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APPLICATION OF IONIC LIQUIDS IN THE DEVELOPMENT OF SUSTAINED DELIVERY SYSTEMS

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APLICACIÓN DE LÍQUIDOS IÓNICOS EN EL DESARROLLO DE SISTEMAS DE ADMINISTRACIÓN SOSTENIDA
APLICAÇÃO DE LÍQUIDOS IÓNICOS NO DESENVOLVIMENTO DE SISTEMAS DE ADMINISTRAÇÃO SUSTENTADA

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Abstract

The development of drug delivery systems, namely for controlled release, present some problems such as the poor solubility and bioavailability of many drugs, also inflexible drug release profiles, the possible side effects, and the low drug selectivity for target tissues. So, finding new strategies and/or excipients to surpass these challenges is crucial and ionic liquids (ILs) may be key materials in this matter. Hence, the aim of this thesis was to explore the applicability of ILs in the development of more effective sustained drug delivery systems, namely polymeric nanoparticles, lipidic implants, and transfersomes, all containing ILs.

Firstly, IL-polymer nanoparticle hybrid systems containing rutin were prepared using the polymer poly(lactic-co-glycolic acid) (PLGA) and two biobased ILs, (2-hydroxyethyl)-trimethylammonium-L-phenylalaninate [Cho][Phe] and the (2-hydroxyethyl)-trimethylammonium-L-glutaminate [Cho][Glu]. Two different ratios of PLGA (50:50 and 75:25), were studied and the hybrid systems showed a particle size between 250 nm and 300 nm, as well as a low polydispersity index, a zeta potential around - 40 mV and a drug association efficiency (AE) ranging from 51% to 76%. Since rutin is a poorly soluble drug, the obtained AE was quite relevant showing that the ILs were crucial to load rutin into the nanosystem. Additionally, the ILs did not interfere with the sustained release properties of the nanosystem and allowed a rutin release of about 85% after 72 h. Also, upon freeze-drying no particle aggregation was observed, exhibiting the stability of the systems containing ILs. Finally, the results also indicated that the developed systems may be suitable for skin topical applications since no relevant skin permeability was seen and no toxicity was shown in the cell viability study in HaCaT, human keratinocytes.

Lipidic implants with caffeine, salicylic acid, or rutin, were also prepared. Various compositions were studied, namely the incorporation of the ILs [Cho][Phe] or [Cho][Glu], and of two different release adjuvants, Gelucire® 50/02 and sucrose. Consequently, 54 different batches of implants were developed to study the formulation procedure, the dye content distribution, the drug content and drug release, the water content, as well as the lipidic erosion. Gelucire® 50/02 and sucrose were not fitting tools to enhance the drug release profile. On the other hand, results showed that ILs were valuable materials by facilitating the formulation procedure, improving drug loading and by allowing a more suitable release profile. Moreover, atomic force microscopy (AFM) analysis displayed that the presence of ILs conveyed a more wrinkled surface to the implants, with the [Cho][Glu] leading to a more noticeable surface wrinkling, agreeing with the observed higher drug release, in the presence of this IL.

Finally, new class of nanovesicles containing ILs (TransfersomILs) were developed, having into account an optimized formulation obtained herein from a 15-run, 3-factor, 3-level Box–Behnken factorial design (BBD). The TransfersomILs were prepared in the presence of 1-ethyl-3-methylimidazolium bromide [Emim][Br], 2-hydroxyethyl-trimethylammonium glycinate [Cho][Gly], or 1-ethyl-3-methylimidazolium glycinate [Emim][Gly] and also using ILs combinations, to incorporate rutin and to verify if the ILs would be able to improve the overall performance of the transfersomes. Initially, it was assessed the impact of the ILs and of the ILs combinations on the cell viability of HaCaT cells and on the rutin's solubility. Then, the physicochemical properties of the new TransfersomILs were evaluated and the results demonstrated that the ILs led to improved systems. Namely, when compared with the tranfersomes without IL, the new TransfersomILs showed a smaller size and, in overall a higher association efficiency, as well as loading capacity, and also a higher total amount of drug released. Additionally, the ILs also contributed to upgrade the storage stability of the nanovesicular systems, by promoting their colloidal stability.

Hence, even at low and safe concentrations, ILs can be crucial to facilitate formulation procedures and improve the overall physicochemical properties of controlled drug delivery systems, namely by leading to a higher drug loading and release, by enhancing the stability of systems and even by improving surface characteristics. Thus, this work showcased ILs as key multifunctional materials to upgrade the performance of sustained delivery systems in multiple ways.

Keywords: Ionic Liquids; Sustained Drug Delivery Systems; Upgrade Performance; IL-Polymeric Nanoparticles; IL-Lipidic Implants; TransfersomILs.

Resumen

El desarrollo de sistemas de administración de fármacos, concretamente para liberación controlada, presenta algunos problemas, como la escasa solubilidad y biodisponibilidad de muchos fármacos, los perfiles de liberación de fármacos inflexibles, los posibles efectos secundarios y la baja selectividad del fármaco para los tejidos diana. Por lo tanto, encontrar nuevas estrategias y/o excipientes para superar estos desafíos es crucial y los líquidos iónicos (LI) pueden ser materiales clave en este asunto. Por ello, el objetivo de esta tesis fue explorar la aplicabilidad de los LI en el desarrollo de sistemas de administración sostenida de fármacos más efectivos, en concreto, nanopartículas poliméricas, implantes lipídicos y transfersomas, todos ellos con LI.

En primer lugar, se prepararon sistemas híbridos de nanopartículas poliméricas-LI que contenían rutina utilizando el polímero poli(ácido láctico-co-glicólico) (PLGA) y dos LI de base biológica, (2-hidroxietil)-trimetilamonio-L-fenilalaninato [Cho][Phe] y el (2-hidroxietil)-trimetilamonio-L-glutaminato [Cho][Glu]. Se estudiaron dos proporciones diferentes de PLGA (50:50 y 75:25), y los sistemas híbridos mostraron un tamaño de partícula entre 250 nm y 300 nm, un índice de polidispersidad bajo, un potencial zeta alrededor de -40 mV y una eficiencia de asociación de fármacos (EA) comprendida entre 51% y 76%. Dado que la rutina es un fármaco poco soluble, el EA obtenido fue bastante relevante y mostró que los LI eran cruciales para cargar la rutina en el nanosistema. Adicionalmente, los LI no interfirieron con las propiedades de liberación sostenida del nanosistema y permitieron una liberación de rutina de alrededor del 85% después de 72 h. Además, al liofilizar no se observó agregación de partículas, lo que demuestra la estabilidad de los sistemas que contienen LI. Finalmente, los resultados también indicaron que los sistemas desarrollados pueden ser adecuados para la aplicación tópica sobre la piel, ya que no se observó una permeabilidad cutánea relevante ni toxicidad en el estudio de viabilidad celular en células HaCaT, de queratinocitos humanos.

También se prepararon implantes lipídicos que contenían cafeína, ácido salicílico o rutina. Se estudiaron diferentes composiciones, concretamente la incorporación de los LI [Cho][Phe] o [Cho][Glu], y de dos adyuvantes de liberación diferentes, Gelucire® 50/02 y sacarosa. En consecuencia, se desarrollaron 54 lotes diferentes de implantes para estudiar el procedimiento de formulación, la distribución del contenido de colorante, el contenido y liberación de fármaco, el contenido de agua, así como la erosión lipídica. Ni el Gelucire® 50/02 ni sacarosa resultaron ser herramientas adecuadas para mejorar el perfil de liberación del fármaco. Por otro lado, los resultados mostraron que los LI eran materiales valiosos al facilitar el procedimiento de formulación, mejorar la carga del fármaco y permitir obtener un perfil de liberación más adecuado. Además, el análisis de microscopía de fuerza atómica mostró que la presencia de LI proporcionó una superficie rugosa a los implantes, siendo el [Cho][Glu] el que proporcionó la

mayor rugosidad a la superficie, en consonancia con la mayor liberación de fármaco observada en presencia de este LI.

Finalmente, se desarrollaron una nueva clase de nanovesículas que contienen LI (TransfersomILs), teniendo en cuenta una formulación optimizada obtenida a partir de un diseño de Box-Behnken (DBB) de 15 experimentos, 3 factores y 3 niveles. Los TransfersomILs se prepararon en presencia de bromuro de 1-etil-3-metilimidazolio [Emim][Br], glicinato de 2-hidroxietil-trimetilamonio [Cho][Gly], o glicinato de 1-etil-3-metilimidazolio [Emim][Gly] y también usando combinaciones de LI, para incorporar rutina y para comprobar si los LI pudieran mejorar el rendimiento general de los transfersomas. Inicialmente, se evaluó el impacto de los LI y de las combinaciones de LI sobre la viabilidad celular en células HaCaT y sobre la solubilidad de la rutina. Después, se evaluaron las propiedades fisicoquímicas de los nuevos TransfersomILs y los resultados demostraron que los LI condujeron a sistemas mejorados. En comparación con los transfersomas sin LI, los nuevos TransfersomILs presentaron un tamaño más pequeño y, en general, una mayor eficiencia de asociación, capacidad de carga, así como una cantidad total de fármaco liberado mayor. Además, los LI también contribuyeron a mejorar la estabilidad de almacenamiento de los sistemas nanovesiculares, al promover su estabilidad coloidal.

Por lo tanto, incluso en concentraciones bajas y seguras, los LI pueden ser cruciales para facilitar los procedimientos de formulación y mejorar las propiedades fisicoquímicas generales de los sistemas de administración controlada de fármacos, proporcionando a una mayor carga y liberación de fármacos, mejorando la estabilidad de los sistemas e incluso mejorando las características de la superficie. Por lo tanto, este trabajo mostró los LI como materiales multifuncionales clave para mejorar el rendimiento de los sistemas de liberación sostenida de múltiples maneras.

Palabras clave: Líquidos Iónicos; Sistemas de Administración Sostenida de Fármacos; Mejora del comportamiento; LI-Nanopartículas Poliméricas; Implantes LI-Lipídicos; TransfersomILs.

Chapter 1

General Introduction

1.1. Drug delivery systems

Drug delivery systems are an interface between the drug and the body since they allow the introduction of the drug into the body (Hua, 2019; K. K. Jain, 2008). They also enable drug release in the desired tissue, organ, cell, and subcellular organs, contributing to the drug efficacy and safety (K. K. Jain, 2008; C. Li et al., 2019). Basically, drug delivery systems are designed to obtain drug therapeutic concentrations at the target location (C. Li et al., 2019).

1.1.1. Administration routes

The appropriate delivery system needs to be chosen accordingly to the disease and the intended effect, since they may have systemic or local effects (K. K. Jain, 2008). Depending on the purpose, they may also use different routes to deliver the drug, such as the enteral or the parenteral (**Figure 1.1**) (L. V. Allen & Ansel, 2014; Bardal, Warchter, & Martin, 2011; Mignani, El Kazzouli, Bousmina, & Majoral, 2013; Tiwari et al., 2012).

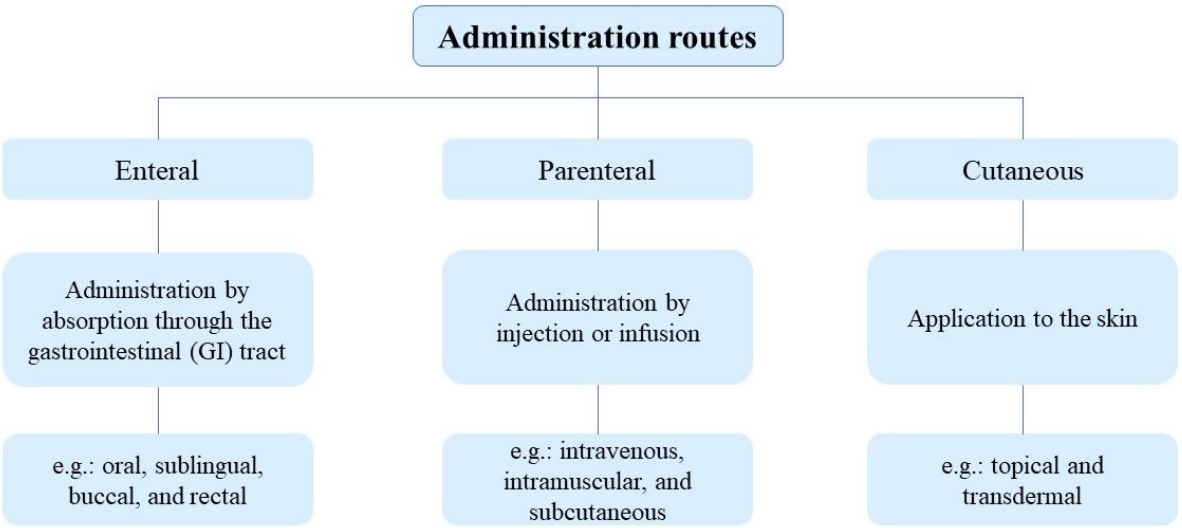

Figure 1.1. Routes of administration in drug delivery systems (L. V. Allen & Ansel, 2014; Bardal et al., 2011; K. K. Jain, 2008; Mignani et al., 2013; Narasimha Murthy & Shivakumar, 2010).

In the enteral route of administration, the drug is absorbed through the gastrointestinal (GI) tract from the mouth until de anus, including for instance the oral, sublingual, buccal, and rectal routes (K. K. Jain, 2008; Mignani et al., 2013). The oral drug delivery is one of the most used routes of administration, since its more convenient, safe, and relatively economical. Consequently, the patient compliance increases (Adepu & Ramakrishna, 2021; Mignani et al., 2013).

In the oral cavity, beyond the oral administration, there are also the sublingual, under the tongue, and the buccal, between cheek and gum, administration routes. Both routes are important to absorb lipophilic drugs with low oral availability (Hua, 2019).

Rectal administration is an alternative route to oral delivery when the patient is unable to take oral medication (e.g., when is unconscious, vomiting, or convulsing) as well as in the childhood. This is also an advantageous route since almost half of the drug dose can surpass the liver, which decreases the first pass effect (Purohit, Hanning, & Wu, 2018).

Nonetheless, there are some disadvantages of the enteral administration, such as: the unpredictable drug concentration in the blood circulation, due to the variable absorption rate in the GI tract; the drug degradation by the digestive enzymes and high acid content; the low pH levels that can difficult the drug dissolution; and the inactivation of drug due to the first pass metabolism (Bardal et al., 2011; K. K. Jain, 2008).

Regarding the parenteral route of administration, this considers the administration not involving the absorption through the GI system and using injections or infusions. This includes the intravenous, the intramuscular and the subcutaneous (Bardal et al., 2011; Soar & Standing, 2019). It may be used for instance when the patient is very ill or in coma and thus the oral administration is not suitable (K. K. Jain, 2008). The advantages of the parenteral route are the rapid onset of action (rapid therapeutic effect), the fact that it allows an almost complete bioavailability, and also because it avoids the drug challenges related to the absorption through GI tract (Bardal et al., 2011; K. K. Jain, 2008). In contrast, the invasive nature of the injectable

routes within the parenteral administration may cause some healthy problems, such as pain in the local of the administration, which decreases the patient compliance (Soar & Standing, 2019).

On the other hand, the cutaneous administration refers to the applicability of the delivery systems to the skin namely for local or for systemic effects (Alkilani, McCrudden, & Donnelly, 2015; Narasimha Murthy & Shivakumar, 2010). This route includes the topical and transdermal routes and consists on the drug being applied directly to the skin, avoiding the systemic effect as well as the first pass effect and gastric and plasma pH variations, but is painless and non-invasive (Alkilani et al., 2015; Narasimha Murthy & Shivakumar, 2010; Singh Malik, Mital, & Kaur, 2016). Moreover, it also enhances patient compliance, since it is easier and convenient to apply (Bardal et al., 2011; Singh Malik et al., 2016). However, the cutaneous delivery should be used for relatively potent drugs since the skin represents an impermeable barrier for the entrance of external compounds (Singh Malik et al., 2016). Also, the skin barrier limits the drug permeability to drugs with molecular weight less than 500 Da, an octanol-water partition coefficient within 1 and 3, and a solubility in water above 1 mg/mL (Paudel et al., 2010; Sahu, Ghosh, & Rath, 2021).

Regardless of the administration route used, it is always needed to find the appropriate drug delivery system (Bardal et al., 2011; K. K. Jain, 2008; Tiwari et al., 2012). For instance, a suitable system could be helpful to overcome some drug challenges, such as poor drug solubility, permeability and biodistribution, problems concerning drug side effects and extravasation (that may cause tissue damage), low drug selectivity for target tissues, not favorable pharmacokinetics, and difficulties in the maintenance of therapeutic concentrations (Adepu & Ramakrishna, 2021; T. M. Allen & Cullis, 2004; C. Li et al., 2019). Thus, the search for new release strategies and drug delivery systems to overcome some of the previously described challenges has been increasingly evident. One of the strategies that has emerged is the use of sustained and controlled delivery systems.

1.1.2. Sustained delivery systems

Sustained delivery systems are important to extend the drug effect, by minimizing drug doses, leading to less adverse effects and fewer fluctuations of the therapeutic window and blood levels (Adepu & Ramakrishna, 2021; K. K. Jain, 2008). They can be distinguished based on their drug release mechanisms (**Figure 1.2**) (Adepu & Ramakrishna, 2021; Siegel & Rathbone, 2012), namely by:

✓Dissolution: This is the case of drugs protected by being coated with or encapsulated within polymeric membranes or matrices that can dissolve.

✓Diffusion: In this case the drugs are trapped within the systems, released by diffusion and drug release is controlled by Fick's laws of diffusion.

✓Water penetration: As the name indicates, in this case the drug release is dependent on the water penetration into the systems.

✓Chemical modification: In this situation the chemical structure of the systems is altered when it is exposed to the biological media allowing for the drug to be released.

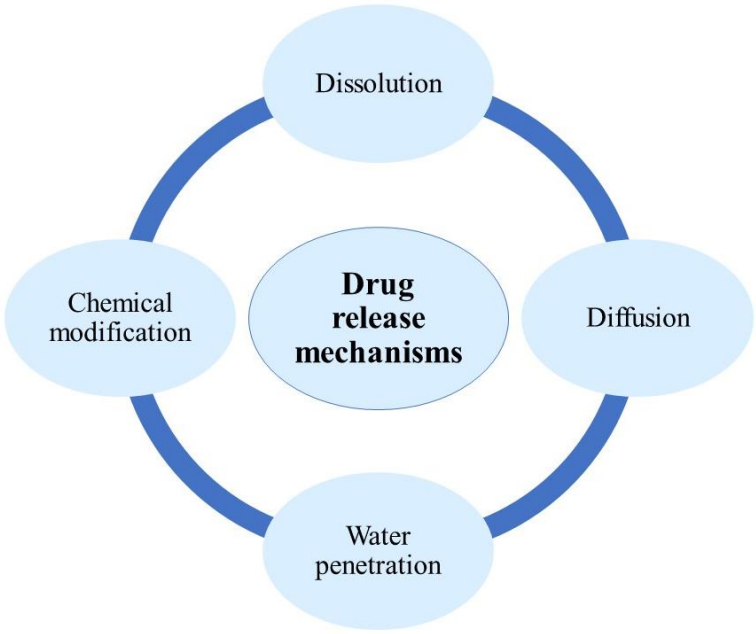

Figure 1.2. Drug release mechanisms in controlled drug delivery systems (Adepu & Ramakrishna, 2021).

Controlled delivery systems present several important advantages, such as the controlled drug release, the possibility of directing the drug to the target, the drug resistance to the pH and osmotic

variations, the protection of the first pass effect and of the metabolization process, the enhancement of the drug bioavailability, the necessity of fewer drug dose, and the better patient compliance (Adepu & Ramakrishna, 2021; Bardal et al., 2011; K. K. Jain, 2008).

Nonetheless, controlled delivery systems may have a high-cost production and sometimes the materials used may be toxic to the human body, which can limit their applicability (Adepu & Ramakrishna, 2021; Bardal et al., 2011; K. K. Jain, 2008). Additionally, some of these systems can be invasive in terms of their application and/or removal (Adepu & Ramakrishna, 2021).

Examples of sustained release systems, that have been increasingly being studied, are for instance nanosystems and implantable systems (Abu-Diak, Andrews, & Jones, 2012; Adepu & Ramakrishna, 2021; Ayub & Wettig, 2022; Stewart, Domínguez-Robles, Donnelly, & Larrañeta, 2018; Tsung & Burgess, 2012).

1.1.2.1. Nanosystems

In the 21st Century, the number of studies considering the applicability of nanosystems in the pharmaceutical field has been increasing. These systems are composed of nanoparticles, which are solid colloidal particles with size up to 1000 nm acting as submicron-sized drug carriers where the drug can be dissolved, adsorbed, attached, encapsulated, or entrapped into them (Adepu & Ramakrishna, 2021; Maghsoudnia, Eftekhari, Sohi, Zamzami, & Dorkoosh, 2020; Santos de Almeida, Júlio, Mota, Rijo, & Reis, 2017). The nanosystems can be tailored by changing the materials or the formulation process accordingly with the drug to be loaded (Adepu & Ramakrishna, 2021; Maghsoudnia et al., 2020).

Overall, nanosystems have some advantages such as increasing patient compliance, by avoiding repetitive bolus injections due to controlled and sustained drug release. Additionally, these systems can improve drug solubility and drug targeting which may enhance drug biodistribution and transportation. Moreover, the properties of nanoparticles can be optimized by

combining them with different materials to surpass the challenges associated with developing this type of targeted delivery systems. Namely, to overcome obstacles such as the body's barriers, like the cell membrane, the extracellular matrix, and vascular endothelium, for a drug to reach a certain location (Almeida, Filipe, Rosado, & Pereira-Leite, 2022; Ayub & Wettig, 2022; Chavda, 2019). Moreover, these systems also have other limitations, particularly when considering their applicability in the pharmaceutical field, such as the possible toxicity of some of the materials used, and the fact that some nanoparticles have more susceptibility for accumulation in the tissues (Chavda, 2019; Santos de Almeida et al., 2017; Vrignaud, Benoit, & Saulnier, 2011). Furthermore, nanoparticles can show particle growth and aggregation tendency (Vrignaud et al., 2011) and the development and production of some nanocarriers can be extremely expensive (Santos de Almeida et al., 2017; Vrignaud et al., 2011). The nanosystems can be classified as metallic, polymeric, and lipid-based nanoparticles.

Metallic nanosystems

The metallic nanoparticles are colloidal particles (Desai et al., 2021), that are composed of inorganic materials, such as gold, iron oxide, and silver (Pinsky et al., 2021). In these systems the drugs can be found in three different positions: dispersed or encapsulated within a shell; covalently attached to the surface; or entrapped within a structure to deliver the drug at the target site (Desai et al., 2021).

Furthermore, the metallic nanoparticles have enormous potential for targeting the drug to the desired local, they present high stability, and the majority of their applications are within the diagnostic and imaging fields (Adepu & Ramakrishna, 2021; Dubey, Sertorio, & Takiar, 2022; Pinsky et al., 2021). According to the intended application, their size, chemical composition, and shape can be altered (Adepu & Ramakrishna, 2021; Sharma, Goyal, & Rath, 2017).

However, the metallic nanoparticles also have disadvantages such as presenting some instability due to the possibility of aggregation, the increment of inflammatory process when the metallic particles degrade and in the case of nanoparticles precipitation, this causes cellular damage (Adepu & Ramakrishna, 2021; Pinsky et al., 2021). It is also important to mention that for this type of system, aggregation can be avoided by supporting the nanoparticles in a solid material, such as ceramics, glasses, carbon-based materials, and other metals (Pinsky et al., 2021).

Polymeric nanosystems

One of the most studied type of nanoparticles are the polymeric ones. These particles are solid matrix systems, where the drug is dispersed within the particle or conjugated to their surface, and they are prepared in the presence of natural, semi-synthetic, or synthetic polymers (G. Calixto, Bernegossi, Fonseca-Santos, & Chorilli, 2014; Heinz et al., 2017; Santos de Almeida et al., 2017). The most common natural polymers used in nanoparticles are chitosan, albumin, and gelatin and the synthetic ones are poly(lactic-co-glycolic) acid (PLGA), polylactic acid (PLA), poly-caprolactone (PCL), and poly-alkyl-cyano-acrylate (PAC) (Biolhassani et al., 2019; G. M. F. Calixto et al., 2016).

