that the obtained results are the result of prolonged release by the carrier (lipid microparticles) and are not the result of the lack of diffusion capacity through the used dialysis membrane. This confirms the ability of SLMs as a dosage form to provide a prolonged release effect. Dilution of the SLM dispersion in the dialysis bag with the acceptor fluid was also considered important. This somewhat mimics the model without a dialysis membrane and the *in vivo* conditions in which direct mixing with body fluids occurs. At the same time, the ratio of this dilution is important (e.g., 1 + 1) so that the amount of added fluid is not too large (as in the case of the method without a membrane).

Other factors that significantly affect the release profile include the solubility of the drug substance in the acceptor fluid and the difference in API concentrations on both sides of the membrane, which is the driving force of the process, as well as the osmotic pressure in both compartments. For this reason, the experiments were continued, taking into account the importance of physiological factors that occur and affect the dosage form at the site of administration.

The use of biorelevant media is considered to be an appropriate approach to mimic *in vivo* conditions because these are fluids with the same physiological components and properties similar to simulated fluids, e.g., tear fluid or gastrointestinal fluid. SLMs are lipid particles that do not melt at human body temperature; therefore, a complete release of the API from the lipid matrix requires its erosion, for example, by lipolytic enzymes. Other physiological factors at the site of administration are also important, such as electrolytes that change enzyme stability or drug solubility, as well as the pH, ionic strength, gastrointestinal motility or type and concentration of bile salts (in the case of oral administration) [89]. A specific site of application is also the conjunctival sac, with a pH of 7.4 and osmolality of about 290 mOsm/kg after waking up [90].

Unfortunately, it is difficult to compare the obtained results [91] because no one has tested the release from SLMs into artificial tear fluid with the addition of the enzyme lysozyme. Currently, there are only a few studies available on the release from lipospheres (SLNs, SLMs), in which some physiological factors *in vitro* were taken into account, and the studies of lipospheres intended for administration to the eye are practically limited exclusively to SLNs. These studies were conducted using a modified Franz diffusion cell, a USP release apparatus with modifications or a modified diffusion technique in a dialysis bag in a paddle apparatus and simulated tear fluid (STF) as the acceptor fluid [92–95]. Importantly, the composition of the STF used in these studies was limited to only 3–4 salts, such as NaCl, $NaHCO_3$, $CaCl_2$ or KCl, which does not reflect the rich composition of tear fluid. Furthermore, no enzymes were used in these studies; therefore, there is a lack of knowledge about the effect of lysozyme on the integrity of SLMs or SLNs and, thus, on the release rate of the API from these formulations. To date, only two SLM studies have been conducted using fluids simulating physiological conditions, and both refer to oral administration [50,96].

The artificial tear fluid used in our study, the composition of which, like the concentration of lysozyme (1.4 mg/mL), was selected based on available data [97–99], contained not only classical electrolytes (sodium, potassium, ammonium, magnesium and calcium chlorides and sodium bicarbonate) but also lactic acid, citric acid and urea, physiologically present in the conjunctival sac. The determined solubility of the API in the artificial tear fluid was higher than in the polysorbate solution, which can be explained by the composition of the artificial tear fluid. The observed effect is due to, among others, sodium citrate and urea due to the enhanced solvation of drug molecules by water [100]. The introduction of polysorbate improved the solubility of model indomethacin in both the artificial tear fluid and sodium chloride solution, in contrast to the enzyme lysozyme. Despite ensuring *sink* conditions during the study, the incomplete release of the model API from SLMs to the acceptor fluid, which was artificial tear fluid, was observed. Both the addition of lysozyme and polysorbate increased the degree of indomethacin release from the SLMs, but a *plateau* effect still occurred. Only the combined use of all these components allowed for achieving a 100% release of the API from the SLMs [91].

Due to its weakly acidic nature, indomethacin has very low solubility in water [101], similar to many tested APIs suitable for administration in the form of SLMs. The use of surface-active compounds (e.g., sodium lauryl sulfate, polysorbate) increasing the solubility of APIs in the composition of acceptor fluids is justified by the position of the European