These nanoparticles allow increasing drug solubility, which enhances the drug concentration around the target tissues. Additionally, they also confer a high level of protection to the drug against rapid degradation, when in contact with the physiological fluids (G. Calixto et al., 2014; Heinz et al., 2017; Santos de Almeida et al., 2017). Polymeric nanoparticles include nanocapsules and nanospheres (G. Calixto et al., 2014; Heinz et al., 2017; Santos de Almeida et al., 2017). Nanocapsules are heterogeneous reservoirs or vesicular systems in which the drug is normally dissolved, in a molecular dispersion or solid/liquid form (Santos de Almeida et al., 2017; Vrignaud et al., 2011). This type of nanoparticles may also carry the drug on their surfaces or imbibed in the polymeric membrane, which can be formed by lipophilic or hydrophobic materials (Santos de Almeida et al., 2017). Regarding the nanospheres, they consist of a homogenous matrix

in which the drug is dispersed, and the drug may be absorbed in the sphere surface or encapsulated within the particle (Santos de Almeida et al., 2017).

The polymeric nanoparticles are highly suitable to be applied in the pharmaceutical area due to several advantages, such as their biodegradability and biocompatibility, their size and morphology, versatility, surface functionalization, easy handing without the loss of their characteristics, high encapsulation efficiency, controlled release with great bioavailability, and are among the most economically acceptable (Chavda, 2019; Leyva-Gómez et al., 2018; Vrignaud et al., 2011).

Lipid-based nanosystems

Besides the polymeric nanosystems, there are also the lipid-based nanoparticles. The lipid-based systems can also increase the therapeutic effect of the drug by prolonging the drug half-life and/or by contributing to a controlled drug release and may encapsulate hydrophobic and hydrophilic drugs (Adepu & Ramakrishna, 2021; García-Pinel et al., 2019; S. Jain, Patel, Shah, Khatri, & Vora, 2017). These systems can be chemically modified to avoid detection by the immune system or to enhance the solubility of the drug (García-Pinel et al., 2019). The lipid-based nanoparticles can be divided into solid liquids nanoparticles (SLNs), nanostructured lipid carriers (NLCs) and vesicular systems (G. M. F. Calixto et al., 2016; García-Pinel et al., 2019; Musielak, Feliczak-guzik, & Nowak, 2022; Sharadha, Gowda, Vishal Gupta, & Akhila, 2020).

The SLNs are spherical nanoparticles made of solid lipids. They have some advantages, such as the fact that they are easier to formulate compared with the polymeric nanoparticles, are well tolerated and biocompatible, have physical stability, protect the drug against degradation, and enhance drug stability. Their surface can also be functionalized with several compounds. However, SLNs also have limitations like drug expulsion during storage time, presenting

considerable water content, and low encapsulation efficiency (G. M. F. Calixto et al., 2016; Sharadha et al., 2020).

With respect to the NLCs, these are prepared by combining a solid lipid with a liquid lipid and they were developed with the goal of surpassing some of the SLNs limitations, such as the low encapsulation efficiency and drug expulsion during storage. They also show higher stability than SLN (G. M. F. Calixto et al., 2016; Sharadha et al., 2020).

Regarding the vesicular systems, these are made of biocompatible lipids and aqueous solvents, where the lipids form concentric lamellae entrapping the aqueous phase which may contain the drug to load (S. Jain et al., 2017). There are several types of nanovesicular carriers, such as liposomes, transfersomes, ethosomes, and niosomes

The liposomes were the first to be developed. These are organized in a bilayer structure of phospholipids (the most common phospholipid is phosphatidylcholine (Adepu & Ramakrishna, 2021)), which confer biocompatibility and biodegradability. Their amphipathic properties make them able to load hydrophobic and hydrophilic drugs (García-Pinel et al., 2019). Additionally, liposomes also improve drug localized delivery. However, they present an high production cost and some formulation issues such as poor chemical and physical stability, high diameter and low encapsulation efficiency (S. Jain et al., 2017).

Regarding the ethosomes, they are a modification of conventional liposomes, constituted of a concentration of ethanol of up to 45% w/w, phospholipids, as well as water (Abdulbaqi, Darwis, Khan, Assi, & Khan, 2016). They also are suitable to load both hydrophobic and hydrophilic drugs and present higher elasticity, small diameter and higher encapsulation efficiency compared with the conventional liposomes (Abdulbaqi et al., 2016; S. Jain et al., 2017). In contrast, they have short structural and chemical stability during storage (S. Jain et al., 2017).

Moreover, the niosomes are bilayer vesicles structurally similar to liposomes formed by the self-assembly of nonionic surfactants in the presence of cholesterol (Yaghoobian, Haeri,

Bolourchian, Shahhosseni, & Dadashzadeh, 2020). Recently, they have shown a good alternative to liposomes as nanocarriers for oral delivery, because of their higher chemical stability and also lower production cost (García-Pinel et al., 2019; Yaghoobian et al., 2020). Niosomes are also biocompatible and have the capacity to encapsulate both hydrophilic and lipophilic drugs. Besides that, they are non-immunogenic and their composition is flexible (Yaghoobian et al., 2020).

Finally, in terms of vesicular systems, the ultra-deformable bilayer vesicles, designated as transfersomes, have also emerged as a way to overcome some of the challenges that the conventional liposomes have (S. Jain et al., 2017; Oliveira, Tavares, Soares-sobrinho, & Chaves, 2022; Yeo, Yoon, & Lee, 2022). Transfersomes are elastic liposomes composed of the phospholipid component and an edge activator (EA), that allows transfersomes to deform (Maji et al., 2021; Opatha, Titapiwatanakun, & Chutoprapat, 2020). EAs are functional excipients that act as membrane destabilizing factors when combined in a fitting ratio with an appropriate lipid. This enables the transfersomes to become deformable and more flexible, resulting in a better permeation ability and consequently more suitable for topical and transdermal applications (Maji et al., 2021; Opatha et al., 2020). Additionally, the EAs can also promote the solubilization of hydrophobic drugs, and thus enhancing the drug encapsulation efficiency (Opatha et al., 2020). Comparatively to the conventional liposomes, they are also smaller and have more elasticity (S. Jain et al., 2017; Maji et al., 2021). Consequently, these systems present several advantages that justify further investing in the study of their applicability.

1.1.2.2. Implantable systems

Implants are another type of controlled drug delivery systems that allow a prolonged, controlled, and localized release of the drug, and may present different sizes, shapes, and delivery mechanisms (Hillery, 2001; Kreye, Siepmann, & Siepmann, 2008; Kreye, Siepmann, Willart, Descamps, & Siepmann, 2011; Stewart et al., 2018; Tsung & Burgess, 2012). They are usually placed transdermally or subcutaneously, in the upper arm, thigh and peritoneum (Kreye et al., 2008; Kreye, Siepmann, Willart, et al., 2011).

In the implantable systems, the drug can be introduced into a reservoir (nucleus), which controls its release, called a reservoir system. The drug can also be incorporated into a matrix, a monolithic system, which conditions its release, or it can be placed in an implantable pump system, which delivers the drug through a flow rate controlled by mechanical or osmotic mechanisms (Hillery, 2001; Kreye, Siepmann, Zimmer, et al., 2011a; Kreye et al., 2008; Pongjanyakul, Medlicott, & Tucker, 2004; Tsung & Burgess, 2012).

Regardless of where the drug is retained, its release from implants can be accomplished by several mechanisms, but the release by diffusion is the most common (Kreye, Siepmann, Zimmer, et al., 2011a). For instance, the drug release begins with the penetration of the solvent, usually water, into the implant core, causing the drug to diffuse into the release medium. However, in systems where the membrane is non-porous, drug diffusion is conditioned either by the size of the drug molecules or by the spaces available between the polymer chains. Moreover, the place where the system will be implanted, as well as the interface between the membrane and the reservoir and the control rate by the membrane, are three factors that also affect the drug release. In these systems the drug release rate is governed by Fick's Law (Hillery, 2001; Stewart et al., 2018).

Regarding the application of the implants, these types of systems have several advantages, such as (Hillery, 2001; Stewart et al., 2018):

✓Potential for controlled release: by releasing the drug in a controlled manner and according to zero-order release kinetics, they may avoid plasma fluctuations that could lead to toxicity issues and/or the subtherapeutic doses. They also reduce the frequency of administration.

✓Improved drug delivery: Implantable systems allow the local or systemic administration of drugs that are susceptible to interferences from biological barriers or metabolic processes, which condition their arrival at the target site.

✓Convenience: Allow the maintenance of plasma drug concentrations for long periods of time without the need for the patient to remain under medical surveillance during drug administration.

✓Compliance: after being inserted, there is no risk of the patient not complying with the therapeutic regimen, thus leading to better adherence to therapy.

✓Flexibility: the possibility of using different materials and manufacturing processes, as well as different drug dosages and release rates allows these systems to be easily adapted to the therapeutic needs.

However, there are also some disadvantages that need to be noted:

✓Invasive system: the use of these systems involves performing a minor surgery for its placement. Additionally, in the case of non-biodegradable implants and implantable pumps after the end of the treatment it is also necessary to undergo surgery to remove them. Regarding biodegradable implants, it may sometimes be necessary to remove them as their degradation over time may affect the end of therapy (Stewart et al., 2018).

✓Possibility of adverse effects: the drug is continuously released in the same site, so high levels of drug concentration can be reached for long periods of time, which can cause adverse local tissue reactions or irritation (Hillery, 2001).

✓Limited to potent drugs: the implants must be reduced in size, so this may limit their dosing capacity, making them only viable for the administration of potent drugs (Hillery, 2001).

✓Commercial: their production requires huge financial resources and its time consuming, so this can delay their commercialization, and their cost may be too expensive for the patient (Hillery, 2001).

✓Release Profile: the preparation processes used may cause some of its constituents to undergo polymorphisms, resulting in erratic drug releases (Kreye, Siepmann, Zimmer, et al., 2011a).

Nonetheless, the advantages of implants compensate their disadvantages, so it continues to be relevant to study these systems, namely, to perform alterations that may allow to improve their performance and/or overcome some of their challenges.

Concerning their formulation, the implants can be prepared with polymers or lipids, depending on their application and/or on the drug release mechanisms that are more adequate for the therapeutic effect (Stewart et al., 2018).

Polymeric implants

Regarding polymeric implants, they can be non-biodegradable or biodegradable. Within non-biodegradable polymeric implants, the commonly used polymers are silicones, poly(urethanes), or poly(acrylares). These types of implants have been used for the delivery of contraceptive drugs since they form implants with structurally resilient and robust over their lifetime. However, once

they are non-biodegradable it is necessary to remove them surgically. Even to avoid the appearance of adverse effects, infection, or damage (Stewart et al., 2018).

As an alternative to these implantable systems appears the biodegradable implants, composed of polymers such as PCL, PLA, or PLGA, which can be divided into smaller fragments that will be excreted or absorbed by the body (Stewart et al., 2018). Concerning these implants, they can be degraded by two different mechanisms. By bioerosion, when the polymer matrix is gradually dissolved by surface erosion towards the centre of the matrix, or by biodegradation, when chemical or enzymatic processes act on the polymer structure causing the homogeneous erosion of the entire polymer matrix (Hillery, 2001; Kreye, Siepmann, Zimmer, et al., 2011b).

The PLGA polymer is the most used copolymer in the production of biodegradable sustained drug release systems (Shrestha, Bala, & Arora, 2014; Stewart et al., 2018). However, implants developed from PLGA during its biodegradation generate acidic environments that can cause the inactivation of some drugs, which is why they are not indicated to deliver protein-based drugs (Guse et al., 2006; Shrestha et al., 2014; Stewart et al., 2018). However, this challenge can be solved by preparing lipidic implants, which have been described as an alternative to deliver of acid-labile drugs and drugs with low solubility in water (Heng, 2018; Kreye, Siepmann, Zimmer, et al., 2011a; Kreye, Siepmann, Willart, et al., 2011; Shrestha et al., 2014; F. Siepmann et al., 2006).

Lipid-based implants

Regarding the lipidic implants, they consist of a lipid matrix in which the drug is incorporated, allowing the formation of delivery systems that also release the drug in a controlled manner. They can be made up of a wide variety of lipid materials and produced by different methods (Kreye et al., 2008). Due to their greater degree of biocompatibility and versatility, they are especially useful for the parenteral administration of drugs used in long-term therapies, namely for chronic

diseases or hormonal treatments (Frari, Guibaud, Christine, Christine, & Alain, 2018; Rawat, Singh, Saraf, & Saraf, 2008).

Hence, lipid implants are biodegradable and versatile systems with high biocompatibility, they allow the delivery of substances with different molecular weights and are especially indicated for the delivery of drugs of a protein nature, since they are produced without the use of organic solvents, and they do not give rise to acidic environments during their degradation, thus allowing to preserve the tertiary structure of proteins. Its use is also advantageous to increase the bioavailability of substances with low solubility in water and to carry hydrophilic and lipophilic drugs (Heng, 2018; Kreye, Siepmann, Zimmer, et al., 2011b, 2011a; Kreye, Siepmann, Willart, et al., 2011; Pongjanyakul et al., 2004; Rawat et al., 2008; Shrestha et al., 2014)

It has been described that this type of implantable systems can be produced by (Kreye et al., 2008):

✓Compression of the drug:lipid powder blend: which is an easy and sometimes convenient development procedure. This procedure can be performed directly between drug and lipid powder and indirectly where the drug powder is dispersed in an organic solution of lipid or an aqueous drug solution in an organic lipid solution.

✓Melting and molding: wherein the drug is suspended and/or dissolved in the molten lipid, therefore this mixture is melted into moulds and cooled under pre-defined conditions. For thermosensitive drugs, this method cannot be used due to the high temperatures of the melting step.

✓Extrusion: this procedure is considered potentially faster and easier to scale up than the other previously described methods. Usually, it is used to load hydrophilic drugs with a short drug time of action (few days).

In overall, it becomes clear that all the controlled delivery systems previously described present advantages and disadvantages. To take full advantage of their value, their individual characteristics should always be considered, to choose between them according to the best cost-benefit ratio and considering which system will be most suitable for the disease to be treated. Concerning their limitations, namely in terms of production cost, inflexible drug release profiles and the stability of the developed systems (Adepu & Ramakrishna, 2021; K. K. Jain, 2008), it is quite relevant to continue to invest in the development and particularly in the upgrading of this type of formulations. In this sense, studying new types of materials, such as ionic liquids, to be incorporated into these systems, could be a valuable strategy to overcome the mentioned drawbacks.

1.2. Ionic liquids

Ionic liquids (ILs) are composed of an organic cation as well as an organic or inorganic anion. These ions are poorly coordinated, giving solvents liquid below 100 °C (Santos de Almeida et al., 2017; W. Silva, Zanatta, Ferreira, Corvo, & Cabrita, 2020). Some are even liquid at room temperature, designated room temperature ILs (RTILs) (Adawiyah, Moniruzzaman, Hawatulaila, & Goto, 2016).

In the last years, the research related to ILs, particularly their application in the pharmaceutical area, has been increasing. This is due to their interesting properties, repeatedly mentioned in numerous studies, such as their high thermal and also chemical stability, large electrochemical window, low volatility, as well as nonflammability, and re-usability (T. B. V. Dinis, e Silva, Sousa, & Freire, 2021; Gadilohar & Shankarling, 2017; Gomes, Silva, & Reis, 2019; Pedro, Freire, Silvestre, & Freire, 2020). Furthermore, ILs may be synthetically modified accordingly with a desired use, by altering the ions contained within them. For example, their miscibility can be modified by altering their chemical structure, allowing them to become miscible, partially miscible or even immiscible, with water (McDaniel & Verma, 2019). This characteristic may be quite relevant when considering the inclusion of ILs in drug delivery systems.

There are several ways to classify ILs, depending on what is considered as a differentiating factor (**Figure 1.3**).

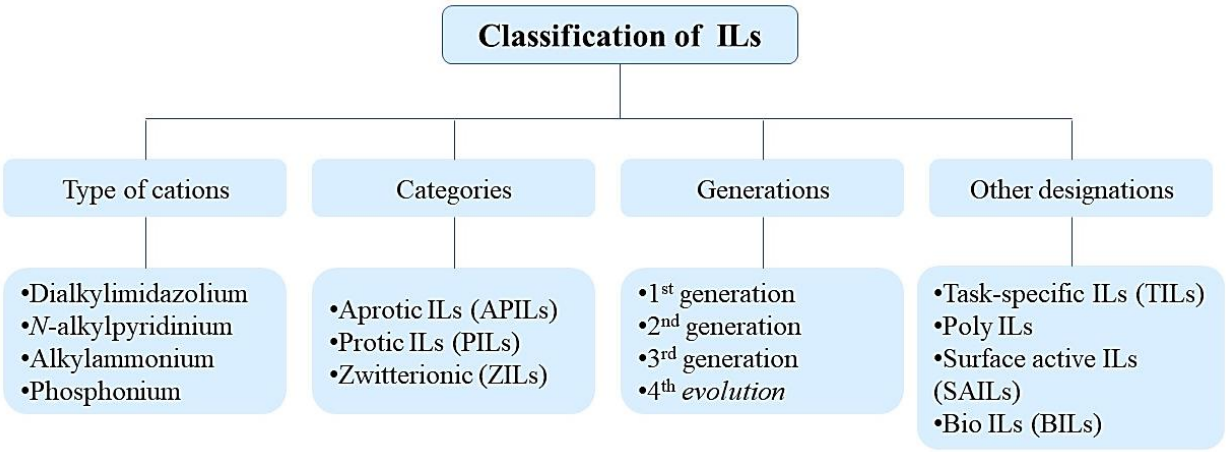

Figure 1.3. Different classifications of the ionic liquids (ILs) considered in the literature (Balk, Holzgrabe, & Meinel, 2015; Egorova, Gordeev, & Ananikov, 2017; Frizzo et al., 2013; Gomes et al., 2019; Hajipour & Rafiee, 2015; X. Li, Ma, Zhang, Ling, & Zhang, 2021; MacFarlane et al., 2018; Pan et al., 2020; Santos de Almeida et al., 2017; A. T. Silva et al., 2021; Welton, 2018).

One of the most used classifications is related to the different cations that can be included in ILs, such as the dialkylimidazolium cations, the *N*-alkylpyridinium cations, the phosphonium cations or more recently the alkylammonium cations (Santos de Almeida et al., 2017).

Among the four classes that arise from the type of cations used, the imidazole-based ILs and the alkylammonium-based ILs, particularly choline-based, will be the ILs considered in this thesis. The imidazole-based ILs were the first to be studied and consequently are amongst the most studied ILs. Although, their toxicity may be a concern for their applicability in drug delivery, they may still be useful, for instance to enhance drug permeability (Santos de Almeida et al., 2017), as long as toxicity studies are not neglected (Pandolfi et al., 2022; Santos de Almeida et al., 2017). In contrast, in terms of applicability in the pharmaceutical field the ILs belonging to the class containing alkylammonium cations, particularly choline-based ILs, have increasingly being studied, for being considered less toxic (Santos de Almeida et al., 2017). Nonetheless, all the four classes have been studied in different fields of applicability, and they have all shown promising features, namely as solvents, catalysts, reactional means (Santos de Almeida et al.,

2017). Moreover, concerning their applicability in Health, the choline cation continues to stand out since this cation is a natural essential micronutrient and is considered safe by the U.S. Food and Drug Administration and by the European Food Safety Agency (X. Li et al., 2021). Therefore, there has been an increasing number of studies investing in the evaluation of choline-based ILs as suitable functional excipients in drug delivery systems (Gadilohar & Shankarling, 2017; X. Li et al., 2021; Pedro et al., 2020; Santos de Almeida et al., 2017; Welton, 2018).

ILs can also be divided into three categories (MacFarlane et al., 2018; Ohno, Yoshizawa-Fujita, & Kohno, 2018; Pan et al., 2020; Santos de Almeida et al., 2017; W. Silva et al., 2020): **a)** aprotic ionic liquids (APILs): that are obtained by a quaternization reaction and then by anion exchange, as they are constituted by non-protonated cations that react with anions; **b)** protic ionic liquids (PILs): that are obtained by proton transfer synthesis from a Brönsted acid to a Brönsted base (neutralization reaction), with low vapor pressure; and **c)** zwitterionic ionic liquids (ZILs): Compared to ordinary ILs, in ZILs the ions are not independently mobile, and in contrast they also present lower ionic conductivity and higher melting points.

Another way of classifying ILs is by generations, based on their physicochemical properties, which divides them into three different generations and a fourth evolution generation:

✓1st generation: ILs resulting from the junction of dialkylmidazolium or alkylpyridinium cations with metal iodide anions, which are mostly used as solvents, mostly because of their low vapor pressure and thermal stability, however they present air and water sensibility and high toxicity (Egorova et al., 2017; Frizzo et al., 2013).

✓2nd generation: Result from combining dialkylmidazolium, alkylpyridinium, ammonium, or phosphonium cations with halide, tetrafluoroborate, or hexafluorophosphate anions (Egorova et al., 2017) and leads to ILs that are stable to air and water, that may be used in separation and extraction processes (Balk et al., 2015; Egorova et al., 2017; Tang, Bi, Tian, & Row, 2012), as lubricants (Balk et al., 2015; Bermúdez, Jiménez, Sanes, & Carrión, 2009).

✓3rd generation: corresponds to ILs that derive from biodegradable ions or with known biological activity, such as choline and amino acids, so they are mostly applied in the fields of biology and ecology (Balk et al., 2015; Egorova et al., 2017).

✓4th evolution generation: this generation is the most recent and was suggested due to the less predictable properties offered by ionic liquids in solutions or mixtures with molecular liquids. This evolution allowed to expand the scope of these materials, because other salts involved in these solutions, may not normally be considered as ionic liquids and thus this recent evolution encourages studies of salts outside the limits of the room temperature melting point (Gomes et al., 2019; MacFarlane et al., 2018).

In addition, and in consequence of the continuous evolution and investment in the synthesis of new ILs, there are other designations still arising that do not fit into a specific type of classification, but instead are created to designate a specific function or property of ILs. Among these are, task-specific ionic liquids (TSILs), poly ILs, surface-active ionic liquids (SAILs), and bio ionic liquids (BILs).

Concerning the TSILs, these act as reaction medium, and as reactants or catalysts. They are organic salts known to have a functional group covalently bonded to the cation and/or the anion within them. (Hajipour & Rafiee, 2015; Quijada-Maldonado, Sánchez, Pérez, Tapia, & Romero, 2018).

PILs are polyelectrolytes in which the cationic or anionic regions are present in the repeating polymer chain units. These ILs, can be used in polymer chemistry and also in materials science due to their physicochemical properties, that include the thermal and chemical stability, ionic conductivity, and also versatile anion exchange (Hajipour & Rafiee, 2015; Zhao, Sheng, & Zhou, 2022).

The surface-active ILs (SAILs), also designated ILs-based surfactants (ILBSs), are ILs that act as surfactants, due to their amphipathicity and self-assembly properties. These ILs derived

from the 1-alkyl-3-methylimidazolium cation, thus because of the imidazole ring, they present a higher surface activity than the conventional surfactants with hydrocarbon chains of equal length. Additionally, compared with the conventional surfactants, SAILs are easier to handle, thermal and chemically stable, and generally not flammable. They also present tuneable properties, for instance depending on the ions that constitute them, these SAILs may lead to an easier formation micelle, and they are also known to be miscible in different types of solvents. Thus, these ILs can be particularly relevant in the pharmaceutical field (A. T. Silva et al., 2021; W. Silva et al., 2020).

Moreover, when preparing ILs, it is crucial to consider the toxicity and biodegradability of these salts. In fact, several ionic liquids present high cytotoxicity and low biodegradability, which are challenges that need to be overcome to improve their applicability, particularly when considering the health area. Because of that, the bio-ILs (BILs) have emerged, which are ILs synthesized from renewable and non-toxic natural products. Here, are included the already mentioned ILs containing choline cation and having amino acids as the anions present (Egorova et al., 2017; Gadilohar & Shankarling, 2017; Gomes et al., 2019; Hajipour & Rafiee, 2015; X. Li et al., 2021; Pedro et al., 2020; W. Silva et al., 2020; Welton, 2018).

Finally, in terms of classifying ILs considering their applications, many more designations will certainly continue to arise as a result of the growing investment from the scientific community in developing new and more targeted ILs.

In summary, it becomes clear that ILs have been used for many different applications and, more concretely in the pharmaceutical field, ILs have been applied: to extract pharmaceutical substances from aqueous solutions (Bernardo et al., 2020; T. B. V. Dinis, E Silva, Sousa, & Freire, 2021; Vraneš, Panić, Gadžurić, Bešter-Rogač, & Tot, 2021); as drug solubility and permeation enhancers (Jadhav et al., 2021; Jesus et al., 2021; Marrucho, Branco, & Rebelo, 2014; Sidat et al., 2019; Yuan, Wu, & Yin, 2020); as solvents or catalysts of the synthesis of active pharmaceutical ingredients (Marrucho et al., 2014; Wu et al., 2021). Several studies have also considered the use of ILs as water or oil alternatives, and also as additives or surfactants in both emulsions or

microemulsions (Adawiyah et al., 2016; Correia et al., 2021; A. T. Silva et al., 2021). Nonetheless, in terms of the possible functionalities that ILs may confer, there is still much that may be done and consequently the number of studies considering the multiple functionalities of ILs will most certainly continue to grow.

The development of new sustained delivery systems is one of the areas that may gain from the incorporation of ILs. Consequently, in this thesis it was explored the applicability of ILs, at non-toxic concentrations, in the development of sustained delivery systems. Hence, herein IL-polymeric nanoparticles, IL-lipidic implants, and TransfersomILs were developed, and their formulation procedures were optimized, and their physicochemical properties and performance were evaluated.

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Chapter 2

Hypothesis & Aims

2.1. Hypothesis

Could ionic liquids be multitalented materials to upgrade sustained delivery systems?

2.2. General Aim

The overall goal of this thesis was the development of new and improved sustained delivery systems incorporating ionic liquids.

The preparation of more efficient drug delivery systems has been a main concern for the pharmaceutical industry mainly because of, poor drug solubility in water, systemic side effects, limited stability of some delivery systems, and difficulties in drug loading. ILs have been studied as functional excipients in different types of delivery systems, to successfully overcome some of these challenges. Nonetheless, the use of ILs in sustained delivery systems is less explored and consequently this was the main goal of this thesis.

Hence, the aim of this work was to explore the applicability of ILs towards the development of improved sustained delivery systems. For this purpose, different types of formulations were developed using ILs, namely IL-polymeric nanoparticles (**Chapter 3**), IL-lipidic implants (**Chapter 4**) and transfersomILs (**Chapter 5**). Then, the physicochemical properties and performance of the newly developed formulations were evaluated to establish the impact of ionic liquids on the delivery systems.

2.3. Specific objectives

2.3.1. Chapter 3

The aim of the study presented in **Chapter 3** was to develop and characterize IL-nanoparticle hybrid systems containing rutin, by combining choline-based ILs with polymeric nanoparticles and evaluate the impact of ILs on the performance of the new hybrid system.

In this Chapter, the defined goals were to:

- ✓ Synthesise ILs and develop PLGA nanosystems containing ILs via a water/oil/water double emulsion technique.
- ✓ Determine how the ILs impact the physicochemical properties of the developed hybrid systems, namely the size, the polydispersity index, the zeta potential, and as well as the association efficiency.
- ✓ Perform the Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC) and the Scanning Electron Microscopy (SEM) analysis of the hybrid systems.
- ✓ Establish if the ILs may contribute to a higher rutin loading into the hybrid IL-nanosystem.
- ✓ Study the impact of the ILs on the drug release profile from the new hybrid system.
- ✓ Perform permeation studies and evaluate the cytotoxicity characteristics of the developed IL-nanocarrier in HaCaT human keratinocytes, using the MTT assay.

This chapter will disclose if the incorporation of ILs into polymeric nanoparticles could lead to a more efficient nanosystem. Namely, this work will uncover if ILs can improve the loading of the poorly water soluble rutin and reveal in what ways may ILs upgrade the physicochemical properties and performance of the developed hybrid system, while considering safety.

2.3.2. Chapter 4

In this chapter the main goal was to use ILs towards the development of another controlled drug delivery system, namely lipidic implants containing three different drugs. Different compositions of the implants were studied and the impact of these compositions and of the studied ILs, on the performance of the developed implants, was evaluated.

Different objectives were stablished in this Chapter, namely to:

- ✓ Develop lipid-based implants by a fusion and melting methodology, containing different compositions, namely incorporating two different release adjuvants, Gelucire® 50/02 or Sucrose, and the ILs under study.
- ✓ Incorporate a hydrophilic and a lipophilic dye within the implants to assess if the formulation procedure and the used excipients, namely the ILs, may lead to a uniform distribution of both types of dyes.
- ✓ Prepare implants in the presence and absence of each of the following three drugs, the hydrophilic caffeine, and the lipophilic salicylic acid or rutin and study and compare the drug content within the prepared implants.
- ✓ Comprehend the influence of the different compositions, namely the presence of the ILs, on the drug release profile, as well as on the water content and lipid erosion of the developed implants, for a period of 140 days.
- ✓ Analyse the surface topography and roughness of the implants through 3D imaging, using Atomic Force Microscopy.

The results obtained in this study will contribute to the continuously evolving field of drug delivery, particularly by revealing if ILs could help surpass some of the challenges associated with the development of implantable systems. This chapter will lead to data concerning the impact of ILs on the preparation procedure, drug release profile and surface properties of lipidic-implants.

2.3.3. Chapter 5

The main goal of **Chapter 5** was the development of novel class of lipid-based nanovesicular systems containing rutin, more specifically transfersomes, produced in the presence of ILs or IL:IL combinations. Then, the physicochemical properties and the performance of the newly developed TransfersomILs were assessed.

So, in the Chapter the various aims pursued were to:

- ✓ Conduct previous exploratory studies to establish different factors that are fundamental for the development of new transfersomes containing the synthesised ILs either alone or as IL:IL combinations, namely to:
    - o Study the cytotoxicity of the ILs alone, and of the studied combinations of ILs, in human keratinocytes (HaCaT), using the MTT assay.
    - o Determine the influence of the IL:IL combinations on the solubility of rutin.
    - o Optimize a transfersomal system to load rutin, using a Box-Behnken factorial design (BBD), to determine the best formulation for the incorporation of ILs.
- ✓ Develop new TranfersomILs systems containing rutin and to:
    - o Evaluate the impact of the ILs on the physicochemical properties of the produced systems, namely the hydrodynamic diameter, polydispersity index, and zeta potential, as well as the association efficiency and loading capacity.
    - o Study the drug release profile of the formulations and determine how ILs and their combinations affect this feature.
    - o Preliminarily assess if the ILs may influence the storage stability of the prepared transfersomes.

This chapter may lead to a further breakthrough in the transdermal delivery field, namely by disclosing the development of a new class of nanovesicular systems, the TransfersomILs, that may combine the noteworthy properties of both transfersomes and ILs.

Chapter 3

Ionic Liquid-Polymer Nanoparticle Hybrid Systems as New Tools to Deliver Poorly Soluble Drugs

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Ionic Liquid-Polymer Nanoparticle Hybrid Systems as New Tools to Deliver Poorly Soluble Drugs

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Abstract: The use of functional excipients such as ionic liquids (ILs) and the encapsulation of drugs into nanocarriers are useful strategies to overcome poor drug solubility. The aim of this work was to evaluate the potential of IL-polymer nanoparticle hybrid systems as tools to deliver poorly soluble drugs. These systems were obtained using a methodology previously developed by our group and improved herein to produce IL-polymer nanoparticle hybrid systems. Two different choline-based ILs and poly (lactic-co-glycolic acid) (PLGA) 50:50 or PLGA 75:25 were used to load rutin into the delivery system. The resulting rutin-loaded IL-polymer nanoparticle hybrid systems presented a diameter of 250–300 nm, with a low polydispersity index and a zeta potential of about −40 mV. The drug association efficiency ranged from 51% to 76%, which represents a good achievement considering the poor solubility of rutin. No significant particle aggregation was obtained upon freeze-drying. The presence of the IL in the nanosystem does not affect its sustained release properties, achieving about 85% of rutin released after 72 h. The cytotoxicity studies showed that the delivery system was not toxic to HaCat cells. Our findings may open a new paradigm on the therapy improvement of diseases treated with poorly soluble drugs.

Keywords: ionic liquid; polymer; PLGA; nanoparticle; poorly soluble drug; rutin; drug delivery; hybrid system

1. Introduction

Ionic liquids (ILs) are organic salts [1,2], which combine an organic cation and an organic/inorganic anion [1,3], that are liquid below 100 °C [4,5]. These salts have been classified into four categories, accordingly to the cation present in these ILs, namely as, dialkylimidazolium, *N*-alkyl-pyridinium, phosphonium, or alkylammonium cation [6–9]. Amongst these classes, imidazolium-based ILs are the most studied, due to their high stability, low viscosity [6–8] and it is relatively easy synthesis [6–8]. However, in the drug delivery field, they have exhibited some limitations, since they have shown high toxicity [8,10,11]. In contrast, the quaternary ammonium-based ILs, such as the choline-based ILs, have been described as the less toxic [4,8,11,12], so they have been considered in the literature

as "green" ILs [7,8,12,13]. This fact puts them at the forefront as more suitable for applications in the pharmaceutical field [8,11–13].

In general, ILs have several distinct and valuable properties, such as high thermal and chemical stability, low vapour pressure, non-volatility, the possibility of being recycled and the ability to be solubilized in several solvents [7,8,12,14]. Due to these properties and to the ability to be modified according to a desired physicochemical property, they may be used for different purposes [1,6,8]. For instance, ILs have been applied in different fields, including in several organic reactions [15–20], in extractions and separation reactions [15,21–24], in electrolysis and electrochemistry [25–28] and in nanotechnology [9,28–31]. Another emergent application of ILs is as functional excipients, due to their ability to increase drug solubility and/or permeation and as drug stabilizers [1–3,5,8,12,32–34]. Some studies have established the value of ILs as solubility enhancers for topical formulations, like gels and emulsions [8,12,33,35,36]. However, only a few studies have considered the incorporation and functionality of these ILs at non-toxic concentrations [8,12,34]. Hence, evaluating the maximum concentration of these ILs that does not impact cell viability, and prove their functionality, at these concentrations is crucial to prove safety alongside with the ability to increase the efficiency of the delivery systems.

The encapsulation of drugs into nanoparticles have been also a useful strategy to protect the drug while allowing a controlled and/or targeted drug delivery to a specific tissue, improving its bioavailability and decreasing adverse side effects [37–41]. In addition, nanoencapsulation also enables the incorporation of hydrophobic and hydrophilic drugs while being well tolerated through different routes of drug delivery [42,43]. Several types of polymer nanoparticles have been used due to its good biocompatible and biodegradable properties [42,44–46]. Such carriers have shown the ability to increase the absorption, bioavailability, solubility, and stability of drugs [9,47–49]. Furthermore, they may also be tailored according to the required application [9,47–49].

The poly(lactic-co-glycolic acid) (PLGA) is a polymer approved by the Food and Drug Administration (US FDA) and by the European Medicine Agency (EMA) for application in nanomedicine [44–46,50]. It has high biodegradability and biocompatibility and shows a good controlled release profile over time [44–53]. The common PLGA ratios used in drug delivery are 50% lactic acid and 50% glycolic acid (PLGA 50:50) and 75% lactic acid to 25% glycolic acid (PLGA 75:25) [44,46]. These ratios are commonly used due to their easier degradation in the human body since the combination of lactic acid and glycolic acid is quickly hydrolyzed to the monomers [44]. The PLGA nanoparticles are used with several applications, such as vaccination, cancer, and other diseases [44].

Therefore, the combination of polymeric nanoparticles with ILs may be a valuable strategy by taking advantage of the synergistic effects of both materials, leading to delivery systems with higher physicochemical and colloidal stability [9,54–56], and increased the drug loading. More importantly, this combination may improve the therapeutic effect of poorly soluble drugs while decreasing possible adverse side effects. A good example of a poorly soluble drug is rutin, with a solubility in water of 0.2 mg/mL [12,19,35,37]. Rutin is a polyphenolic bioflavonoid extracted from fruits and plants [57,58]. Its antioxidant, antidiabetic activity, antihypertensive, and antilipidemic activity are widely reported in the literature [58,59]. Additionally, in vitro studies also demonstrated that rutin may have an anticancer effect (as leukaemia preventive agent and as inhibitor of human adenocarcinoma in HT-29 and Caco-2 cells), an anti-inflammatory effect (against cyclooxygenase and lipoxygenase) and a neuroprotector effect [12,57–60]. However, its applicability is limited since its bioactivity is impaired by the poor solubility, stability, and permeability [12,58–60].

Previously, we developed a new IL-polymer nanoparticle hybrid system to load poorly soluble drugs [39]. Herein, the aim of this work was to take this approach further by improving this new nanocarrier and deeper evaluate its performance in terms of its physicochemical features, drug delivery profile, and cytotoxicity characteristics. Such drug delivery system may be a valuable tool to deliver poorly soluble drugs by combining the advantages of both ionic liquids and nanocarriers.

2. Materials and Methods

2.1. Materials

Concerning the synthesis of ILs, choline hydroxide in methanol [Cho][OH]/MeOH 45% was purchased from Sigma-Aldrich (St. Louis, MO, USA), acetonitrile was from VWR (Fontenay-sous-Bois, France) and methanol was from Sigma-Aldrich Chemie Gmbh (Munich, Germany). The amino acids used L-phenylalanine was obtained from PanReac AppliChem® ITW Reagents (Barcelona, Spain) and L-glutamine from Sigma-Aldrich (St. Louis, MO, USA). The rotatory evaporator used was an IKA RV06-ML from IKA®-Werke GmbH and Co. (Staufen, Germany) and the centrifuge was a Hermle Z 32 HK from Hermle LaborTechnik (Wehingen, Germany).

Regarding the production of the nanoparticles, two different ratios of PLGA were used, 50:50 (Purasorb® PDLG 5002A) and 75:25 (Purasorb® PDLG 7502A), that were kindly supplied by Corbion Purac (Amsterdam, The Netherlands). Dichloromethane and polyvinyl alcohol (PVA) from Sigma-Aldrich (St. Louis, MO, USA) were also used. Rutin was purchased from Fagron (São Paulo, Brazil) and trehalose was from PanReac AppliChem® ITW Reagents (Barcelona, Spain). The bidistilled water was prepared in-house.

For cytotoxicity studies, trypsin, penicillin-streptomycin solution, fetal bovine serum, dimethyl sulphoxide (DMSO), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Dulbecco's modified Eagle's medium (DMEM) was provided by Biowest (Nuaillé, France). Finally, in the permeation study, it was also used absolute ethanol from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Synthesis of ILs

Two choline-based ILs, (2-hydroxyethyl)-trimethylammonium-L-phenylalaninate [Cho][Phe] and (2-hydroxyethyl)-trimethylammonium-L-glutaminate [Cho][Glu] were synthesized as previously described in the literature [8]. Briefly, an aqueous solution of 57.79 mmol of the amino acid was added to 15.6 mL of [Cho][OH]/MeOH 45%, previously evaporated at 50 °C under vacuum. The obtained mixture was cooled in an ice bath under stirring (17 h) and subsequently the water was evaporated at 60 °C under vacuum. After that, the unreacted amino acid was precipitated using a mixture of acetonitrile:methanol (9:1), under vigorous stirring. The obtained solid was removed by centrifugation at 10,080× *g* for 20 min, followed by a gravimetric filtration. The solvents were then evaporated under vacuum at 60 °C. The synthesized IL was stored under moisture-free conditions until use. The ILs were characterized by ^{1}H NMR and ^{13}C NMR at 400 MHz using D_2O as a solvent, in a Brucker Avance 400 from Bruker Corporation (Billerica, MA, USA).

2.3. Production of the IL-Polymer Nanoparticle Hybrid Systems

The IL-polymer nanoparticle hybrid system was produced using a water in oil in water (W/O/W) double emulsion technique described by Júlio et al. [39] with slight modifications. Briefly, 200 µL of an aqueous solution of rutin in 0.2% (*v*/*v*) of each IL [8], [Cho][Phe] or [Cho][Glu], was prepared, dissolving the drug proximal to its saturation point (1.15 mg/mL for [Cho][Phe] and 0.68 mg/mL for [Cho][Glu] [12]). Then, dichloromethane was added to 200 mg of PLGA (50:50 or 75:25) and the water:IL solution containing rutin was blended with the PLGA mixture. These samples were then sonicated for 30 s at 70% of amplitude using a Q125 Sonicator from QSonica Sonicators (Newtown, CT, USA). This first emulsion was immediately poured into 25 mL of PVA 2% (*w*/*v*) [39] and directly sonicated under the same previous conditions. Finally, the formulation was placed under magnetic stirring until organic solvent removal. All formulations were produced in triplicate.

2.4. Particle Size, Polydispersity Index and Zeta Potential

The particle size and polydispersity index (PdI) were analyzed by dynamic light scattering and its zeta potential was evaluated by the electrophoretic mobility technique using a Delsa™ Nano C

from Beckman Coulter, Inc. (Brea, CA, USA). All samples were run in triplicate at room temperature (23 ± 2 °C), after proper dilution with bidistilled water.

2.5. Association Efficiency (AE) of Rutin

The AE is a parameter that quantifies the amount of drug associated with nanoparticle systems. The formulations were centrifuged at 16,350× *g* for 15 min at 4 °C, and the supernatant was collected. The rutin present in the supernatant was quantified by UV spectroscopy using a UV–VIS Spectrophotometer Evolution® 300 from Thermo Scientific (Hertfordshire, England) at its maximum absorption wavelength (354 nm).

The AE of rutin was calculated using the Equation (1):

$$\text{AE} = \frac{\text{Total amount of rutin} - \text{Free amount of rutin in the supernatant}}{\text{Total amount of rutin}} \times 100 \quad (1)$$

2.6. Freeze-Drying of the IL-Polymer Nanoparticle Hybrid Systems

The formulations were centrifuged at 12,600× *g* for 15 min at 4 °C in Hermle Z 32 HK centrifuge to remove the supernatant containing PVA at 2% (*w*/*v*), and the particles were redispersed in a solution of trehalose at 3% (*w*/*v*). Samples without lyoprotectant were also prepared.

The formulations were frozen overnight at −80 °C, and freeze-dried in a Labconco FreeZone 25® (Kansas City, MO, USA) at a surface condenser temperature of −50 °C and 400 mTorr for 48 h.

2.7. Reconstitution of the Lyophilizates and Freeze-Drying Ratio

The freeze-dried samples were reconstituted by adding bidistilled water in the inside wall of the glass flask and maintained for 5 min. to ensure the cake wetting, and slowly shaken until complete homogenization. Then, the particle size was characterized using the methodology described above and the freeze-drying ratio was calculated using the Equation (2). The freeze-drying ratio is a parameter that allows to understand the maintenance of the physicochemical features of the nanoparticles upon freeze-drying:

$$\text{Freeze} - \text{drying ratio} = \frac{\text{Mean particle size after freeze} - \text{drying}}{\text{Mean particle size before freeze} - \text{drying}} \quad (2)$$

2.8. Fourier Transform Infrared Spectroscopy (FTIR)

The IL-polymer nanoparticle hybrid systems obtained after freeze-drying was evaluated by FTIR in a PerkinElmer® Spectrum 400 (Waltham, MA, USA) equipped with an attenuated total reflectance (ATR) device. The spectra were obtained collecting 100 scans of each sample, between 4000 and 600 cm^{-1}, with a resolution of 4 cm^{-1}. The FTIR analysis was also performed for rutin and other control samples.

2.9. Differential Scanning Calorimetry (DSC)

The thermograms of freeze-dried formulations were obtained using a Differential Scanning Calorimeter DSC 200 F3 Maia Netzsch® (Selb, Germany). Samples were weighed (1 mg) and placed into Netzsch® aluminum pans (Selb, Germany), which were hermetically sealed, and the thermal analysis was performed in a temperature range between 20 °C and 100 °C, with a rate of 10 °C per minute.

2.10. Scanning Electron Microscopy (SEM)

The SEM analysis of the resuspended freeze-dried samples was performed on a JSM-7001F from JEOL (Tokyo, Japan) after they were put onto metal stubs and vacuum-coated with a layer of gold/palladium during 20 s with a current of 25 mA. The samples were previously resuspended

in bidistilled water and washed at 12,600× *g* for 15 min at 4 °C in a Hermle Z 32 HK centrifuge from Hermle LaborTechnik (Wehingen, Germany) to remove the surfactant, that is dissolved in the bidistilled water.

2.11. In Vitro Release Study

For in vitro release study of rutin, the nanoparticle suspension was centrifuged at 12,600× *g* for 20 min at 4 °C, and the obtained pellet was resuspended in 10.0 mL of pH 7.4 phosphate buffer saline (PBS) solution. These solutions were incubated at 37 °C with stirring at 100 rpm in a Heidolph® 1000 incubator with a motor Heidolph® Unimax 1010 (Schwabach, Germany). Aliquots of each sample were taken at predetermined time intervals (30 min, 1, 2, 4, 6, 8, 12, 24, 48, and 72 h) and replaced with the same volume of PBS. After samples centrifugation at 12,600× *g* for 15 min in a Hermle Z 32 HK centrifuge, rutin in the samples was quantified in a UV–VIS Spectrophotometer Evolution® 300 from Thermo Scientific (Hertfordshire, England) at a fixed wavelength of 354 nm.

2.12. Permeation Study

The permeation studies ($n = 5$) were performed on vertical diffusion glass cells (Franz cells) with a receiver volume of approximately 4 mL and a diffusion area of 0.95 cm^2, using a polydimethylsiloxane (PDMS) membrane. Thus, 500 µL of the nanoparticle suspension was placed in the donor compartment, which was then occluded with microscope coverslips. The receptor compartment was immersed in a thermostatic bath at 37 °C and was filled with a mixture of PBS pH 7.4:ethanol (80:20).

At predetermined time intervals (3, 6, 9, 12, and 24 h), the medium in the receptor compartment was collected and replaced with a previously thermostated mixture of PBS pH 7.4:ethanol (80:20). After the collection, rutin was quantified using a UV–VIS Spectrophotometer Evolution® 300 from Thermo Scientific (Hertfordshire, England) at a fixed wavelength of 354 nm.

2.13. MTT Cytotoxicity Studies

HaCat human keratinocytes were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were maintained at 37 °C, under a humidified air atmosphere containing 5% of CO_2 in air and seeded at a density of 5×10^3 per well in 200 µL culture medium in 96-well plates and incubated for 24 h. The cells were then exposed to the nanoparticle formulations for a 24 h period. The MTT reduction assay was then carried out, according to a previously described protocol [61–63]. The absorbance values for cultures incubated only with vehicle (PBS 5% *v*/*v*) corresponds to 100% cell viability. DMSO (5% *v*/*v*) was used as positive control and decreased cell viability to 2.97 ± 1.32%. For this assay, two independent experiments were performed, and at least four replicate cultures were used in each experiment.

2.14. Statistical Analysis

The obtained results were evaluated by one-way analysis of variance, ANOVA, followed by Tukey's multiple comparison tests. The values are expressed as mean ± standard deviation (SD). The differences between samples were significant at $p < 0.05$ level. Results were treated using a GraphPad Prism 5® from GraphPad Software (San Diego, CA, USA).

3. Results and Discussion

In this work, we produced an IL-polymer nanoparticle hybrid system following an adapted W/O/W double emulsion technique [39] to load rutin. This system was composed of a choline-amino acid IL, [Cho][Phe] or [Cho][Glu], and PLGA 50:50 or PLGA 75:25 as polymers following a production method developed by our group [39] and improved herein.

It was our aim to demonstrate the ability of ILs to be placed within polymer nanoparticle matrices, achieving robust and stable hybrid delivery systems for multifunctional applications.

The developed formulations result from the combination of the two ratios of PLGA (50:50 or 75:25) with each of the choline-based ILs. Hence, from now on, the formulations combining PLGA 50:50 with [Cho][Phe] or [Cho][Glu], will be referred as PLGA 50:50/[Cho][Phe] and PLGA 50:50/[Cho][Glu], respectively. Furthermore, the formulations combining the PLGA ratio 75:25 with each IL will be assigned as PLGA 75:25/[Cho][Phe] and PLGA 75:25/[Cho][Glu]. Additionally, when these formulations contain rutin the denotation, + Rutin, will be added to account for the presence of the drug.

The amino acid-based ILs were chosen since previous studies have shown that they are able to enhance drug solubility at concentrations where cell viability is maintained—0.2% (*v*/*v*) [8]. Hence, [Cho][Phe] and [Cho][Glu] were synthesized, according to the literature [8], their structure was confirmed by ^{1}H-NMR and ^{13}C-NMR using D_2O as a solvent, and the results were found to be in agreement with the literature [4,12]. After the synthesis of the ILs and preparation of the IL-polymer nanoparticle hybrid systems, the obtained delivery systems were evaluated.