Medicines Agency [102]. The purposefulness of such guidelines and the addition of a non-physiological component to the acceptor fluid can also be confirmed by the studies described by Siepmann et al. [103], according to which limited solubility of the drug may play a major role in controlling release. The drug released *in vivo* is absorbed, distributed and metabolized, so it is continuously removed from the site of application. The experimental *in vitro* model is most often a "closed system" in which an artificial effect of drug saturation in the surrounding release medium may occur, "falsifying" the resulting release kinetics [103]. Despite the *sink* conditions, the dissolution rate is proportional to the concentration gradient, according to the Noyes–Whitney equation [104]. In addition to the surface effect, this is also one of the effects influencing the better release of the API in the presence of polysorbate. In this way, the use of a surfactant *in vitro*, to some extent, balances the described *in vivo* effects, which cannot be perfectly imitated in a closed-release model. Thus, although both the artificial tear fluid and the tear fluid with the addition of polysorbate provided *sink* conditions and did not differ in pH, a combination of these components should be used because solubility (which was higher in the presence of surfactant) should be considered the driving force for the release of the API from SLMs. The surfactant, by increasing solubility, balanced *in vitro* the effects that normally occur *in vivo*. Such a composition is, therefore, not an artificial stimulation of release but only allows the carrier to behave "naturally" as in real conditions.

Microscopic observations have shown that the surface of the microparticles is irregular and corrugated. Interacting with this surface, the surfactant from the acceptor fluid in the release study causes its further enlargement by deepening the folds and grooves as a result of the dissolution and release of the surface-localized API. This affects the action of other components of the acceptor fluid on SLMs. The lysozyme enzyme is not a typical lipolytic enzyme, and its interaction with lipids is still a subject of research [105]. The activity of the enzyme is a function of both pH and ionic strength [106]. As a result of the conducted studies, its effect on the release of APIs from SLMs, similarly to other components of tear fluid, was confirmed. At the same time, this effect did not consist in increasing the solubility of the API and was independent of the type of acceptor fluid to which lysozyme was added, which indicates a different mechanism directly related to SLMs. Therefore, it was considered to what extent lysozyme affects lipid microparticles by influencing the release of the fraction of the substance incorporated into the lipid matrix. For this purpose, SLMs were incubated with natural tear fluid [91]. The changes observed in SLMs with indomethacin were very similar to those after testing the residues from the bag after release and were even more pronounced and intense when the placebo SLMs were incubated with natural tears. Then, even aggregates of particles with clearly disturbed individual structures were visible. In the placebo preparations, many particles were deformed and seemed to be cracked and losing their integrity. Differences in the shape of the particle surface were also visible. When comparing placebo SLMs and SLMs with indomethacin, before and after incubation with tears, larger "wrinkles" and sharp edges on the particle surface were clearly observed in comparison to the smooth and more spherical microspheres with soft edges before incubation. In summary, it should be stated that the effect of lysozyme on SLMs has been confirmed, and it is not related to the degradation of the SLM matrix by the enzyme but only to the surface effects, which, given the established location of the drug substance, guarantees its good release.

Polysorbate and lysozyme were found to be important components of the acceptor fluid despite the lack of direct interaction with the microparticle core. Their slow interaction resulted in the release of the fraction of the drug permanently bound to the lipid (more than the amount quantified in the interphase). It is obvious that after the *in vivo* ocular administration there will be no contact of the formulation with polysorbate. However, the

changes observed due to its presence on the microparticle surface were sufficient for the complete release of the API *in vitro*, such as transformations and deformations, which were observed after the incubation of SLMs with natural tears without polysorbate.

The use of different release testing methods (without or with a membrane) leads to different release profiles. The *burst effect*, characteristic of the membrane-free method, correlated with the amount of API determined on the surface in SLMs, but due to the high ratio of acceptor fluid to dispersion, in most cases, it does not reflect the physiological conditions and thus does not simulate the real release process that could occur *in vivo*. The methods using membranes resulted in obtaining similar results, but the dialysis bag method was less demanding and troublesome. The key factors determining the release rate were identified as the dialysis membrane, type of acceptor fluid and the difference in API concentrations between compartments. The influence of the lysozyme on SLMs can also be confirmed. The structural changes observed in SLMs incubated with natural tears confirmed the influence of the physiological components on microparticles and were the same as those resulting from the simulation of physiological conditions during the release test. Therefore, the use of biorelevant media as an acceptor fluid in the release study is fully justified and has a significant impact on the obtained results, and the simultaneous use of physiological components in combination with a surfactant guarantees better solubility of poorly soluble APIs, imitating the processes leading to the elimination of APIs from the application site *in vivo*.

In summary, all of the instrumental methods characterized above, together with the release and distribution studies described in the following sections, are summarized in Figure 3. The graphical layout presents all of the techniques in order, from the most to the least valuable and useful for describing the properties and assessing the quality of the studied SLM formulations, according to the authors' assessment and own experience. The primary goal of all analyses and the presented summary was the most accurate qualitative and quantitative identification of APIs and the characterization of their distribution in SLM formulations.