3.1. Particle Size, Polydispersity Index, and Zeta Potential

First, the IL-polymer nanoparticle hybrid system without rutin was produced to understand the interaction of the choline-based ILs with the polymers and evaluate the possibility to obtain nanoparticles. Thus, the diameter, PdI and zeta potential of these formulations were evaluated. The diameter ranged between 200–250 nm, the PdI was around 0.3, whereas the zeta potential was about −40 mV (Figure 1), which are appropriate properties for the administration of drugs by different administration routes. The robustness of the production method to obtain nanoparticles with similar characteristics was also confirmed. It was also demonstrated, for the first time, that the choline-based ILs placed within unloaded polymer nanocarriers allow to obtain stable and robust nanoparticles, since the results of the IL-polymer nanoparticle hybrid systems were similar those previously described for polymer nanoparticles without ILs [51,52,64].

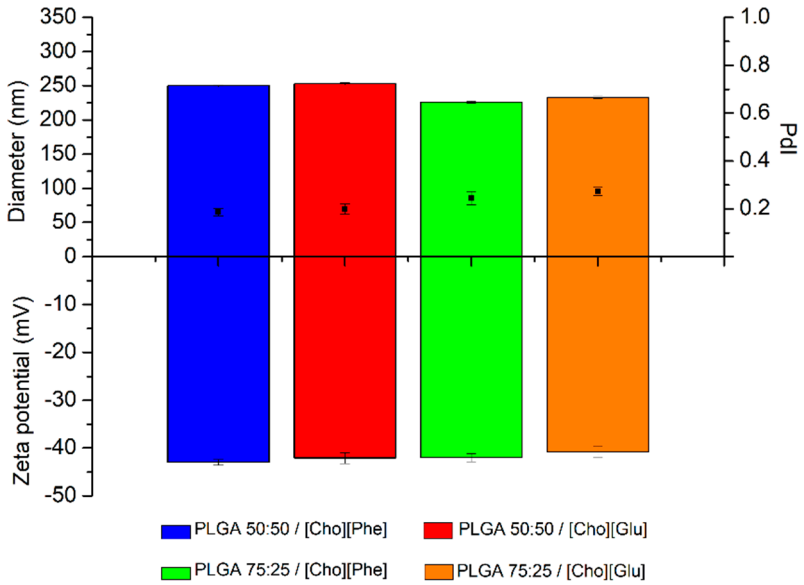

Figure 1. Diameter (nm) (top bars), PdI (black squares), and zeta potential (mV) (bottom bars) of IL-polymer nanoparticle hybrid systems (n = 3, mean ± SD).

Then, the rutin-loaded IL-polymer nanoparticle hybrid systems were prepared in the presence of ILs since the low aqueous solubility of the drug (around 0.2 mg/mL [12,57,60]) hampers its encapsulation in the absence of the ILs. Hence, the ILs were used, separately, to achieve maximum solubility for rutin in 0.2% (*v*/*v*) of [Cho][Glu] or [Cho][Phe], which corresponds to 0.68 mg/mL and 1.15 mg/mL, respectively [12]. These rutin-loaded formulations showed a particle size ranging from 250 to 300 nm with a PdI between 0.3 and 0.4 and a zeta potential of about −40 mV (Figure 2). These results show the

robustness of the developed hybrid systems since they are similar to those obtained without the drug (Figure 1). Additionally, it was observed a slight increase in diameter and in the PdI, that indicates the presence of rutin in the nanocarrier. Which is also corroborated by the FTIR and DSC results.

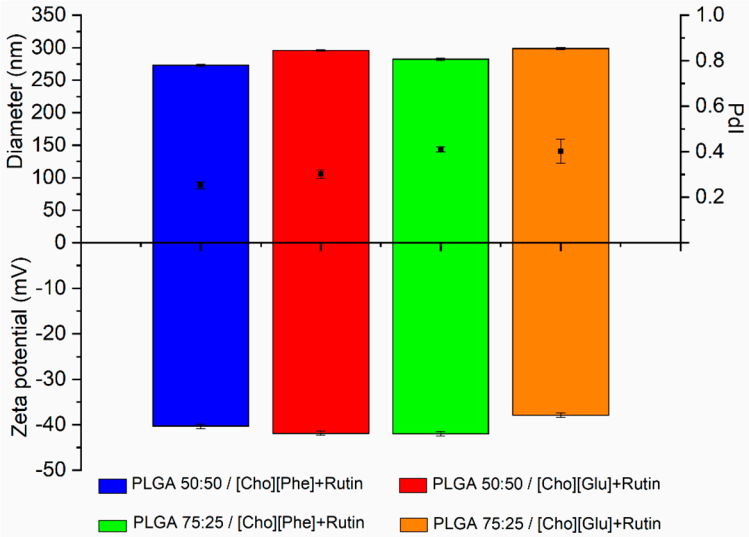

Figure 2. Diameter (nm) (top bars), PdI (black squares), and zeta potential (mV) (bottom bars) of rutin-loaded IL-polymer nanoparticle hybrid systems (n = 3, mean ± SD).

The IL-polymer nanoparticle hybrid system, with and without rutin, demonstrated good colloidal stability, and high negative charge (Figures 1 and 2). Additionally, no significant differences were detected between the analyzed parameters, thus confirming that the encapsulation of rutin does not change the physicochemical properties of the IL-polymer nanoparticle hybrid systems (compare data in Figures 1 and 2).

To extend the shelf life of the delivery system [51,52], the IL-polymer nanoparticle hybrid system was freeze-dried using trehalose 3% (*w*/*v*) as lyoprotectant. To evaluate the impact of this process in the nanocarrier system, the freeze-drying ratio was calculated according to Equation (2). The results showed that the diameter of the IL-polymer nanoparticle hybrid system did not significantly change in the presence of trehalose, since the ratio was close to 1.00 in all samples (Table 1). This may indicate that the incorporation of the IL in the nanocarriers does not interfere with the lyoprotectant effect of trehalose [51].

Table 1. Freeze-drying ratio for the rutin-loaded IL-polymer nanoparticle hybrid systems, in the presence and in the absence of trehalose results, obtained from Equation (2). Data represented as mean ± SD (n = 3).

Polymer	IL	Freeze-Drying Ratio	
		No Lyoprotectant	Trehalose at 3% (*w*/*v*)
PLGA 50:50	[Cho][Phe]	1.31 ± 0.01	1.01 ± 0.01
	[Cho][Glu]	1.45 ± 0.05	1.04 ± 0.01
PLGA 75:25	[Cho][Phe]	1.28 ± 0.04	1.01 ± 0.02
	[Cho][Glu]	1.50 ± 0.04	0.98 ± 0.02

Despite the formulations without trehalose had a freeze-drying ratio higher than 1, no particle aggregation was observed showing once again the stability of the system. This result is also indicative that the presence of the IL in the nanoparticle matrix contributed to decrease the stress effect of the freeze-drying process on the nanoparticles, since all formulations had a ratio around 1.13 and 1.50,

which is much lower to what happens in the absence of the ILs [47,65]. This can be explained by the possibility of ILs act as water substitutes [66], which is a protective mechanism of lyoprotectants.

Moreover, since all samples presented a freeze-drying ratio closed to 1.00, this shows that the hybrid nanosystems, with and without the lyoprotectant did not suffer from stress [65], since they kept their particle size after freeze-drying, without any noticeable signs of particle aggregation or damage.

3.2. Association Efficiency (AE) of Rutin

Comparatively to our previous work, an improvement of about 20% in the AE of rutin in the IL-polymer nanoparticle hybrid systems was obtained for both ILs (Table 2). These results represent a considerable achievement in the incorporation of a poorly soluble drug into the nanoparticles without compromising the particle size. This shows that the presence of 0.2% (*v*/*v*) of a choline-based IL in the nanoparticle is determinant to load rutin in the hybrid system with a high AE. Additionally, the results also showed that there are no statistically significant differences in the AE for the two ratios of PLGA. More importantly, the formulations with [Cho][Phe] demonstrated significantly higher AE values than formulations containing [Cho][Glu] ($p < 0.05$), which indicates it might be a better IL to allow the incorporation of high amounts of poorly soluble drugs. These results may be explained by the higher aqueous solubility of rutin in the presence of [Cho][Phe], compared to [Cho][Glu], which explains the higher AE of the drug in the presence of the phenylalaninate IL.

Table 2. Association Efficiency (AE) of rutin-loaded ILs-polymer nanoparticle hybrid system. Data represented as mean ± SD ($n = 3$). Results are significantly different ($p < 0.05$) between ILs for each polymer, when marked with *.

Polymer	IL	AE (%)
PLGA 50:50	[Cho][Phe]	75.6 ± 1.0 *
	[Cho][Glu]	53.8 ± 2.4
PLGA 75:25	[Cho][Phe]	73.2 ± 0.9 *
	[Cho][Glu]	51.3 ± 1.3

3.3. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR spectra were collected for rutin, and both PLGA ratios (50:50 and 75:25) to serve as controls (Figure 3A) and for the rutin-loaded IL-polymer nanoparticle hybrid systems (Figure 3B). All analyses were performed after samples freeze-drying. The PLGA spectrum showed the presence of the characteristic peak between 1750–1760 cm^{-1}, which corresponds to the C=O stretching (Figure 3) [67–69] and the peak at 3000 cm^{-1}, corresponding to the C–H stretching (Figure 3) [67–69]. The comparison between the spectra of PLGA (Figure 3A) and of the rutin-loaded IL-polymer nanoparticle hybrid systems (Figure 3B) reveals an increase in the intensity of the C=O and C–H bands, which may be caused by the superimposition of the rutin peaks at the same wavenumber [70–72]. Furthermore, the IL-polymer nanoparticle hybrid systems also display a band between 3250 and 3400 cm^{-1} (Figure 3B), which is similar to the characteristic broad peak of rutin [70–72], also observed at the individual spectrum for this drug (Figure 3A). This observation points to the existence of an interaction between the drug and the IL-polymer nanoparticle hybrid systems, which may show that rutin was efficiently encapsulated. It was also observed that this broad peak was increased in the formulations with higher AE (Table 2), namely in PLGA 50:50/[Cho][Phe] and PLGA 75:25/[Cho][Phe].

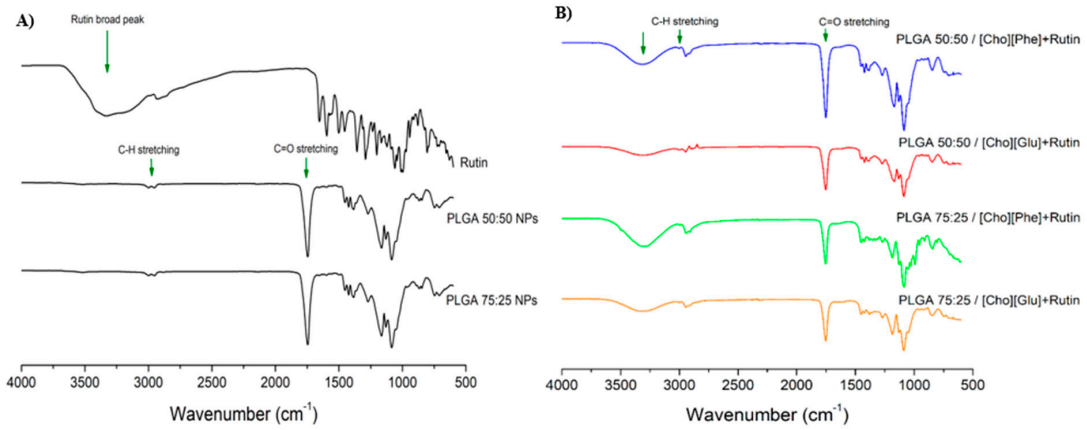

Figure 3. FTIR spectra of controls (Rutin, PLGA 50:50 and PLGA 75:25) (**A**) and rutin-loaded IL-PLGA nanoparticle hybrid systems (**B**), all obtained after freeze-drying.

3.4. Differential Scanning Calorimetry (DSC) Analysis

Polymer-drug and/or polymer-IL interactions were evaluated by DSC analysis. DSC analysis was performed both for rutin-loaded IL-polymer nanoparticle hybrid systems and for the controls—PLGA 50:50, PLGA 75:25, [Cho][Phe], [Cho][Glu] and rutin. The thermograms showed that the melting profiles of all samples are similar (Figure 4) and that the interactions between the compounds do not interfere with the behavior of each compound under increasing temperature (Figure 4B). Moreover, the characteristic endothermic peak of PLGA at 47.2 °C for 50:50 ratio and 45.0 °C for 75:25 ratio [34,69,71,73], appears on the control samples (Figure 4A). Additionally, the incorporation of the ILs in the polymer nanoparticles seems to stabilize the nanocarriers, since the endothermic peak of PLGA is less pronounced or even insubstantial in the IL-polymer nanoparticle hybrid systems (Figure 4B) than in the PLGA controls (Figure 4A). This phenomenon is also verified in the unloaded IL-polymer nanoparticle hybrid systems (data not shown). Yet, rutin, at the analyzed temperature, was not altered in the presence of the developed delivery system when compared with the thermogram of the rutin control (Figure 4). Finally, the DSC profiles of both ILs, in the hybrid nanocarriers, were also similar (Figure 4B) to the respective controls (Figure 4A).

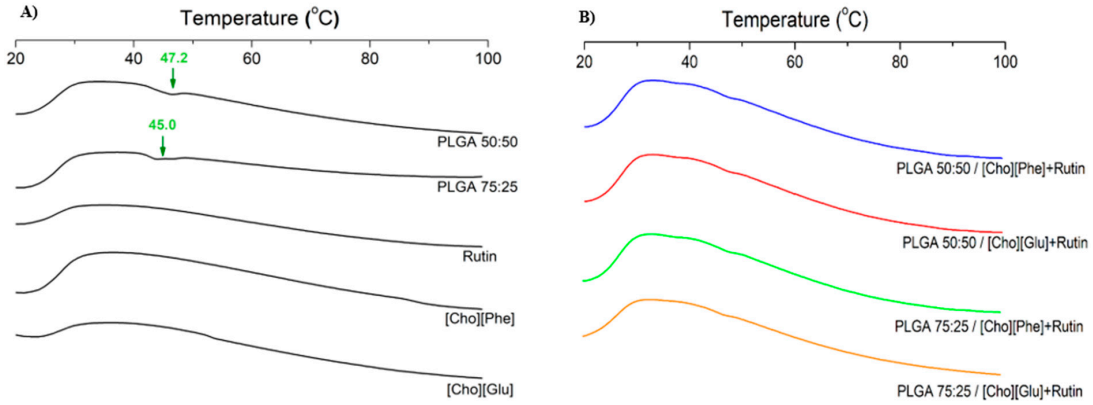

Figure 4. DSC thermogram of controls (PLGA 50:50, PLGA 75:25, rutin, [Cho][Phe] and [Cho][Glu]) (**A**), rutin-loaded IL-PLGA nanoparticle hybrid systems (**B**), all obtained after freeze-drying.

3.5. Scanning Electron Microscopy (SEM) Analysis

The unloaded and rutin-loaded IL-polymer nanoparticle hybrid systems were analyzed by SEM, after freeze-drying without lyoprotectant. Analysis of the PLGA nanoparticles produced by the same method, but in the absence of ILs, was also performed to be used as control.

The SEM analysis showed that the incorporation of the IL on the inner phase of the nanosystem does not interfere with the nanoparticle morphology since the PLGA nanoparticles and the IL-polymer nanoparticle hybrid systems display a similar spherical morphology with a smooth surface (Figure 5), which is in agreement with the literature for this type of nanoparticles without IL [39,51,52]. Furthermore, the similarity in particle size observed in these analyses (Figure 5) was also concordant with the physicochemical properties previously determined herein (Figures 1 and 2).

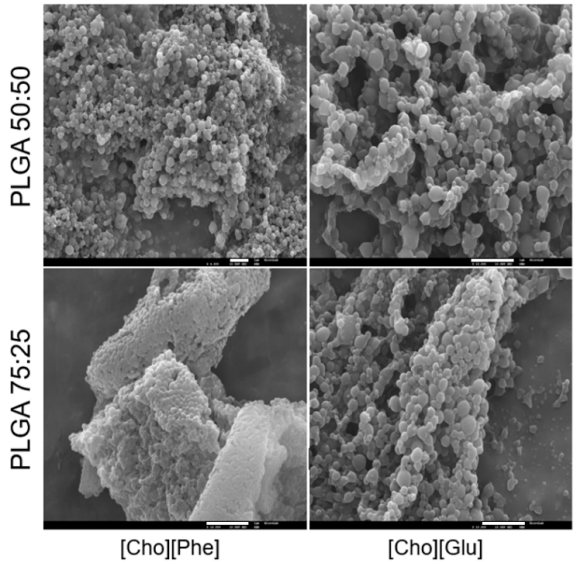

Figure 5. SEM microphotographs of rutin-loaded IL-polymer nanoparticle hybrid systems after freeze-drying at a magnification of 4000× (PLGA 50:50/[Cho][Phe]) and 10,000× (remaining images). The scale bar of the microphotographs at the bottom right of the images corresponds to 2 μm.

3.6. In Vitro Rutin Release Study

The release of rutin from the IL-polymer nanoparticle hybrid systems was studied using freshly prepared formulations. All formulations demonstrated a controlled release profile of rutin (Figure 6), which is a typical PLGA pattern [47,52].

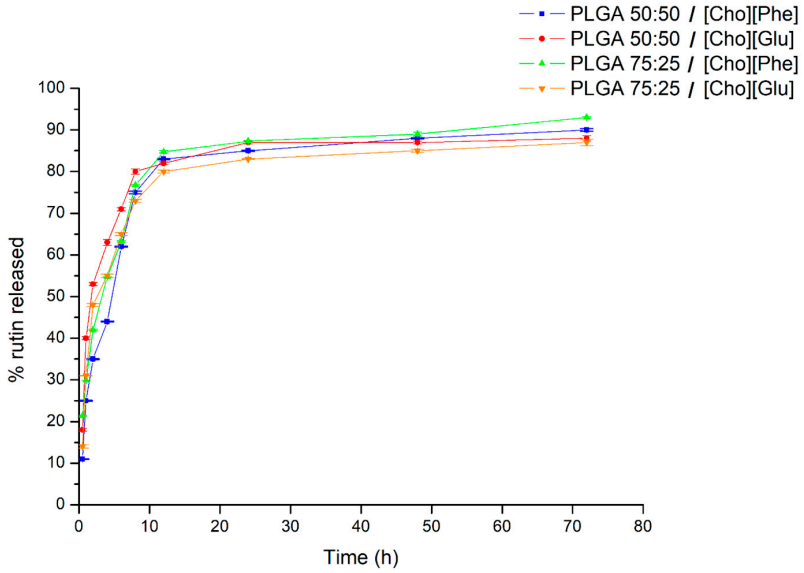

Figure 6. Release profile of the rutin-loaded IL-PLGA nanoparticle hybrid systems during 72 h in phosphate buffer saline at pH 7.4. Data represented as mean ± SD (n = 3).

There is an initial burst in the first 2 h of the study (Figure 6), which is likely to be caused by rutin adsorbed to the surface of the nanoparticles [51,74,75]. After this initial burst, the hybrid systems presented a sustained drug release over time (Figure 6), reaching values between 85% to 95%, after 72 h (Figure 6). Additionally, there is not significant differences in the release profile between both PLGA ratios neither between choline-based ILs. These results demonstrate the ability of the IL-polymer nanoparticle hybrid systems to deliver rutin in a sustained manner up to 72 h. More importantly, it was demonstrated that the presence of ionic liquids in the nanoparticles does not hamper the drug release by keeping the characteristic sustained release.

3.7. Permeation Study

The permeation study was performed in vitro with a PDMS membrane. Taking into account the low water solubility of rutin and according to the method described in the literature [76], the receptor fluid was a mixture of phosphate buffer pH 7.4 and ethanol (80:20), to guarantee that the assay was done in sink conditions [76].

The permeation assay was performed using all formulations and an aqueous solution of rutin, containing each IL, at the same concentration as in the nanocarrier formulation was used as control. After 24 h, the results showed that free and encapsulated rutin presented a low skin permeation, with the permeation flux between 0.48 and 0.55 μg/cm^2/h (Table 3), which may be explained by the high molecular weight and the high hydrophobicity of rutin [57].

Table 3. Permeation flux of rutin IL solution, at 0.87 mg/mL for [Cho][Phe] and 0.37 mg/mL for [Cho][Glu], and rutin-loaded IL-polymer nanoparticle hybrid systems. Data represented as mean ± SD (n = 5).

Formulation	IL	Flux (μg/cm^2/h)
Rutin solution	[Cho][Phe]	0.50 ± 0.09
	[Cho][Glu]	0.52 ± 0.08
PLGA 50:50	[Cho][Phe]	0.55 ± 0.13
	[Cho][Glu]	0.51 ± 0.11
PLGA 75:25	[Cho][Phe]	0.50 ± 0.06
	[Cho][Glu]	0.49 ± 0.12

Although no significant differences between the sample and the control were found, the higher lipophilicity of rutin [57] may contribute to the permanence of the drug on the PDMS surface or decrease in its release to the medium receptor, since PDMS membrane has also higher lipophilic properties [77,78]. These results may indicate that these systems can be used for topical administration.

3.8. MTT Cytotoxicity Assay

Given that rutin may be used topically [12,57,58], a study was performed with HaCat human keratinocytes to check the impact of the IL-polymer nanoparticle hybrid systems on the cell viability. The MTT reduction assay, which is a colorimetric assay that quantifies the mitochondrial function, measured the effect of the system [62].

To understand if the presence of the IL in the nanocarrier interferes with the viability of HaCat cells, a comparative analysis was performed with unloaded PLGA nanoparticles in the absence of the IL and unloaded IL-polymer nanoparticle hybrid systems. Furthermore, we also aimed to investigate whether drug encapsulation in the IL-polymer nanoparticle hybrid systems could cause changes in the interaction with HaCat. Therefore, a rutin-loaded IL-polymer nanoparticle hybrid system was also evaluated alongside with an aqueous solution of rutin containing ILs. Additionally, to go even further, we also tested the samples obtained at the end of the release study, which we refer to hereafter as leachable.

It is also important to mention that, previous studies in our group already showed that both choline-based ILs used maintained the cell viability in these cells at the same tested conditions until 0.2% (*v*/*v*) [8].

Regarding the obtained results, they showed that, under our experimental conditions, there was no significant difference between the unloaded PLGA nanoparticles and the PLGA nanoparticles combined with the ILs (Figure 7(A1,B1)). This indicates that the presence of the ILs as the solubility promotor in the inner phase of the nanoparticles does not decrease cell viability. Additionally, viability remained unaffected after cell exposure to IL-polymer nanoparticle hybrid systems and to an aqueous solution of rutin with 0.2% (*v*/*v*) of ILs. The experiments performed with the leachable further showed that the components leached from a 72 h contact with rutin-loaded IL-polymer nanoparticles do not cause damage to cells (Figure 7(A2,B2)), suggesting the biocompatibility of the new developed hybrid systems.

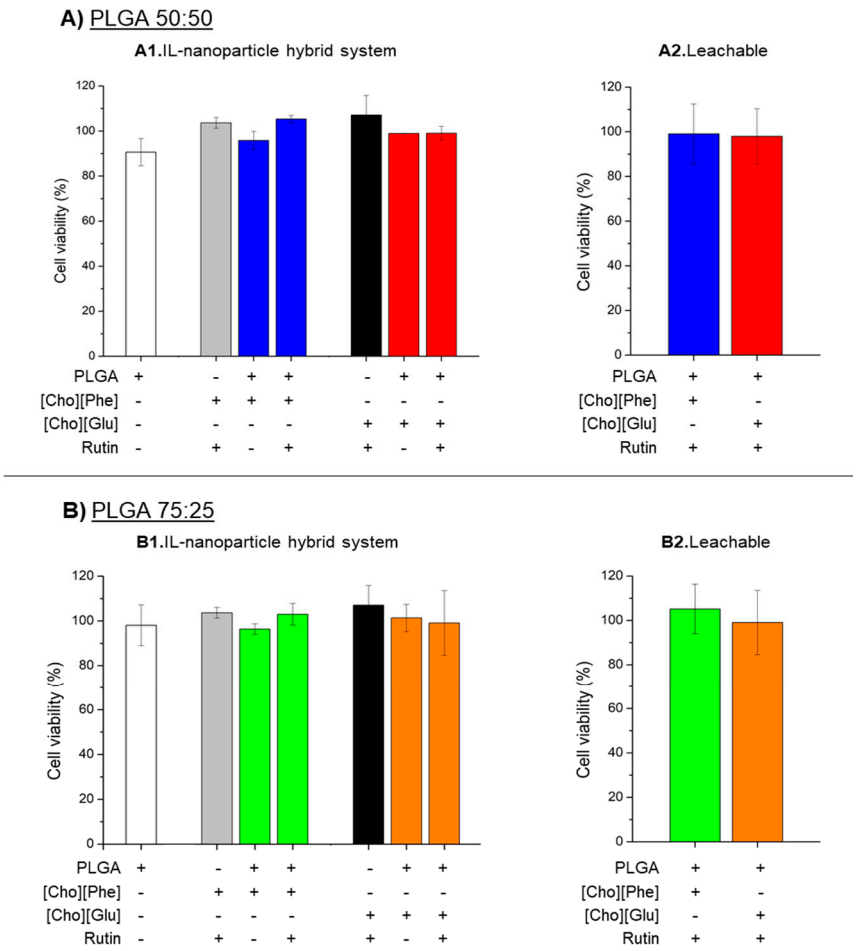

Figure 7. Cell viability of HaCat cells exposed to unloaded PLGA nanoparticles, rutin-loaded choline-based IL-polymer nanoparticle hybrid systems, an aqueous solution of rutin with 0.2% (*v*/*v*) of choline-based IL (**1**) and leachable (**2**). The presented ratios PLGA were 50:50 (**A**) and 75:25 (**B**) and concentration of rutin is 0.29 µM and 0.69 µM for samples containing [Cho][Glu] and [Cho][Phe], respectively. In all samples, the cell viability after 24 h was evaluated by MTT reduction assay. Values represent mean ± SD (n = 2) and are expressed as a percentage of the non-treated control cells.

4. Conclusions

The development of drug delivery systems faces several challenges, such as the low aqueous solubility and permeability of some drugs. Then, the combination of ILs and the nanoencapsulation may be a strategy to overcome these drawbacks. Thus, in this work, two different amino acid-based

ILs were used, [Cho][Phe] and [Cho][Glu], as solubility enhancers of a poorly soluble drug, rutin, for the development of IL-polymer nanoparticle hybrid systems.

The physicochemical characterization of the hybrid nanosystems proved that this combination contributes to a particle size between 250 and 300 nm, with good polydispersity and high colloidal stability. Besides that, it was possible to obtain an association efficiency higher than 50% for both ILs and up to about 76% in the presence of [Cho][Phe], which is a significant achievement considering the low aqueous solubility of rutin. This significant improvement was attained while maintaining the stability and particle size of the developed hybrid systems. Furthermore, the nanosystems did not shown any significant particle aggregation upon freeze-drying. Such results demonstrated the robustness of the delivery system

It was observed that the developed nanocarriers had a sustained release up to 72 h, demonstrating that the presence of the IL within the nanoparticles, does not interfere with rutin release. In addition, since no relevant skin permeability was observed, and no toxicity was verified in the study of cell viability in HaCat, human keratinocytes, these nanocarriers may be suitable to be used for skin topical applications.

Additionally, all results obtained in this work seem to indicate that the IL-polymer nanoparticle hybrid system with [Cho][Phe], as choline-based IL, and with PLGA 50:50 is the best formulation for the delivery of rutin. The results also revealed the high potential of these new delivery systems to deliver poorly soluble drugs, since the incorporation of the ILs in the nanocarrier contributed positively to the stability of the polymer nanoparticles as well as allowed higher incorporation of the drug in the nanocarrier.

In conclusion, the produced IL-polymer nanoparticle hybrid systems may be used as a strategy to overcome the low solubility of some drugs, contributing to a higher drug loading and to a controlled and/or targeted delivery. The findings in this work, may open a new paradigm on the use of IL-polymer nanoparticle hybrid systems to deliver poorly soluble drugs, with clear benefits to the therapy of different diseases and health problems.

Author Contributions: Conceptualization: T.S.d.A. and P.F.; methodology: all authors; software: A.J. and R.C.; validation: T.S.d.A. and P.F.; formal analysis: T.S.d.A. and P.F.; investigation: A.J., R.C., A.S.F., T.S.d.A., and P.F.; resources: A.S.F., D.M.F.P., S.A.C.L., S.R., C.R., T.S.d.A., and P.F.; writing—original draft preparation: A.J., T.S.d.A., and P.F.; writing—review and editing: all authors; supervision: T.S.d.A. and P.F.; project administration: T.S.d.A. and P.F.

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Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 4

Biobased Ionic Liquids as Multitalented Materials in Lipidic Drug Implants

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Biobased Ionic Liquids as Multitalented Materials in Lipidic Drug Implants

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Abstract: Lipidic implants are valuable controlled delivery systems that present good biocompatibility and are useful for long-lasting therapies. However, these promising systems can present inflexible drug release profiles that limit their performance. Thus, finding new materials to overcome this drawback is crucial. Herein, lipidic implants containing caffeine and poorly soluble salicylic acid and rutin were developed. The inclusion of Gelucire® 50/02, sucrose, and two biobased ionic liquids, [Cho][Phe] and [Cho][Glu], were evaluated as a mean to improve the performance of the systems. The formulation procedure, dye content distribution, drug content, drug release, water content, and lipidic erosion of the developed systems were assessed. AFM analysis of the implants containing ILs was also performed. The results demonstrated that neither Gelucire® 50/02 nor sucrose were suitable tools to improve the drug release profile. In contrast, the ILs proved to be promising materials for multiple reasons; not only did they facilitate the formulation and incorporation of the studied drugs into the implants, but they also allowed a more suitable release profile, with [Cho][Glu] allowing a higher drug release due to its ability to increase surface wrinkling. Hence, this study showcases ILs as multitalented materials in lipid-based drug implants.

Keywords: lipidic implants; caffeine; salicylic acid; rutin; ionic liquids; improved performance

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1. Introduction

The pharmaceutical industry has always sought to develop drug delivery systems that allow for a prolonged and effective therapeutic effect to ensure less adverse effects and less frequency of administration. Among these controlled delivery systems are implants that are particularly useful in prolonged therapies [1,2]. These systems are inserted subcutaneously (into the interstitial tissues of the arm, thigh, or abdomen) when a systemic effect is sought. In contrast, if a localized effect is desired, they may be placed in the target organ through surgical procedures (for example, in the vitreous cavity of the eye or intraperitoneally) [3].

Moreover, there are three types of implants including polymeric, mini-pumps, or lipidic. The polymeric implants have different shapes and are composed of biodegradable or non-biodegradable polymers, while mini-pumps are prepared by combining a polymer and titanium, thus containing an osmotic system [3–6]. In contrast, the lipidic implants are produced with lipids that contribute to a higher biocompatibility and low toxicity compared to polymeric implants [3–6]. Hence, there are various studies focused on lipidic implants due to their attractive properties compared to polymeric ones [3–6]. Apart from their low toxicity and good biocompatibility, due to the presence of lipids that are constituents of the organism, their ability for drug protection and flexibility in choosing different excipients in the formulation process also represent advantages [3–8]. However, they may present

inflexible drug release profiles such as incomplete total drug release that then leads to a low efficiency of the system [3,6]. Additionally, they may also present some issues concerning degradation [3,6,9].

Hence, when developing new implants, it should be considered that while the lipidic implants are biocompatible and have low toxicity, these delivery systems must also be prepared through a straightforward and efficient method that allows for uniform drug distribution and leads to the desired drug release profile [3,5,6,10]. To achieve these goals, the type of materials selected to be incorporated in the implants may be crucial. Considering this, ionic liquids (ILs) were used in this study to evaluate their possible applicability as multifunctional components in lipidic implants.

ILs are poorly coordinated organic salts that have been defined as a liquid below 100 °C or even at room temperature [11–15]. They have high thermal and chemical stability, low vapor pressure, are negligibly volatile, and present good ionic conductivity [16,17]. ILs have been used in pharmaceutical research for several applications [18] such as solubility promotors [12,13,15,19–22], as catalysts in the synthesis of active pharmaceutical ingredients [19,23,24], as oil or water substitutes [24–27], as surfactants in emulsions and micro emulsions [12,15,19,20,25–27], or even integrated with nanomaterials [28–31]. Hence, ILs may potentially be key materials to be incorporated in different delivery systems such as lipidic implants to improve their efficiency. Specifically in this study, two choline-based ILs were incorporated into lipidic implants at non-toxic concentrations [13,15] and the impact of these materials on the formulation procedure and in the performance of the developed systems was evaluated. The lipidic implants were produced in the presence and absence of ILs with variable compositions and with or without the studied drugs, namely caffeine and poorly water-soluble salicylic acid and rutin.

The three incorporated drugs were chosen as they each have pharmaceutical interest. Caffeine, a natural methylxanthine alkaloid, salicylic acid, a natural β-hydroxy acid, and rutin, a polyphenolic bioflavonoid, each have activity at the level of the central nervous system which may be useful in the treatment of some neurodegenerative diseases as a stimulant [32–35], anticonvulsant [35–38], sedative [35,36,39], and possibly as a suppressor of the neurological pathway that contributes to the onset of tardive dyskinesia, common in Alzheimer's and Parkinson's [35–38]. In the case of neurodegenerative diseases, the application of lipidic implants for the purpose of achieving a controlled delivery may be an added value by allowing for a higher adherence to therapy and reducing the number of doses, while decreasing plasma oscillations of drug concentrations and avoiding toxic levels [40–42]. Thus, due to the importance that lipidic implants may represent in terms of controlled delivery and the relevance of finding resourceful and multi-talented materials that may improve the efficiency of these systems, herein, ionic liquids were incorporated into lipidic implants. The outcome of this inclusion was evaluated in multiple ways. To achieve this, several implants were developed, and the impact of the different compositions were studied including: (1) the efficiency of the development procedure; (2) the distribution of different dyes; (3) the drug content; (4) the water content; (5) the lipidic erosion; and (6) the drug release profile.

2. Materials and Methods

2.1. Materials and Reagents

The lipidic implants were produced using Dynasan® 118; glyceryl tristearate from Cremer Oleo GmbH (Hamburg, Germany); Gelucire® 50/02 which is a mixture of glycerol monoesters, diesters, and tri-esters with polyethylene glycol monoesters and diesters from GatteFossé (Saint-Priest, France); and sucrose from Sigma-Aldrich (Darmstadt, Germany). The chosen drugs were, caffeine and salicylic acid, both from Sigma-Aldrich (St. Louis, MO, USA), and rutin from Fagron (São Paulo, Brazil). To evaluate the dye content distribution, the developed lipidic implants were produced in the presence of one lipophilic dye, Sudan III, or one hydrophilic dye, Methylene Blue, purchased from Sigma-Aldrich (St. Louis, MO, USA) and Sigma-Aldrich (Darmstadt, Germany), respectively. The incorpo-

rated ILs, namely (2-hydroxyethyl)-trimethylammonium-L-phenylalaninate [Cho][Phe] and (2-hydroxyethyl)-trimethylammonium-L-glutaminate [Cho][Glu] were synthesized and characterized within the context of other studies developed by our group [12,15].

For the drug release, water content, and the lipidic erosion studies, phosphate-buffered saline (PBS) pH 7.4 was used and prepared in house with 0.01% (*w*/*w*) sodium azide from Sigma-Aldrich (St. Louis, MO, USA) used as release medium. This medium was also used to assess the drug content and both the diethyl ether from PanReac AppliChem® ITW Reagents (Barcelona, Spain) and the ethanol absolute from Sigma-Aldrich (St. Louis, MO, USA). As equipment, a Heidolph 1000® incubator with a stirring Heidolph Unimax 1010® (Schwabach, Germany), a multipoint plate IKA® RT 15P (Staufen, Germany), and an Evolution® spectrophotometer (Thermo Scientific, Hertfordshire, England) was used.

2.2. Implants Preparation

The lipidic implants were prepared manually by fusion and melting through a technique modified from the method previously described by Kreye et al. [3] with the aim of improving the preparation procedure and reducing material loss. Firstly, the drug was sprayed and sieved with a diameter under 100 µm, before being used. Then, the lipid (Dynasan® 118), each release adjuvant of sucrose or Gelucire® 50/02 (the first value denotes the melting point of the substance and the second the value notes the hydrophilic lipophilic balance-HLB), and/or the IL used were weighed.

The drug was also weighed and added to the respective mixtures. All mixtures were heated with stirring (100 rpm) in a water bath at 80 °C in the heating and stirring plate IKA® 45 (Staufen, Germany) until the melting, complete fusion, and homogenization (with stirring) of the samples was achieved. The fused mixtures were pipetted with a sterile disposable cylinder. All batches presented a total batch weight of 3 g. After cooling to room temperature, the implants were removed from the containers and stored under moisture-free conditions. Prior to being used, the implants were cut to equally determined sizes (0.5 cm). For the implants containing the studied drugs, the lipid:drug ratio was 90:10% (*w*/*w*). The composition of all prepared implants is described in Table 1. The total weight of the prepared implants range between 26 mg to 29 mg and the drug content and release studies were performed based on the total weight of each implant.

2.3. Dye Content Distribution

To assess the distribution of the two dyes, the lipidic implants were produced as previously described but now also containing a lipophilic or hydrophilic dye solution of 2.5% *w*/*w* of Sudan III [43] or Methylene Blue [44] (Table 1).

2.4. Drug Content

2.4.1. Implants Containing Caffeine

The samples (n = 3) containing caffeine were dissolved in a mixture of 1.5 mL of PBS pH 7.4 with 0.01% (*w*/*w*) of sodium azide and 0.5 mL of diethyl ether and were then placed under stirring at 37 °C in the multipoint plate IKA® RT 15P (Staufen, Germany). After 90 min and 180 min, an aliquot of the aqueous phase (100 µL) was removed and quantified using an Evolution® spectrophotometer (Thermo Scientific, Hertfordshire, England) at 273 nm (maximum absorption wavelength in PBS solution). The percentage of the drug was calculated based on the total weight of each implant.

2.4.2. Implants Containing Salicylic Acid or Rutin

Implants (n = 3) were crushed and dispersed in 25 mL of absolute ethanol. The drug (salicylic acid or rutin) was completely dissolved, whereas implant excipients were dispersed. An aliquot (1 mL) was filtered, diluted, and then the drug quantification was performed by UV-Vis spectrophotometry (Evolution® spectrophotometer, Thermo Scientific, Hertfordshire, England) at the maximum absorption wavelength for each drug

(295 nm for salicylic acid or 348 nm for rutin) in absolute ethanol. The drug content was calculated based on the total weight of each implant.

Table 1. Composition, % (*w*/*w*), of all the Dynasan® 118 lipidic implants containing variable compositions of Gelucire® 50/02, sucrose, and each IL ([Cho][Phe] or [Cho][Glu]). Implants (A–I) were prepared without (controls) or in the presence of each dye (methylene blue or sudan III) or each drug (caffeine, salicylic acid, or rutin).

Drug	Formulations	% *w*/*w*				
		Dynasan® 118	**Gelucire® 50/02**	**Sucrose**	**[Cho][Phe]**	**[Cho][Glu]**
Controls (without drug)	A_0	100	-	-	-	-
	B_0	90	10	-	-	-
	C_0	90	-	10	-	-
	D_0	99.8	-	-	0.2	-
	E_0	99.8	-	-	-	0.2
	F_0	89.8	10	-	0.2	-
	G_0	89.8	10	-	-	0.2
	H_0	89.8	-	10	0.2	-
	I_0	89.8	-	10	-	0.2
Dye2.5% *w*/*w* (Methylene blue orSudan III)	A_{Dye}	97.5	-	-	-	-
	B_{Dye}	87.5	10	-	-	-
	C_{Dye}	87.5	-	10	-	-
	D_{Dye}	97.3	-	-	0.2	-
	E_{Dye}	97.3	-	-	-	0.2
	F_{Dye}	87.3	10	-	0.2	-
	G_{Dye}	87.3	10	-	-	0.2
	H_{Dye}	87.3	-	10	0.2	-
	I_{Dye}	87.3	-	10	-	0.2
Drug 10% *w*/*w* (caffeine, salicylic acid, or rutin)	A_{Drug}	90	-	-	-	-
	B_{Drug}	80	10	-	-	-
	C_{Drug}	80	-	10	-	-
	D_{Drug}	89.8	-	-	0.2	-
	E_{Drug}	89.8	-	-	-	0.2
	F_{Drug}	79.8	10	-	0.2	-
	G_{Drug}	79.8	10	-	-	0.2
	H_{Drug}	79.8	-	10	0.2	-
	I_{Drug}	79.8	-	10	-	0.2

2.5. In Vitro Drug Release

The implants (n = 5) were placed in 1.5 mL PBS pH 7.4 with 0.01% (*w*/*w*) of sodium azide at 37 °C and 100 rpm in a Heidolph 1000® incubator with stirring (Heidolph Unimax 1010®, Schwabach, Germany). At predetermined time points, the drug release was measured by UV-Vis spectrophotometry in an Evolution® spectrophotometer (Thermo Scientific, Hertfordshire, England) at the maximum absorption wavelength of each drug in PBS (273 nm for caffeine, 281 nm for salicylic acid, and 354 nm for rutin). To achieve this, after removing the totality of the release medium for analysis, this medium was always completely replaced with fresh PBS pH 7.4 (1.5 mL). This study was performed for 140 days as follows. In the first week, the samples were analyzed every day. In the second and third weeks, these analyses were performed biweekly. In the following days the measurements were done once a week. Sink conditions were kept in all experiments throughout the study. Once again, the percentage of drug release was obtained considering the total weight of each implant.

2.6. Water Content and Lipidic Erosion

The implants (n = 5) were weighed [dry mass (t = 0)] and placed in 1.5 mL PBS pH 7.4 with 0.01% (w/w) of sodium azide at 37 °C and 100 rpm in a Heidolph 1000® incubator with stirring (Heidolph Unimax 1010®, Schwabach, Germany).

At the same predetermined time points of the in vitro release studies, the implants were removed from the medium and carefully dried so that the droplets of medium on the surface were removed. Then, the implants were weighed, obtaining the wet mass (t), and then dried at 37 °C in an oven (Memmert U30® from Memmert, Schwabach, Germany) until a constant mass was obtained which was designated as dry mass (t). The release medium was analyzed by UV-Vis spectrophotometry in an Evolution® spectrophotometer (Thermo Scientific, Hertfordshire, England) at the maximum absorption wavelength of each drug in PBS to measure the drug released at time t.

The water content (WC) and the lipidic erosion (LE), both in percentage (%), were calculated by the following equations:

$$WC\ (\%) = \frac{wet\ mass\ (t) - dry\ mass\ (t)}{wet\ mass(t)} \times 100 \quad (1)$$

$$LE\ (\%) = \frac{dry\ mass\ (t = 0) - drug\ released\ (t) - dry\ mass(t)}{dry\ mass(0)} \times 100 \quad (2)$$

2.7. Atomic Force Microscopy (AFM)

AFM measurements were conducted in air (23 ± 1 °C) on a Multimode 8HR microscope coupled to a Nanoscope V (Bruker Corporation, Billerica, MA, USA). The images were acquired in tapping mode using etched silicon probes with a resonance frequency of ca. 75 kHz (FESP, Bruker Corporation, Billerica, MA, USA) and at a scan rate of ~1.3 Hz.

As the implants were previously cut with 0.5 cm, the samples were then sectioned half-longitudinally in length and glued directly onto the AFM magnetic specimens for imaging. At least two regions of each sample were imaged.

2.8. Statistical Analysis

After conducting normality and homogeneity tests, the results were expressed as mean ± standard deviation (SD) and evaluated with the Kruskal–Wallis test, followed by Bonferroni correction test or one-way analysis of variance (ANOVA) and then by Tukey's multiple comparison test. The differences between individual means were significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. The analyses were performed using the SPSS® statistical package (version 25, SPSS Inc. Chicago, IL, USA).

3. Results and Discussion

Several lipidic implants with different compositions, namely different release adjuvants (Gelucire® 50/02 or sucrose), and in the presence or absence of two biobased ILs derived from natural amino acids ([Cho][Phe] or [Cho][Glu]) were prepared. This was performed to assess the impact of the incorporated excipients, particularly the studied ILs, on the performance of the developed delivery systems and to establish if these materials could exhibit several functionalities.

It should be noted that to prove the ILs actually have valid functionalities, these materials were incorporated into the formulations at non-toxic concentrations, namely 0.2% (v/v) that is known to be the maximum percentage where cell viability is maintained in HaCaT cells (human keratinocytes) from previous studies performed by our group [13,15].

One of the initial goals of this study was to improve the implants' preparation technique as it is crucial to have a simple and effective method that allows to attain uniform implants. Hence, a modified melting and fusion method that uses a single container mold was developed. This allowed for a better distribution of the various components of the

prepared delivery systems and led not only to a faster production of the implants but also to a reduction in material loss, decreasing the production cost.

3.1. Dye Content Distribution

Before incorporating the three studied drugs, all implants were prepared in the presence of a hydrophilic (Methylene blue-**a**) or a lipophilic (Sudan III-**b**) dye (Table 1). This was performed to evaluate whether the developed preparation procedure and chosen excipients including the ILs would allow for an even distribution of both model dyes and then to infer if we could effectively incorporate both hydrophilic and lipophilic drugs into the developed implants. In fact, the results revealed (Figure 1) that all batches either containing the dyes of **a** or **b** presented a homogeneous appearance on the surface and on the cross section of the implants, suggesting that the preparation technique performed under the studied compositions leads to a uniform distribution.

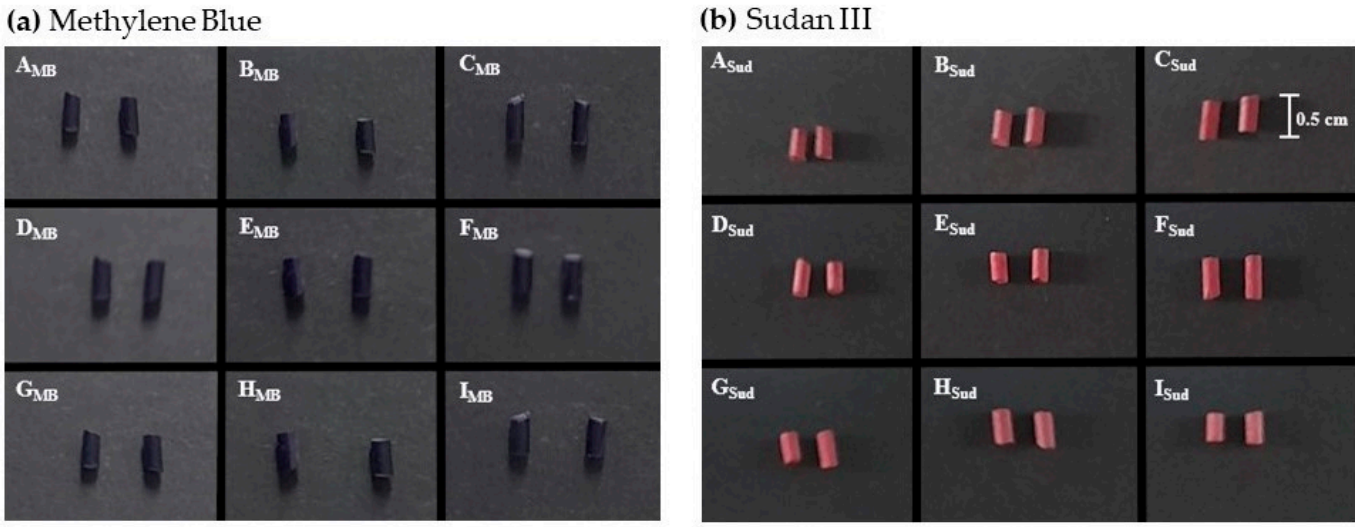

Figure 1. Macroscopic appearance of the developed implants containing (**a**) the hydrophilic methylene blue ($\mathbf{A_{MB}}$–$\mathbf{I_{MB}}$) or (**b**) the lipophilic sudan III ($\mathbf{A_{Sud}}$–$\mathbf{I_{Sud}}$) dyes in the distribution assessment.

Moreover, slight differences in color intensity between different batches were only observed due to the presence of different components that contribute differently to the observed shades. For instance, the implants containing the ILs presented a more intense color. This was not surprising considering the studied ILs are slightly colored.

After demonstrating that the used methodology and chosen components seem to allow a uniform distribution of both hydrophilic and lipophilic compounds, it was justified to move towards the incorporation of different drugs in the implants.

3.2. Implants Containing Each Drugs

The lipidic systems were then prepared in the presence of the three studied drugs, namely caffeine, a more hydrophilic active, and salicylic acid or rutin, both more lipophilic drugs compared to caffeine (Table 1, Figure 2).