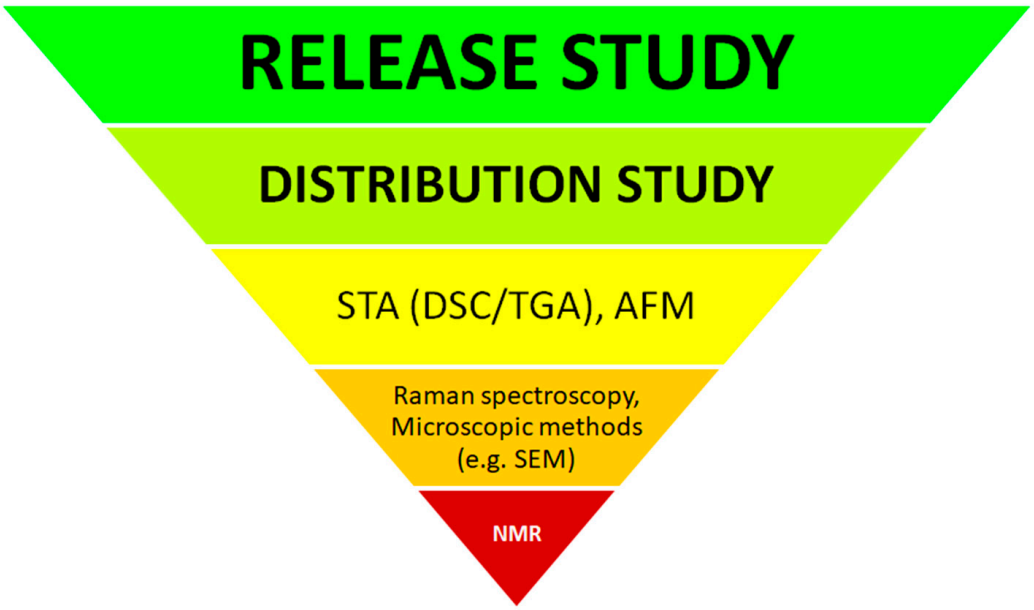


Figure 3. The cascade of methods for assessing the SLM properties in terms of the usefulness and value of the obtained results according to the authors' experience and assessment.

7. Conclusions and Perspectives

This review focuses on two aspects, which are crucial from the point of view of assessing SLMs as a modern, multi-compartment, lipid dosage form—distribution and release of the drug substance. A number of instrumental methods (STA, AFM, Raman spectroscopy, NMR) are presented, indicating their advantages and limitations in their use for analyzing the distribution of APIs in lipid microparticles. This work also focuses on comparing the quantitative method of testing the distribution of APIs in SLMs as a solution developed specifically for the needs of SLM analysis, with the most commonly used parameters EE and DL used for characterizing the liposphere, critically assessing their limitations and imperfections in describing the distribution of the drug substance in individual phases of SLM dispersions. Finally, the methods of testing the release used in practice are presented, emphasizing the importance of using a biorelevant medium and taking into account the physiological factors occurring *in vivo* at the site of application during liposphere studies.

The aim of this review was primarily to identify the advantages and limitations of various instrumental tools and techniques used to assess the properties and development studies of dosage forms such as SLMs. The description of an innovative approach to assessing the distribution of the incorporated APIs in SLMs allows for a quantitative characterization of the API in individual phases of SLM dispersions. A thorough analysis of the release testing methods and understanding of the influence of physiological factors on the course of the process and properties of SLMs should justify the essence of standardization of the procedure and protect the reader from misinterpretation or comparison of the inter-center results obtained with different techniques.

The aim of the presented study is also to critically summarize the experience gained during many years of research, which can help researchers save their time and effort spent on recognizing different types of problems and focus on new aspects and strategies for the analysis, development and improvement of SLMs as a dosage form.

There are undoubtedly many challenges that still need to be overcome in the further stages of the development of SLMs as a lipid dosage form. However, we hope that the presented paper will enable further progress in research on solid lipid microparticles and facilitate an effective assessment of their properties. An important aspect is also the achievement of standardization of presented procedures, as an element necessary for comparing the research and development of registration regulations, which in the future could facilitate the admission of a modern SLM formulation to the market.

In our opinion, the future of solid lipid microparticle technology should focus on the development of reproducible and scalable formulations obtained in the simplest possible—although not necessarily one-step—environmentally friendly production processes to ensure the availability of an excellent lipid carrier for poorly soluble drug substances, capable of being administered by various routes.

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