It should be noted that the incorporation of the studied drugs was facilitated for the implants containing ILs when compared to the implants without ILs. This may be due to the fact that these salts are known to be good solubility promotors [12,13] and consequently they likely allow a better incorporation of the studied drugs as well as a better blend of the different components contained in the studied formulations. These were the first indicators that including ILs into the developed implants could be a valuable strategy to improve the performance of lipid-based formulations.

Furthermore, all implants presented a similar appearance, differing only slightly in color depending on the incorporated drug and/or IL (Figure 2). This reveals once

again that all the used excipients do not seem to interfere with the homogeneity of the developed implants.

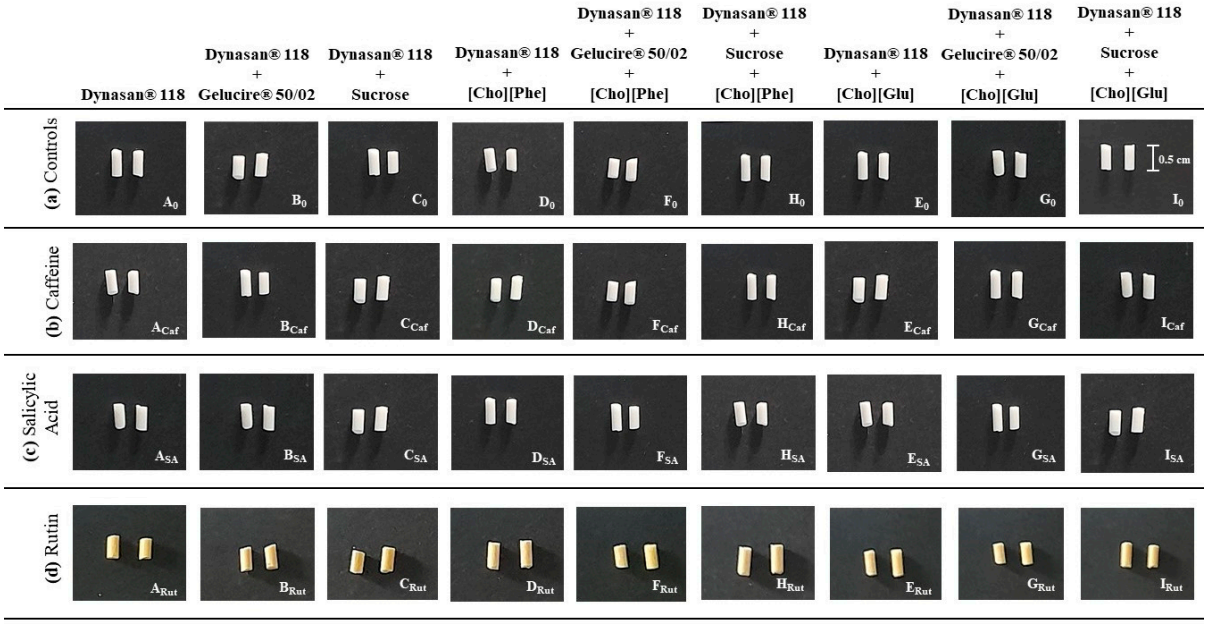

Figure 2. Macroscopic appearance of all prepared implants: without drug ($\mathbf{A_0}$–$\mathbf{I_0}$), containing caffeine ($\mathbf{A_{Caf}}$–$\mathbf{I_{Caf}}$), salicylic acid ($\mathbf{A_{SA}}$–$\mathbf{I_{SA}}$), or rutin ($\mathbf{A_{Rut}}$–$\mathbf{I_{Rut}}$).

3.2.1. Drug Content

To further ensure the efficiency of the developed methodology, the percentage of drug content of all the implants containing each studied drug was also evaluated. In this assessment, results showed that the drug content was greater than 95% for all the implants containing each of the three drugs (Figure 3).

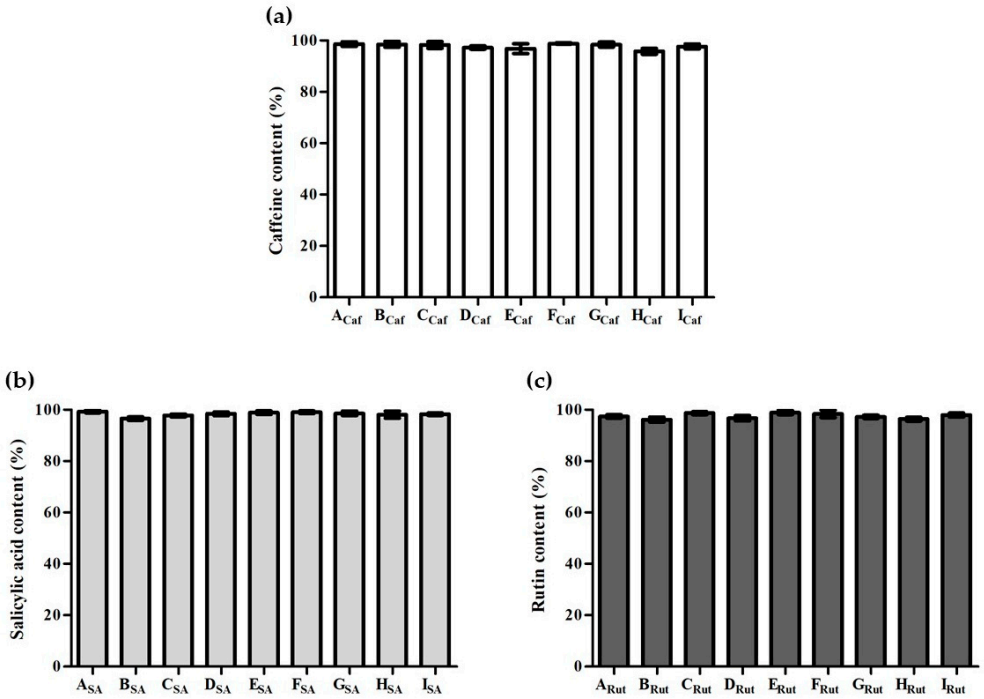

Figure 3. Drug content (%) of the implants containing (**a**) caffeine ($\mathbf{A_{Caf}}$–$\mathbf{I_{Caf}}$), (**b**) salicylic acid ($\mathbf{A_{SA}}$–$\mathbf{I_{SA}}$), and (**c**) rutin ($\mathbf{A_{Rut}}$–$\mathbf{I_{Rut}}$). $n = 3$, mean ± SD.

No statistical differences were observed between the different formulations. This outcome corroborates the robustness of the methodology used to prepare the implants, demonstrating a uniform drug content.

Then, to continue to evaluate the performance of the developed implants and the impact of the used release adjuvants (Gelucire® 50/02 or sucrose) and of the two ILs, several studies were implemented, namely studying the in vitro drug release, water content, and lipidic erosion (Figures 4–7).

3.2.2. In Vitro Drug Release, Water Content, and Lipidic Erosion

When comparing the inclusion of Gelucire® 50/02 or sucrose in the implants, the drug release results revealed that Gelucire® 50/02 is a better drug release promotor (Figure 4).

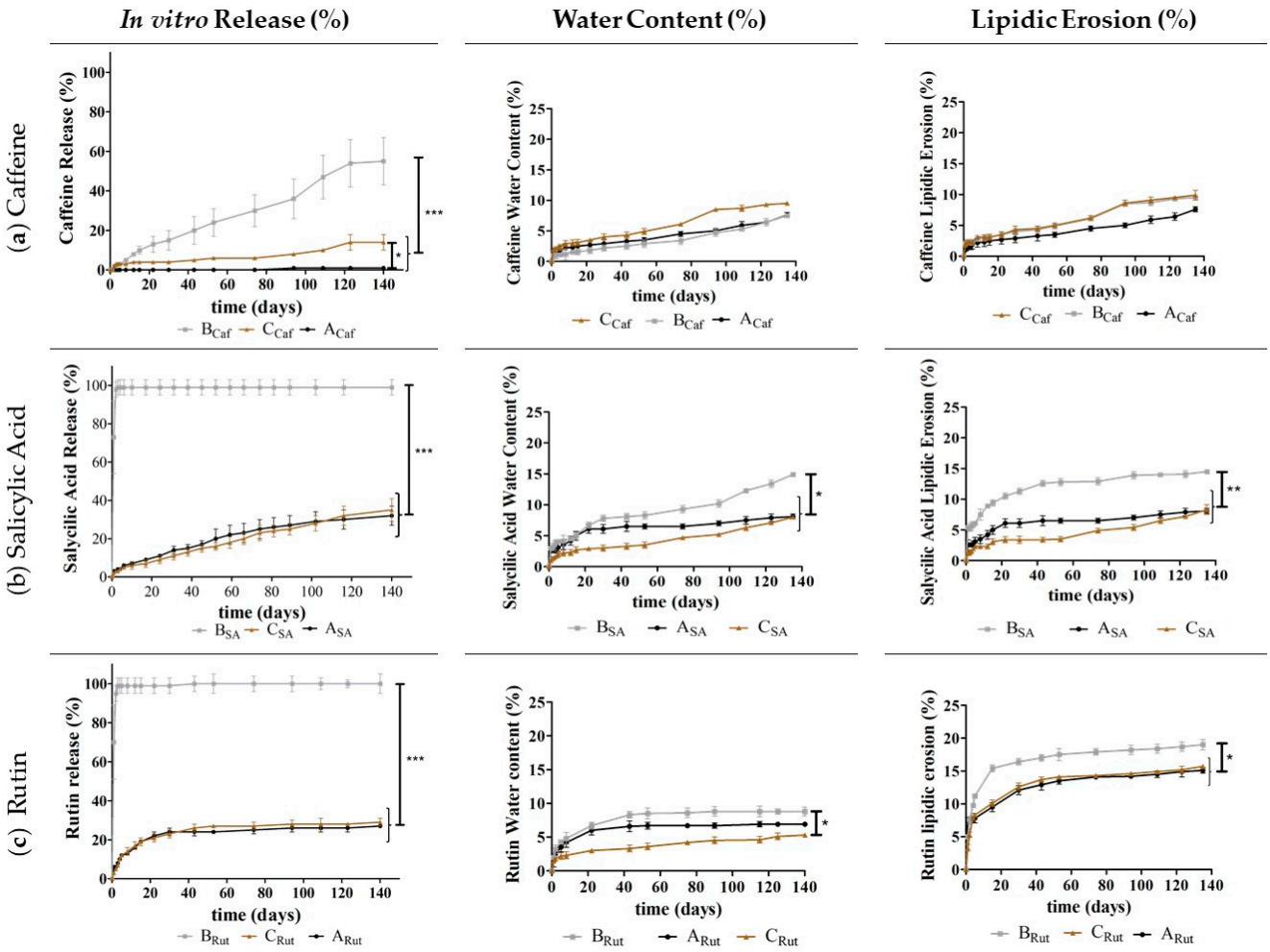

Figure 4. In vitro drug release (%), water content (%), and lipidic erosion (%) of the implants with Dynasan® 118 ($\mathbf{A_{Caf}}$, $\mathbf{A_{SA}}$, and $\mathbf{A_{Rut}}$); Dynasan® 118 and Gelucire® 50/02 ($\mathbf{B_{Caf}}$, $\mathbf{B_{SA}}$, and $\mathbf{B_{Rut}}$); and Dynasan® 118 and sucrose ($\mathbf{C_{Caf}}$, $\mathbf{C_{SA}}$, and $\mathbf{C_{Rut}}$) in the presence of caffeine (**a**), salicylic acid (**b**), or rutin (**c**). $n = 5$, mean ± SD, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

This is particularly evident for the implants in the presence of salicylic acid ($\mathbf{B_{SA}}$) or rutin ($\mathbf{B_{Rut}}$) for which an immediate drug release was observed for the implants containing Dynasan® 118 and Gelucire® 50/02, in opposition to the lower drug release observed for the implants containing Dynasan® 118 and sucrose ($\mathbf{C_{SA}}$ and $\mathbf{C_{Rut}}$) or for the implants containing only Dynasan® 118 ($\mathbf{A_{SA}}$ and $\mathbf{A_{Rut}}$).

With respect to the water content and lipidic erosion, no substantial differences were observed, although for the systems containing salicylic acid and rutin, the implants with Gelucire® 50/02 exhibited a slightly higher lipid erosion compared to the implants with sucrose. Although, this aspect may somewhat contribute to the higher drug release ob-

served, when considering the implants containing Gelucire® 50/02, this difference is not substantial enough to be the sole contributor for the immediate drug release observed for these implants. Conversely, a higher affinity between the two more lipophilic drugs and the more lipophilic carrier Gelucire® 50/02 may facilitate the drug diffusion in this material and thus be a more relevant contributor for the higher drug release.

What is more interesting is that neither Gelucire® 50/02 nor sucrose alone appeared to be the best choice to ensure the desired controlled release over time. For Gelucire® 50/02, a non-desired immediate release was observed for the more lipophilic actives, while with sucrose, even though the release profile was sustained over time, it was quite low after the 140 days, not exceeding a 35% release. For caffeine, a low drug release was observed after the 140 days in the presence of both materials.

Then, we considered the impact of each ionic liquid, [Cho][Phe] or [Cho][Glu], on the performance of the developed implants to assess whether including these materials could be a better strategy.

When evaluating whether combining sucrose (Figure 5) or Gelucire® 50/02 (Figure 6) with each IL could be useful to improve the drug release profile, our results indicated that for caffeine (**a**), both combinations generally allowed for an increase in drug release compared to the implants containing only Dynasan® 118 (Figures 5a and 6a). This increase is more obvious for [Cho][Glu] (Figure 5, $\mathbf{I_{Caf}}$ and Figure 6, $\mathbf{G_{Caf}}$).

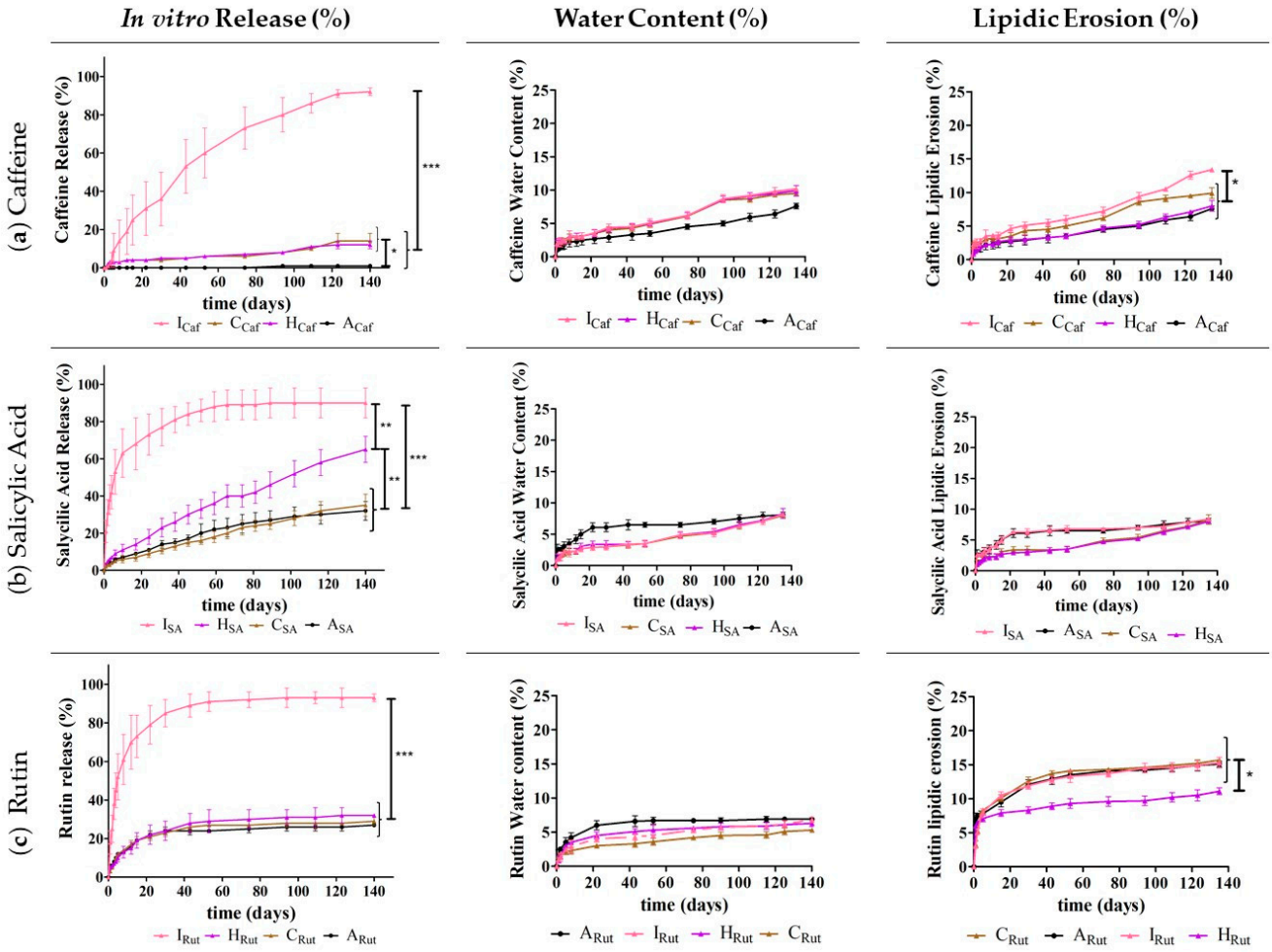

Figure 5. In vitro drug release (%), water content (%), and lipidic erosion (%) of the implants with Dynasan® 118 ($\mathbf{A_{Caf}}$, $\mathbf{A_{SA}}$, and $\mathbf{A_{Rut}}$); with Dynasan® 118 and sucrose ($\mathbf{C_{Caf}}$, $\mathbf{C_{SA}}$, and $\mathbf{C_{Rut}}$); and with Dynasan® 118, sucrose, and the IL [Cho][Phe] ($\mathbf{H_{Caf}}$, $\mathbf{H_{SA}}$, and $\mathbf{H_{Rut}}$) or [Cho][Glu] ($\mathbf{I_{Caf}}$, $\mathbf{I_{SA}}$, and $\mathbf{I_{Rut}}$). Each batch was prepared in the presence of caffeine (**a**), salicylic acid (**b**), and rutin (**c**). $n = 5$, mean ± SD, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

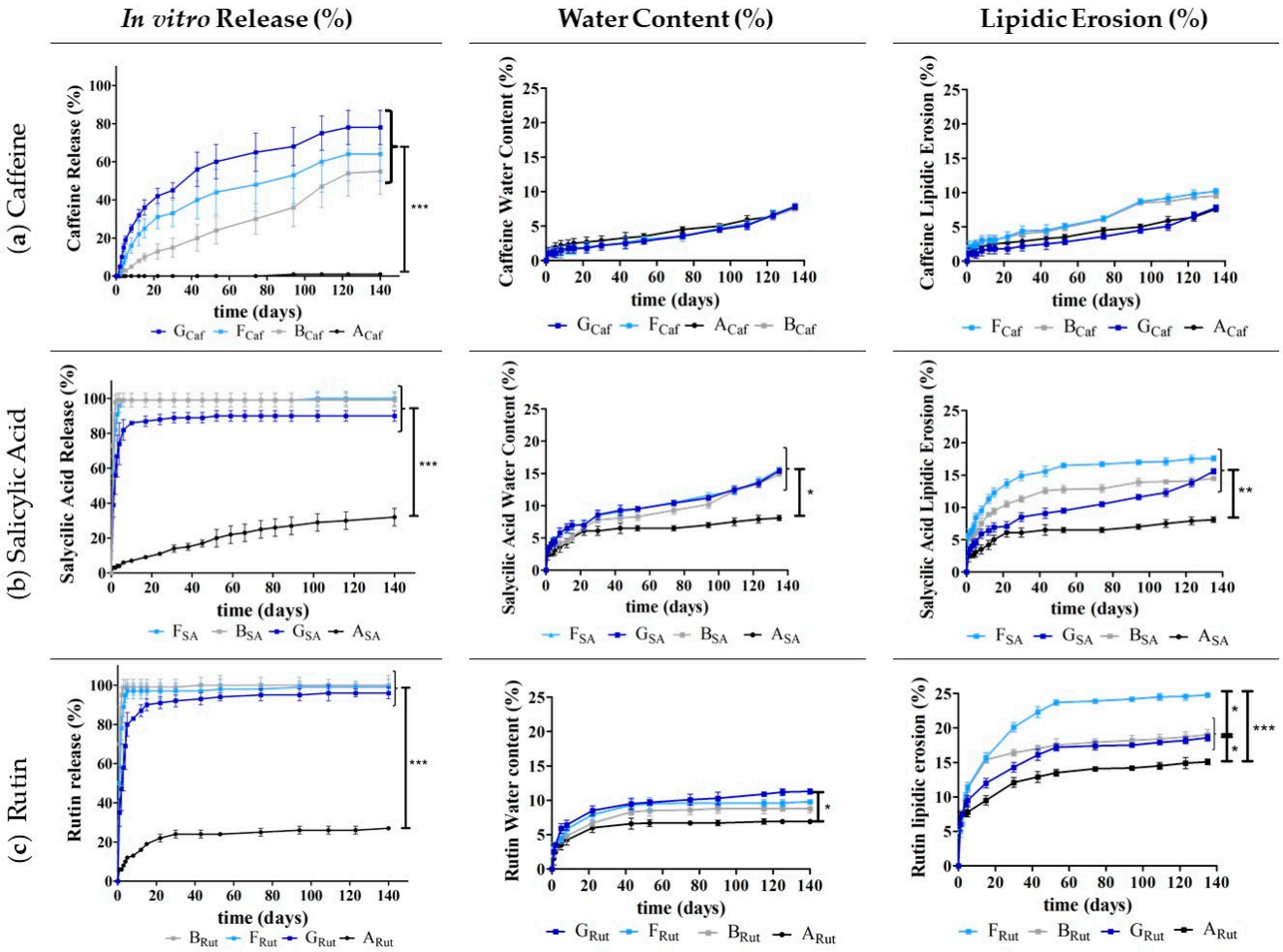

Figure 6. In vitro drug release (%), water content (%), and lipidic erosion (%) of the implants with Dynasan® 118 ($\mathbf{A_{Caf}}$, $\mathbf{A_{SA}}$, and $\mathbf{A_{Rut}}$); with Dynasan® 118 and Gelucire® 50:02 ($\mathbf{B_{Caf}}$, $\mathbf{B_{SA}}$, and $\mathbf{B_{Rut}}$); and with Dynasan® 118, Gelucire® 50:02, and IL [Cho][Phe] ($\mathbf{F_{Caf}}$, $\mathbf{F_{SA}}$, and $\mathbf{F_{Rut}}$) or [Cho][Glu], ($\mathbf{G_{Caf}}$, $\mathbf{G_{SA}}$, and $\mathbf{G_{Rut}}$). Each batch was prepared in the presence of caffeine (**a**), salicylic acid (**b**), and rutin (**c**). n=5, mean ± SD, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

For salicylic acid and rutin, the combination of the IL [Cho][Glu] with either sucrose (Figure 5) or Gelucire® 50/02 (Figure 6) also leads to a higher drug release. Nonetheless, for these compounds, the combination of the ILs ([Cho][Phe] or [Cho][Glu]) with Gelucire® 50/02 ($\mathbf{F_{SA}}$ and $\mathbf{F_{Rut}}$, and $\mathbf{G_{SA}}$ and $\mathbf{G_{Rut}}$) led to a similar immediate release as observed for Gelucire® 50/02 alone ($\mathbf{B_{SA}}$ and $\mathbf{B_{Rut}}$), proving that this combination is not helpful to attain a more controlled and suited release profile.

The next step was to evaluate whether including each IL alone on the implants ($\mathbf{D_{Caf}}$, $\mathbf{D_{SA}}$, and $\mathbf{D_{Rut}}$, and $\mathbf{E_{Caf}}$, $\mathbf{E_{SA}}$, and $\mathbf{E_{Rut}}$) without Sucrose nor Gelucire® 50/02 could be a better approach to improve the performance of the implants (Figure 7).

Compared to the systems containing only Dynasan® 118 (Figure 7, batches $\mathbf{A_{Caf}}$, $\mathbf{A_{SA}}$, and $\mathbf{A_{Rut}}$), the presence of the IL [Cho][Phe] alone led to a slight increase in drug release for the three studied drugs (Figure 7, $\mathbf{D_{Caf}}$, $\mathbf{D_{SA}}$, and $\mathbf{D_{Rut}}$). This release was also superior to what was previously observed in the presence of sucrose (Figure 4). Nonetheless, after the 140 days, the observed drug release was still relatively low for the three drugs (lower than 50%).

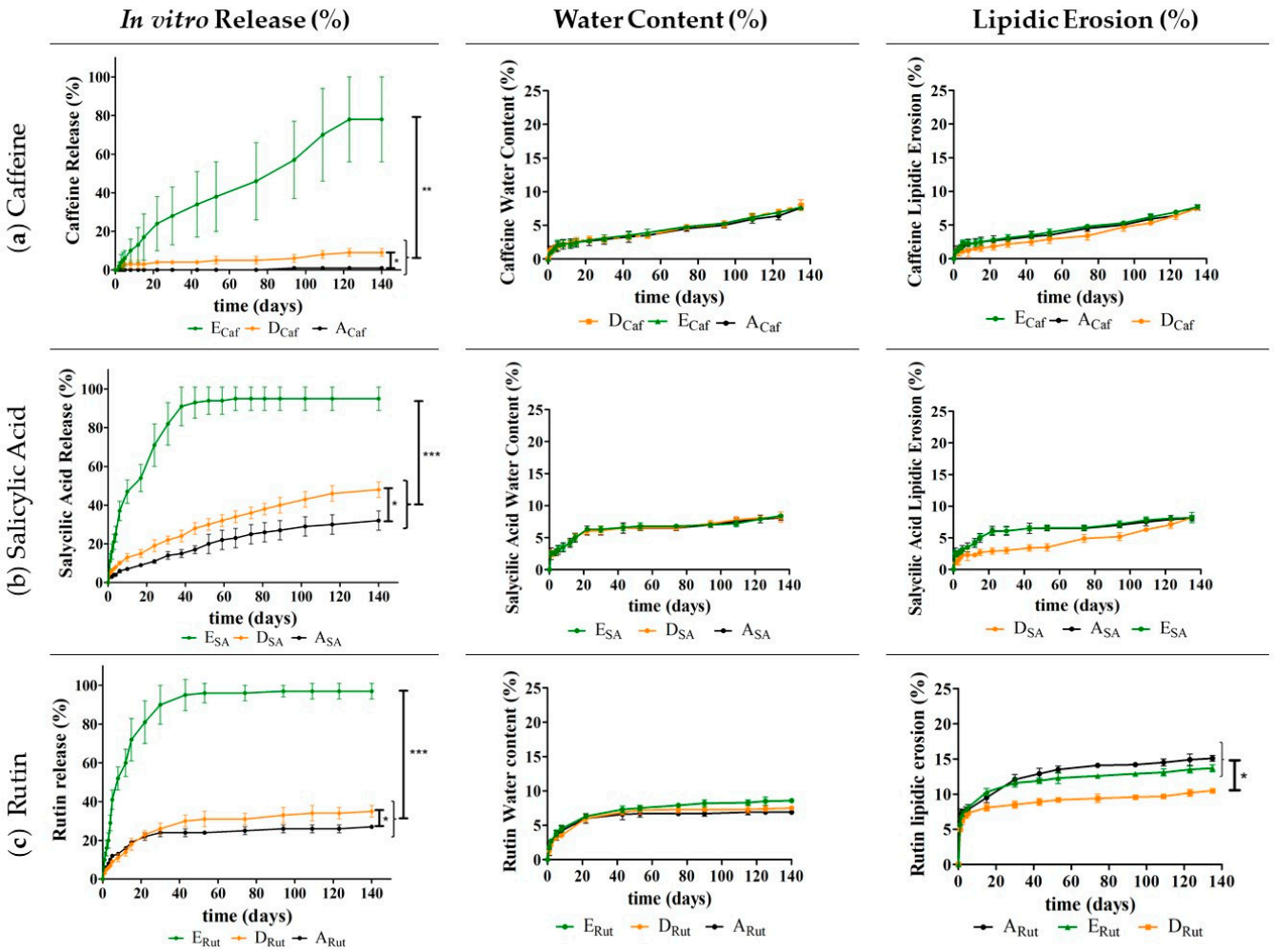

Figure 7. In vitro drug release (%), water content (%), and lipidic erosion (%) of the implants with Dynasan® 118 (**A_{Caf}**, **A_{SA}**, and **A_{Rut}**); Dynasan® 118 and [Cho][Phe] (**D_{Caf}**, **D_{SA}**, and **D_{Rut}**); and Dynasan® 118 and [Cho][Glu] (j**E_{Caf}**, **E_{SA}**, and **E_{Rut}**). Each batch was prepared in the presence of caffeine (**a**), salicylic acid (**b**), and rutin (**c**). $n = 5$, mean ± SD, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

It is also important to note that in the presence of [Cho][Phe], the release was controlled over time, demonstrating that this IL allows for a more suited release profile compared to what was observed in the presence of Gelucire® 50/02 (Figure 4). Thus, including [Cho][Phe] alone into the Dynasan® 118 implants seems to be a better strategy then using sucrose or Gelucire® 50/02, although this improvement is not substantial.

In contrast, the incorporation of [Cho][Glu] proved to be more promising. This IL allowed for a much more pronounced enhancement in drug release for the three drugs (**E_{Caf}**, **E_{SA}**, and **E_{Rut}**).

Specifically, for the more hydrophilic caffeine, the inclusion of [Cho][Glu] alone (Figure 7, **E_{Caf}**) led to an increase in drug release of above 75% (from 2% to 78%) compared to the batch containing only Dynasan® 118 (**A_{Caf}**). Additionally, compared with the results discussed for sucrose and Gelucire® 50/02, [Cho][Glu] allowed for an enhancement in drug release of about 60% (from 14% to 78%) compared to the batch containing sucrose (**C_{Caf}**) and an enhancement of more than 20% (from 55% to 78%) compared to the implants containing Gelucire® 50/02 (**B_{Caf}**). This demonstrates that [Cho][Glu] is a better caffeine release promotor than sucrose and Gelucire® 50/02.

For the implants containing the more lipophilic drugs, salicylic acid and rutin, an upper drug release of about 95% was obtained when [Cho][Glu] was incorporated (**E_{SA}** and **E_{Rut}**). This denotes an increase of more than 70% when compared to the batches

containing only Dynasan® 118 (**A_{SA}** and **A_{Rut}**, Figure 7). Additionally, compared to the implants with Dynasan® 118 and sucrose (**C_{SA}** and **C_{Rut}**, Figure 5) a substantial increase was also observed (above 60%).

It is also noteworthy that in contrast to the implants containing Gelucire® 50/02 (**B_{SA}** and **B_{Rut}**) or Gelucire® 50/02 combined with the ILs (**G_{SA}** and **G_{Rut}**, and **F_{SA}** and **F_{Rut}**), the inclusion of [Cho][Glu] alone (**D_{SA}** and **D_{Rut}**, and **E_{SA}** and **E_{Rut}**) allowed for a more controlled drug release profile, similar to what was observed for [Cho][Phe].

Bearing all this in mind, the inclusion of [Cho][Glu] seems to be particularly advantageous as it not only leads to a high drug release but also this release occurs in a more controlled manner over time (throughout 140 days for caffeine and 80 days for salicylic acid and rutin). This result is aligned with what is desirable for controlled release systems as not only a long-term drug release must be attained but also this release should be as high as possible to ensure therapeutically effective plasma drug concentration levels. The improved drug release may be due to the ability of ILs to be miscible with a wide variety of solvents and solutes [21], and due to the fact that they are known to enhance drug solubility, namely of the studied compounds [13,15], which may facilitate the drug release.

In terms of the water content and lipidic erosion, generally no considerable differences were observed between the implants with and without ILs, revealing that the ILs do not seem to have much impact on these parameters. Moreover, it should be noted that our results for Dynasan® 118 are consistent with a previous study [45] that also presented low values of water content and erosion for Dynasan® 118 implants containing theophylline (a member of the xanthine family such as caffeine) after seven days at 37 °C in a phosphate buffer 7.4.

In terms of the drug release mechanism, it has been described that this aspect may be controlled by water or drug diffusion [45]. Our results seem to suggest that for the developed implants, drug diffusion is likely more relevant than water diffusion as for all the studied compositions, a clear difference in drug release was observed depending on the type of drug incorporated with the more lipophilic drugs presenting a higher diffusion rate. This is likely due to a higher affinity with the lipophilic implant matrix. Conversely, the water content studies did not demonstrate clear differences between the implants, thus indicating that this parameter possibly has a lower impact on the release mechanism.

3.2.3. Atomic Force Microscopy (AFM)

Considering that the isolated incorporation of ILs seems to be the better strategy, AFM images were captured from the Dynasan® 118 implants containing either the [Cho][Phe] or the [Cho][Glu] and each drug (**D_{Caf}**, **D_{SA}**, and **D_{Rut}**, and **E_{Caf}**, **E_{SA}**, and **E_{Rut}**), and from the respective controls with and without each drug (**A_{Caf}**, **A_{SA}**, and **A_{Rut}**, and **A_0**, **D_0**, and **E_0**). Figure 8 displays representative images of each of these implants.

The AFM technique is quite helpful to analyze surface topography and roughness through three-dimensional imaging. Interestingly, the results demonstrated that the implants containing the ILs presented a more wrinkled surface compared to the implants without an IL. Nonetheless, this wrinkling was much more prominent in the presence of [Cho][Glu], independently of the incorporated drug which is consistent with the higher drug release observed for the implants containing this IL. This result may be explained by considering that wrinkling allows for a higher surface area and may consequently lead to an increased drug release. Thus, the obtained wrinkling may also be a determinant driving force that impacts drug release in addition to drug diffusion. In the presence of each active, it was also possible to observe that the size of the crystalline structures was smaller for the implants containing the ILs, demonstrating that the ILs promote a better distribution of the drugs. Furthermore, designing wrinkle delivery systems has been considered a valuable strategy to improve release and in consequence, there has been a growing interest in designing such delivery systems [46]. Hence, this result presents a new and valuable functionality of ILs when incorporated in lipid-implants.

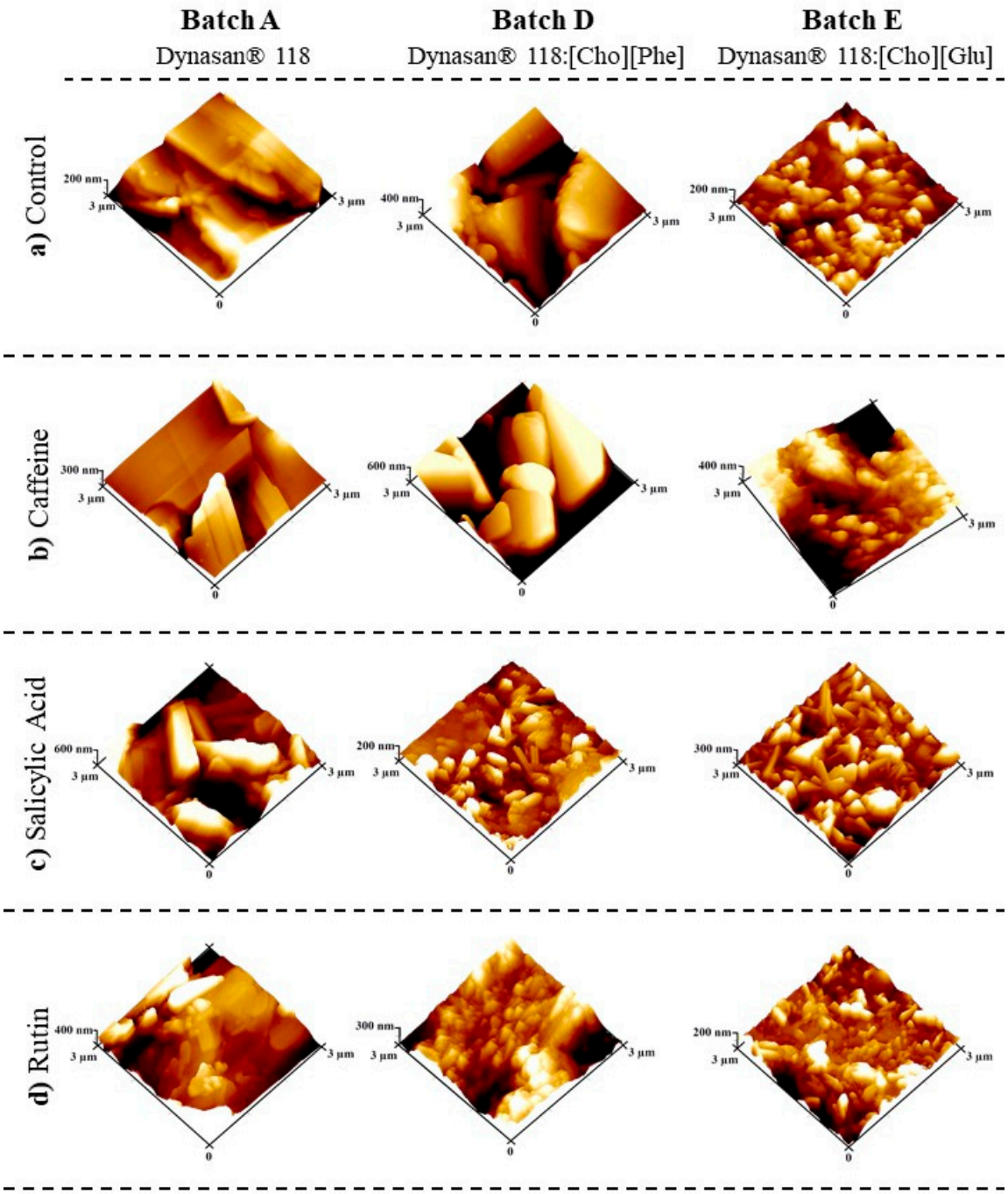

Figure 8. AFM images (3.0 × 3.0 µm^2) of the implants containing only Dynasan® 118 (**Batch A**), Dynasan® 118 and [Cho][Phe] (**Batch D**), and Dynasan® 118 and [Cho][Glu] (**Batch E**). The denoted batches were studied in the absence of drugs (**a-control**) or in the presence of caffeine (**b**), salicylic acid (**c**), or rutin (**d**).

In terms of surface functionalization, ILs have been used for this purpose in nanoparticles [47,48] due to the effect of various intermolecular interactions between the ILs and the components present in the delivery systems that are responsible for the singular distribution of various solutes within ILs, in comparison to other solvents. This may explain the impact of the ILs on the wrinkling observed for the developed implants.

It is the ionic nature of the ILs in addition to their heterogenous structure that provides them a unique combination of strong Coulombic interactions, van der Waals, inductive and dispersion interactions, and hydrogen bond interactions [47]. For instance, it has been described that the anions present in the ILs may have a greater ability to form hydrogen

bonds with drug molecules [49] and thus lead to improved drug solubility [15]. This interaction may justify both the improved drug release into the aqueous medium and the differences observed between the two studied ILs that differ only in terms of the anion present.

Thus, our results demonstrate that including ILs into lipidic implants, particularly the studied [Cho][Glu], may be a quite advantageous strategy. Specifically, ILs proved to be a talented material that improves the performance of the developed systems through a multipurposed functionality.

4. Conclusions

Lipidic implants may be a quite advantageous type of drug delivery system by allowing for a controlled, sustained, and localized delivery. Ensuring that these systems are produced in an easy and efficient manner that allows content and drug uniformity while achieving the desired release profile is fundamental.

Hence, this study had multiple aims including improving the preparation procedure and evaluating the influence of different compositions on this procedure, in addition to their impact on the performance of the developed implants. To achieve this, implants containing Dynasan® 118 and a variable composition in terms of other constituents were prepared. Namely, Gelucire® 50/02, sucrose, and two biobased ILs, [Cho][Phe] or [Cho][Glu], were included in the developed implants, either alone or in different combinations.

A modified and improved fusion and melting method was described herein that allowed for a faster and easier procedure with less material loss. Moreover, the implants containing ionic liquids proved to be much easier to blend both in the absence or presence of the studied drugs, demonstrating that the ILs further improved the preparation of the developed systems.

To evaluate whether the preparation technique and the various excipients used would lead to a homogeneous distribution, the lipophilic dye, Sudan III, and the hydrophilic dye, Methylene Blue, were incorporated into the various implants with different compositions. The prepared implants presented a uniform distribution of each dye, indicating that the preparation technique leads to an even incorporation of both lipophilic and hydrophilic substances. Following this, the three studied drugs, caffeine, salicylic acid, and rutin were included in the developed systems and the drug content was assessed. All implants exhibited a drug content superior to 95% without statistical differences between them.

It was also clear that the type of excipient included in the formulations had a considerable impact on the performance of the implants. In fact, for the implants containing sucrose, generally the drug release was lower compared to the implants containing Gelucire® or ILs. Nonetheless, in the presence of Gelucire®, the implants containing the lipophilic drugs salicylic acid or rutin presented an immediate drug release which is not desirable. In contrast, the incorporation of each IL alone, particularly [Cho][Glu], proved to be the better choice in terms of performance. In fact, the incorporation of these biobased materials led to implants acquiring a more wrinkled surface, allowing for a higher and more suitable release profile for almost 3 months without having impact on the water content or lipidic erosion. The results suggest that drug diffusion and surface wrinkling may be the key factors for drug release.

Hence, the studied ILs proved to be multitalented materials by demonstrating various functionalities when included in lipidic implants. Namely, at non-toxic concentrations, these biobased compounds allowed for an easier formulation of the implants and facilitated the incorporation of both lipophilic and hydrophilic drugs, while allowing to alter the surface properties of the implants and refining the drug release, especially in the case of [Cho][Glu].

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Chapter 5

TransfersomILs: From Ionic Liquids to a New Class of Nanovesicular Systems

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TransfersomILs: From Ionic Liquids to a New Class of Nanovesicular Systems

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Abstract: Ionic liquids (ILs) have increasingly been studied as key materials to upgrade the performance of many pharmaceutical formulations. In controlled delivery systems, ILs have improved multiple physicochemical properties, showing the relevance of continuing to study their incorporation into these formulations. Transfersomes are biocompatible nanovesicular systems, quite useful in controlled delivery. They have promising characteristics, such as elasticity and deformability, making them suitable for cutaneous delivery. Nonetheless, their overall properties and performance may still be improved. Herein, new TransfersomILs systems to load rutin were developed and the physicochemical properties of the formulations were assessed. These systems were prepared based on an optimized formulation obtained from a Box–Behnken factorial design (BBD). The impact of imidazole-based ILs, cholinium-based ILs, and their combinations on the cell viability of HaCaT cells and on the solubility of rutin was initially assessed. The newly developed TransfersomILs containing rutin presented a smaller size and, in general, a higher association efficiency, loading capacity, and total amount of drug release compared to the formulation without IL. The ILs also promoted the colloidal stability of the vesicles, upgrading storage stability. Thus, ILs were a bridge to develop new TransfersomILs systems with an overall improved performance.

Keywords: ionic liquids; nanosystems; rutin; Box–Behnken factorial design; TransfersomILs; cutaneous delivery

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1. Introduction

Over the years, ionic liquids (ILs) have generated a growing interest concerning their applicability in the pharmaceutical field, particularly due to the fact of their multifunctionality [1–5]. In fact, these compounds have remarkable properties, such as a high thermal and chemical stability [3], non-flammability [3], wide liquid range [5], and remarkable dissolution properties [5,6], all of which are characteristics that distinguish them from other organic solvents, namely, for applicability in the pharmaceutical area [3,5,7]. Consequently, ILs have been used as solvents [2,8], as excipients in various formulations [2,4,9–14], as solubility [2,10,11,13–16] and permeability [10,13,17–19] promoters, as surface active ILs [20–23], to improve the functionality of biomolecules such as proteins and enzymes [24–27], and also in drug delivery [4,15–17,28–31], amongst others [7]. Some of them have also been called green solvents, due to the fact of their low toxicity (compared to other ILs), making them key materials, particularly in drug delivery strategies [2–5,29,32,33]. Thus, ILs present great potential and have gained relevance in the development of innovative drug delivery

systems. In fact, ILs have been used as part of various types of delivery systems to improve their performance and some studies have shown that their inclusion may even lead to multiple upgrades in developed systems and not only to a single functionalization [2–4,29,32]. For instance, ILs may not only be valuable to enhance the incorporation of poorly soluble drugs into topical formulations, such as oil-in-water (O/W) emulsions, but they may also increase the viscosity and the stability of these thermodynamically unstable systems [2].

ILs may also be key to improve multiple characteristics of controlled drug delivery systems such as lipidic implants [4] and IL–nanoparticles hybrid systems [16,28,32,34–36]. In fact, since controlled delivery systems are quite relevant for long-term therapies, investing in the study of new materials that may lead to more effective formulations of this type, remains essential.

Transfersomes are among this type of formulations. They are nanovesicular systems with a remarkable skin permeation capability due to the incorporation of edge activators (EAs) within the phospholipid bilayer that surrounds an aqueous compartment [37]. EAs act as bilayer-destabilizing agents, thereby pronouncedly increasing the elasticity and deformability of the nanovesicles, allowing the nanocarriers to transport therapeutic agents to deeper layers of the skin [38]. The most used EAs in transfersomal formulations include non-ionic surfactants and bile acids, as recently reviewed [37,38]. It is noteworthy that it is crucial to optimize the phospholipid–EAs ratio to ensure that the nanovesicles display the penetration-enhancing feature and maintain their structural integrity. In this sense, quality-by-design strategies are an asset to direct the product development towards the predefined objectives, while reducing the manufacturing costs as well as time and materials waste [37]. This type of strategy is particularly relevant when designing transfersomes, as the raw materials used, namely, phospholipids, are quite expensive. This aspect justifies the use of various quality-by-design strategies, such as Box–Behnken factorial design and Plackett–Burman design, in recent publications concerning transfersomes development [39–41]. Another well-known drawback of transfersomal formulations is their poor storage stability, due to the fact of their tendency to aggregate, oxidize, and their high water content, the reason why they are usually stored at low temperatures [38]. To overcome this limitation, it would be interesting to include novel excipients in these formulations with antioxidant or antimicrobial properties. Despite these two less attractive points, the potential of transfersomes to load both hydrophilic and hydrophobic compounds and to provide a sustainable delivery of these agents across the skin justifies the continuous interest of the scientific community in translating these nanovesicular systems into clinical applications.

Rutin, a hydrophobic polyphenolic bioflavonoid found in many natural sources, has shown pharmaceutical interest. Several studies have been describing its potential as anti-inflammatory, antioxidant, and anticancer [42–44]. In this context, rutin has been included in topical formulations for photoprotective and anti-aging purposes [45,46] and for treating atopic and allergic contact dermatitis [47]. The anti-melanoma activity of rutin has also been demonstrated in cell-based studies [48,49]. Thus, the development of rutin-loaded topical formulations may be valuable for cosmetic and therapeutic applications. Nonetheless, this compound has a high molecular weight that impairs its penetration through the skin, with this being the reason why various nanosystems have been developed to load rutin [16,28,32,50–53]. Consequently, improving the incorporation of this compound into transfersomes could be a profitable strategy to further enhance the cutaneous permeability of rutin.

Hence, we aimed to develop new TransfersomILs (transfersomes containing ILs) to load rutin for cutaneous applications and to evaluate the impact of the studied ILs and IL:IL combinations on the physicochemical properties of the prepared formulations. The ILs were chosen since they have previously shown promising features that justify further investigation [2]. The solubility of rutin in aqueous solutions containing IL:IL combinations of imidazole-based ILs and choline-based ILs was determined as well as their impact on the cell viability of human keratinocytes. Secondly, the developed transfersomal system was optimized using a Box–Behnken factorial design (BBD). Then, the imidazole-based ILs,

the choline-based ILs as well as their combinations were incorporated into the optimized formulation. The physicochemical properties and the performance in terms of in vitro drug release and storage stability of the developed systems were assessed. This allowed to evaluate if the TransfersomILs could represent an innovative and advantageous strategy in skin drug delivery.

2. Materials and Methods

2.1. Materials and Reagents

For the synthesis of the ILs, choline hydroxide in methanol [Cho][OH]/MeOH 45%, methanol, Amberlite® IRA-400 chloride form, 1-bromoethane, and glycine from Sigma–Aldrich (Saint Louis, MO, USA) were used as well as 1-methylimidazole and acetonitrile from VWR (Fontenay-sous-Bois, France) and sodium hydroxide (NaOH) from PanReact AppliChem (Barcelona, Spain). Rutin was obtained from Fragron (São Paulo, Brazil).

Regarding the cell viability studies, trypsin, penicillin–streptomycin solution, fetal bovine serum, dimethyl sulfoxide (DMSO), and thiazolyl blue tetrazolium bromide (MTT) were acquired from Sigma–Aldrich (Saint Louis, MO, USA) and Dulbecco's modified Eagle's medium (DMEM) was purchased by Biowest (Nuaillé, France).

For the preparation of transfersomes, methanol was from Carlo Erba Reagenti SpA (Rodano, Italy), chloroform from Sigma–Aldrich Chemie Gmbh (Munich, Germany), Tween® 80 from Sigma–Aldrich (Saint Louis, MO, USA), and soy phosphatidylcholine from Alfa Aesar (Kandel, Germany). For the release study, phosphate-buffered saline (PBS, pH 7.4) was prepared as previously described [54].

2.2. Synthesis of ILs

In this work, the studied ILs were 1-ethyl-3-methylimidazolium bromide [Emim][Br], 2-hydroxyethyl-trimethylammonium glycinate [Cho][Gly], and 1-ethyl-3-methylimidazolium glycinate [Emim][Gly], which were synthesized and characterized within the scope of another recently published study from our group [2]. Briefly, for [Emim][Br] 135 mmol of 1-bromoethane was added, drop by drop, to 45 mmol of 1-mehtylimidazol. This blend remained overnight under stirring at room temperature and was then evaporated under vacuum. For [Cho][Gly], 57.79 mmol of glycine, solubilized in water, was added to 57.76 mmol of the evaporated choline hydroxide. This mixture was stirred overnight, the solvent was evaporated and then an acetonitrile:methanol (9:1) mixture was added. The blend was centrifuged (at 1500 rpm for 30 min) and after filtration, the solvent was evaporated. Concerning the [Emim][Gly], first the [Emim][OH] was prepared by ion exchange chromatography using 10.47 mmol of [Emim][Br] as described in the literature [2]. The obtained combined fractions were added drop by drop to an aqueous solution of glycine in molar excess. The mixture was stirred overnight in an ice bath, then evaporated under vacuum and the unreacted amino acid precipitated with acetonitrile was removed by centrifugation at 450× *g* followed by filtration. The solvent was removed by evaporation.

After being synthesized, all ILs were stored under moisture-free conditions.

2.3. Cell Viability Study

The cell viability was performed in human keratinocytes (HaCaT). Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. HaCaT cells were maintained at 37 °C under a humidified air atmosphere containing 5% of CO_2 in air.

Approximately 6×10^3 cells were seeded per well in 96-well plates in 200 µL of culture medium and incubated for 24 h. Afterwards, HaCaT cells were exposed to the aqueous solutions containing each IL (0.1% or 0.2% *v*/*v*) and the IL combinations (99.8:0.1:0.1% *v*/*v*), for a 24 h period. The cell viability was assessed by MTT reduction assay, according to previously described protocols [55,56]. Absorbance values for the untreated control cells correspond to 100% of cell viability. For this assay, three independent experiments were performed, and four replicate cultures were used in each experiment.

2.4. Solubility Studies

The solubility studies were performed as previously described in the literature [2]. Briefly, rutin saturated solutions were prepared in water, water:IL mixtures (99.9:0.1 or 99.8:0.2% *w*/*w*) or water:IL:IL combinations (99.8:0.1:0.1% *w*/*w*). The studied ILs were [Emim][Br], [Cho][Gly], and [Emim][Gly] and their combinations [Cho][Gly]:[Emim][Br] and [Cho][Gly]:[Emim][Gly].

All the solutions were prepared in triplicate and stirred on a horizontal shaker (IKA VIBRAX VXR®, LTF Labortechnik GmbH & Co., Bodensee, Germany) for 72 h at 25 ± 2 °C. Then, to determine drug solubility, the samples were filtered and analyzed using an UV-Visible spectrophotometer (Evolution® 300, Thermo Scientific, Hertfordshire, UK) at 353 nm (the maximum absorption wavelength for rutin in water).

2.5. Transfersomes Preparation

The transfersomes were produced by the thin-film hydration method followed by sonication as previously described with some modifications [39]. Briefly, soy phosphatidylcholine and Tween® 80 were dissolved in chloroform:methanol (3:1, *v*/*v*). The mixture was placed in a rotary evaporator at 40 °C for 45 min to evaporate the organic solvents and remained under vacuum to remove their traces, forming the EA:lipid films. Then, they were hydrated with a rutin solution in water, in water:IL mixtures, or in water:IL:IL combinations, vigorously vortexed, and sonicated at 50% amplitude using a Q125 Sonicator from QSonica Sonicators (Newtown, CT, USA). Finally, the produced transfersomes were allowed to equilibrate at 200 rpm/min for 30 min in a horizontal shaker (IKA VIBRAX VXR®, LTF Labortechnik GmbH & Co., Bodensee, Germany).

2.6. Box–Behnken Factorial Design

The optimization of the rutin-loaded transfersomes was based on a 15-run, 3-factor, 3-level Box–Behnken factorial design (BBD). The independent variables, also called factors, were lipid concentration (X_1), the EA:lipid ratio (X_2), and the sonication time (X_3). Three levels of each factor were tested, as detailed in Table 1 and they were selected according to our preliminary results and literature research [39–41]. The responses or dependent variables were the hydrodynamic diameter, D_h (Y_1), the polydispersity index, PDI (Y_2), the association efficiency, AE (Y_3), and the loading capacity, LC (Y_4). The corresponding desirable criteria were defined for each dependent variable (Table 1).

Table 1. Factors and responses considered in the Box–Behnken factorial design and the corresponding tested levels and defined desirable criteria (respectively).

Factors	**Levels**		
	−1	**0**	**1**
X_1 = Lipid concentration	4	6	8
X_2 = EA:lipid ratio	5:95	10:90	15:85
X_3 = Sonication time	10	15	20
Responses	**Desirability**		
	Low	**Medium**	**High**
Y_1 = Hydrodynamic diameter, D_h	120	110	100
Y_2 = Polydispersity index, PDI	0.3	0.25	0.2
Y_3 = Association efficiency, AE	70	85	100
Y_4 = Loading Capacity, LC	0.2	0.35	0.5

The obtained results were analyzed in STATISTICA® software (Statsoft, Inc., Tulsa, OK, USA) to predict the optimum levels of factors to produce the optimized formulation. To validate the experimental design, three replicates of the optimized formulation were produced and characterized to compare the experimental responses with the theoretical values predicted by the BBD.

2.7. Physicochemical Characterization of the Transfersomes

The produced formulations were characterized by the analysis of the hydrodynamic diameter (D_h) and the polydispersity index (PDI) using Delsa™ Nano C from Beckman Coulter, Inc. (Brea, CA, USA), after their dilution (50×) with bidistilled water. The zeta potential (ZP) was also measured after diluting the sample 25× in bidistilled water, using a ZetaPALS/ZetaPotential Analyzer (Brookhaven Instruments, Holtsville, NY, USA). All samples were analyzed in triplicate at 23 ± 2 °C.

The AE and LC were also evaluated using an indirect method. After a dilution of 1:10 (*v*/*v*), 500 µL of each sample was placed in a VIVASPIN® 500 centrifuge tube (Sartorius, Goettingen, Germany), prior to centrifugation at 12,000× *g* for 30 min in a Hermle Z 32 HK centrifuge, from Hermle LaborTechnik (Wehingen, Germany). The supernatant was then diluted in an ethanol:water mixture (8.5:1.5) and the non-loaded fraction of rutin was quantified using an UV–Visible spectrophotometer (Evolution® 300, Thermo Scientific, Hertfordshire, England), at its maximum absorption wavelength (353 nm).

The percentage of AE (1) and LC (2) was calculated using the following equations:

$$\%AE = \frac{Total\ (rutin) - Non - loaded\ (rutin)}{Total\ (rutin)} \times 100 \quad (1)$$

$$\%LC = \frac{Total\ (rutin) - Non - loaded\ (rutin)}{Total\ (lipid)} \times 100 \quad (2)$$

2.8. In Vitro Release Studies

In vitro release studies were performed according to a dialysis bag diffusion method. The transfersomal formulations (1.5 mL) were incorporated in a dialysis bag (CelluSep® H1 with a nominal molecular weight cutoff of 2000 Da from Uptima® of Interchim, Montluçon, France). Then, to simulate the physiological conditions, the systems were placed in the PBS buffer at 37 ± 2 °C under stirring. An aliquot of the external medium was collected and immediately replaced by the same volume of PBS at various time intervals. Sink conditions were kept throughout the study. To quantify the released amount of rutin at each time point, UV–Visible spectrophotometry (Evolution® 300, Thermo Scientific, Hertfordshire, UK) was used at the drug's maximum absorption wavelength (353 nm). The release profiles were obtained by considering the cumulative amount of rutin released, in percentage, versus time.

2.9. Preliminary Stability Studies

Immediately after preparation, all produced transfersomal formulations were stored in refrigerated conditions (5 ± 2 °C) for 90 days. To evaluate the storage stability, the transfersomes were submitted to D_h and PDI analyses after preparation and at days 15, 30, 45, 60, and 90 as described in Section 2.7.

2.10. Statistical Analysis

The results are expressed as the mean ± standard deviation (SD). After conducting normality and homogeneity tests, data from solubility studies were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The following results from other studies were evaluated by two-way ANOVA, followed by Bonferroni post hoc test. The differences between individual means were significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. The analyses were performed using the SPSS® statistical package (version 25, SPSS Inc. Chicago, IL, USA).

3. Results and Discussion

Although transfersomes present many advantages, such as being biocompatible, suitable for delivering both lipophilic and hydrophilic drugs, and being deformable, these systems may still be improved, namely, in terms of their physicochemical properties and

their storage stability. Hence, transfersomal formulations containing rutin (Figure 1A) were prepared in the absence and in the presence of ILs to evaluate the impact of these materials on the physicochemical properties of the vesicular systems. The studied ILs (Figure 1B) were 1-ethyl-3-methylimidazolium bromide [Emim][Br], (2-hydroxyethyl)trimethylammonium glycinate [Cho][Gly], 1-ethyl-3-methylimidazolium glycinate [Emim][Gly], and their combinations. These ILs were chosen because they have shown promising characteristics for use in topical drug delivery such as drug solubility and loading enhancement [2].

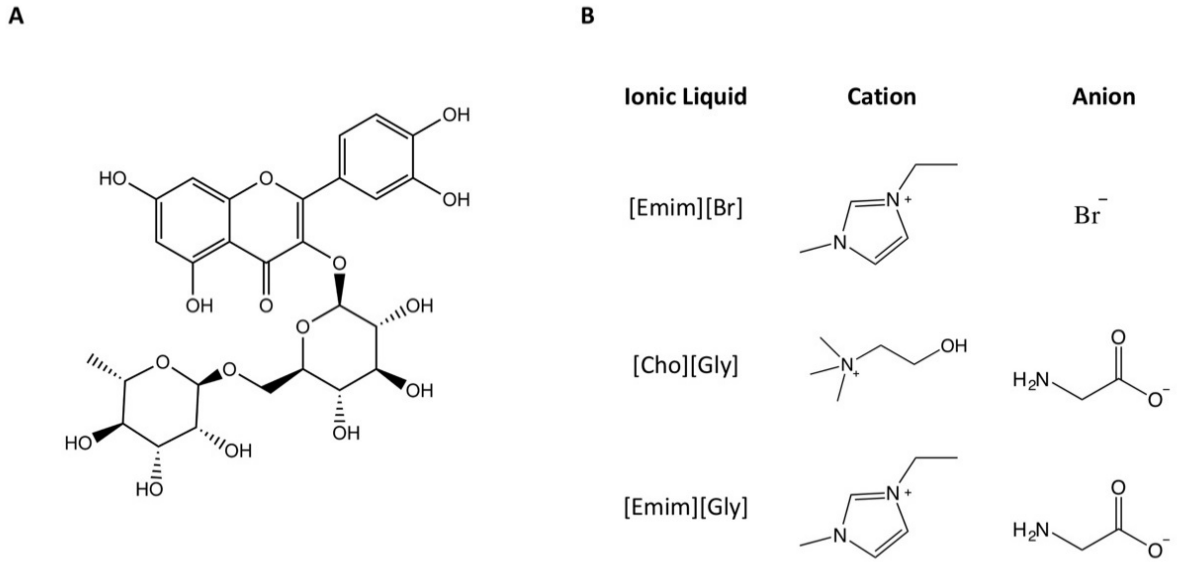

Figure 1. Chemical structures of rutin (**A**) and the studied ionic liquids (**B**).

3.1. Viability and Solubility Studies

It is known that [Emim][Br], [Cho][Gly], and [Emim][Gly] improve the stability of O/W emulsions containing rutin, and that [Cho][Gly] and [Emim][Gly] improve the aqueous solubility of this and other phenolic compounds [2]. Thus, incorporating these ILs alone and their combinations into transfersomes containing rutin may be key, and to the best of our knowledge, this is the first time that this approach has been evaluated.

To achieve this, the ILs should be included at non-toxic concentrations and, thus, initially, the MTT assay was used to evaluate the impact of each IL and of each combination of ILs on the viability of HaCaT cells (0–0.2% v/v; 24 h). The combinations of ILs studied were [Cho][Gly]:[Emim][Br] and [Cho][Gly]:[Emim][Gly] (both at 0.1:0.1% v/v). The impact of these combinations on HaCaT cells' viability was studied herein for the first time. Our present results (Table 2) confirm the previously published data concerning the impact of the ILs alone in this cellular model [2] and reveal that the studied combinations of ILs also maintain the cell viability of HaCaT cells (above 90% for all ILs). Thus, this indicates that the evaluated concentrations may be safely incorporated into the transfersomal systems.

Following this, the impact of the combination of ILs on the aqueous solubility of rutin needed to be assessed. To understand this impact, the solubility of rutin was studied in several solutions. Namely, in water alone, in water:IL mixtures, and in the water:IL:IL combinations (Table 2). Our results showed that the solubility in water and in water:[Emim][Br], water:[Cho][Gly], and water:[Emim][Gly] are all in agreement with the literature [2]. Concerning the impact of the combinations of ILs on the drug solubility, our results unveiled for the first time that both [Cho][Gly]:[Emim][Br] and [Cho][Gly]:[Emim][Gly] combinations enhanced the solubility of rutin, although this enhancement was slightly lower when compared to [Cho][Gly] or [Emim][Gly] alone. The observed enhancement in drug aqueous solubility may be due to the hydrotropic character of the studied ILs, as it has been shown for other imidazolium- and cholinium-based ILs [20,57–59]. For instance, Sintra et al. [57] have shown that a mechanism based in drug-IL aggregation is behind the solubility en-

hancement observed for ibuprofen, another poorly water-soluble drug. Moreover, it has also been reported that ILs containing amino acids play a key role in hydrotropic solubilization [60]. Additionally, the results also showed that the [Cho][Gly]:[Emim][Gly] blend led to a higher increase, proving to be a more successful strategy to improve rutin's solubility. This result is somewhat expected, since individually [Emim][Br] did not impact the solubility of rutin (Table 2). Consequently, a combination of ILs that includes [Emim][Br] could be expected to be less efficient in terms of solubility. Still, since imidazolium-based ILs are known to impact other properties, such as skin permeation [13,18,61], their inclusion (as well as the inclusion of different combinations of ILs) into the transfersomes may still be relevant to upgrade other functionalities, because different cations and anions may affect distinct properties.

Table 2. Cell viability of HaCaT cells exposed to [Emim][Br], [Cho][Gly], [Emim][Gly] (0.1 or 0.2% *v*/*v*; 24 h), and [Cho][Gly]:[Emim][Br] and [Cho][Gly]:[Emim][Gly] (0.1:01% *v*/*v*, 24 h) evaluated by MTT assay (n = 3, mean ± SD, expressed as percentages of the non-treated control cells). Results from rutin's solubility studies at 25 ± 2 °C in water, in water:IL mixtures, and in water:IL:IL combinations are also presented (n = 3, mean ± SD, ** $p < 0.01$, *** $p < 0.001$).

Solvent	Ionic Liquid (%)	Cell Viability (%)	Rutin Solubility (mg/mL)
Water	0	100.0	0.21 ± 0.05
Water:[Emim][Br]	0.1	98.7 ± 3.3	0.21 ± 0.09
	0.2	94.4 ± 4.6	0.22 ± 0.05
Water:[Cho][Gly]	0.1	99.6 ± 5.1	0.84 ± 0.04 **
	0.2	97.1 ± 5.7	1.50 ± 0.08 ***
Water:[Emim][Gly]	0.1	99.3 ± 5.3	0.99 ± 0.04 **
	0.2	93.6 ± 6.9	1.60 ± 0.06 ***
Water:[Cho][Gly]: [Emim][Br]	0.1:0.1	94.0 ± 5.8	0.79 ± 0.03 **
Water:[Cho][Gly]: [Emim][Gly]	0.1:0.1	92.0 ± 5.7	0.92 ± 0.07 **

After studying the impact of the ILs and of their combinations on cell viability and on rutin's solubility, we started by optimizing a transfersomal system to load rutin in the absence of ILs, using a Box–Behnken factorial design (BBD).

3.2. Optimization of Rutin-Loaded Transfersomes: Box–Behnken Design

The development of transfersomes to load rutin was based on a 15-run, 3-factor, 3-level BBD, meaning that we chose three levels for each selected factor, as described in Table 1, resulting in 15 formulations to be produced according to the STATISTICA® software. After being prepared, each formulation was characterized in terms of the selected responses to be evaluated, namely, hydrodynamic diameter (D_h), polydispersity index (PDI), association efficiency (AE), and loading capacity (LC) as described in the Supplementary Materials (Table S1). Overall, D_h varied between 104 and 116 nm and PDI between 0.25 and 0.28, suggesting that all 15 formulations displayed diameter (D_h < 300 nm) and size distribution homogeneity (PDI < 0.3), which are favorable properties to be successfully applied on the skin [37,38]. Moreover, AE and LC values were satisfactory, varying from 74% to 91% and from 0.21% to 0.44%, respectively. Considering the desirability criteria initially chosen for each response (Table 1), the obtained results indicate that the factors and respective levels initially selected were appropriate.

The regression analysis of the aforementioned results, considering a 95% confidence level, was based on the two-way interactions (linear × quadratic) model, since it was the fitting model yielding the highest R^2 values for each evaluated response: 0.95565 for D_h, 0.94318 for PDI, 0.99287 for AE, and 0.99915 for LC. These values indicate that the

experimental and theoretical values were well correlated and confirm that a cubic regression model is needed to fit the experimental data to ultimately predict the most suitable factor levels for producing the optimized formulation. In order to evaluate the effects of each factor or of their linear or quadratic relationships on the obtained responses, coefficients and *p*-values were calculated from the regression analyses as detailed in the Supplementary Materials (Table S2).

Regarding D_h and PDI, no significant linear or quadratic effects were observed for any factor, in agreement with the narrow interval of D_h and PDI values obtained with the 15 produced formulations. In contrast, both synergistic (i.e., positive coefficient) and antagonistic (i.e., negative coefficient) significant effects were observed for AE and LC. Concerning AE, a positive and linear effect of the lipid concentration was found, showing that the higher the lipid concentration used, the higher the AE of transfersomes. Moreover, a synergistic (i.e., linear and quadratic) effect of sonication time was also observed for AE, since increasing the sonication time also increased AE. In contrast, the interaction effects of lipid concentration and EA:lipid ratio (X_1X_2) as well as of lipid concentration and sonication time (X_1X_3) were antagonistic, showing that the increase of X_1X_2 or X_1X_3 values decreased the obtained values of AE. Regarding LC, an antagonistic, linear, and quadratic effect of lipid concentration was observed, meaning that LC increased by decreasing the lipid concentration. In contrast, the sonication time caused a synergistic effect in a linear and quadratic manner as reported for AE. Finally, it should be noted that only one interaction effect was statistically significant, namely, the linear relationship between lipid concentration and sonication time (X_1X_3), which caused an antagonistic effect.

3D–response surface analyses (Figure 2) were determined to better show the statistically significant effects of two factors (i.e., lipid concentration and sonication time), while maintaining constant (at level 0) the EA:lipid ratio. It is noteworthy that AE values superior to 85% can be obtained when using lipid concentrations higher than 6.5% and sonication times higher than 13 min. In contrast, the highest LC values (>0.45%) can be obtained when using low lipid concentrations (<4%) and high sonication times (>16 min).

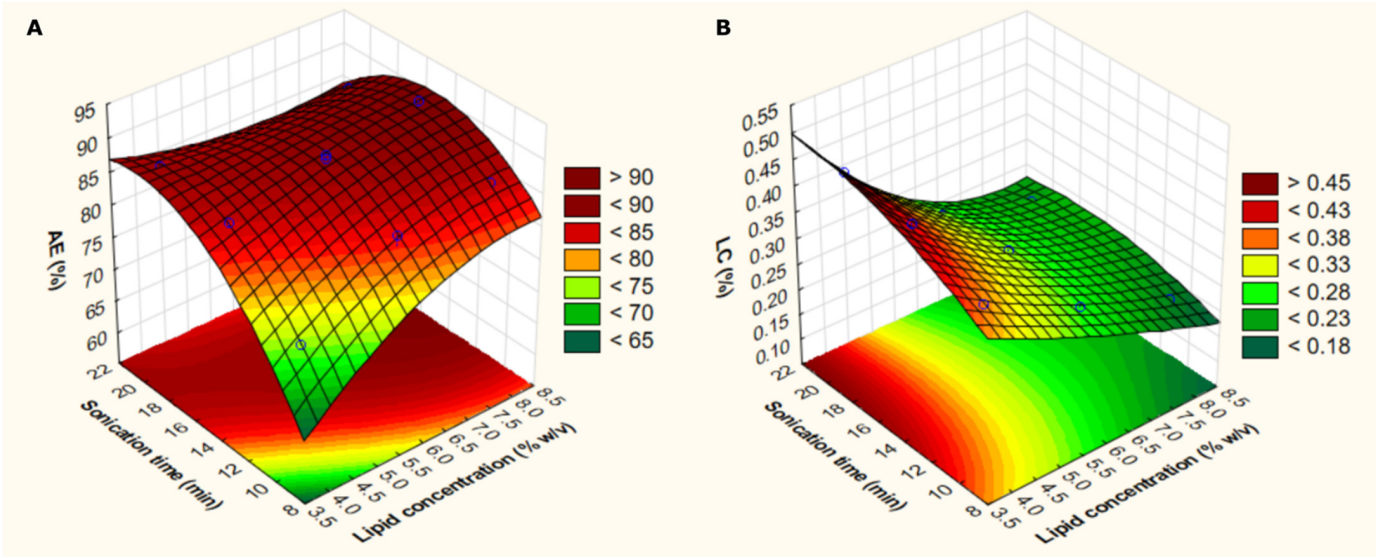

Figure 2. 3D–response surface plots considering lipid concentration and sonication time according to the analyzed response (association efficiency: AE—(**A**); or loading capacity: LC—(**B**)). Light green indicates the lowest response level and dark red indicates the highest response level.

Considering the performed regression analyses and the desirability criteria initially selected (Table 1), the STATISTICA® software was used to calculate the response desirability profile and to predict the most suitable levels of each factor to produce the optimized transfersomes (Table 3). To verify the validity of the prediction ability of the model, the optimized formulation was produced in triplicate, and it was characterized in terms of

D_h, PDI, AE, and LC. The obtained experimental values were further compared with the theoretical values predicted by the model as detailed in Table 3. As the experimental and theoretical data were similar, the implementation of this BBD was a valid quality-by-design approach to optimize rutin-loaded transfersomes.

Table 3. Optimum levels of the selected factors to prepare the optimized formulation and experimental (n = 3, mean ± SD) and theoretical values obtained for the selected responses. The 95% confidence interval (CI) obtained from the theoretical data is also presented.

Optimized Formulation	Response	Experimental Data	Theoretical Data	−95% CI	+95% CI
4:5:95:20 (X_1:X_2:X_3)	D_h	102 ± 3	107.4	95.9	118.8
	PDI	0.26 ± 0.01	0.25	0.22	0.28
	AE	86 ± 2	83.3	77.4	89.2
	LC	0.43 ± 0.01	0.43	0.39	0.46

X_1, lipid concentration (% w/v concentration); X_2, EA:lipid ratio (w/w); X_3, sonication time (min); D_h, hydrodynamic diameter (nm); PDI, polydispersity index; AE, association efficiency (%); LC, loading capacity (%).

By comparing the physicochemical properties of the optimized transfersomes obtained herein with other published studies concerning nanovesicular systems to load rutin [62–66], it is clear that this work is a remarkable breakthrough. This transfersomal formulation displayed low particle size combined with low PDI in contrast with other liposomal [62,63,65] or ethosomal [64] formulations, while maintaining the highest value reported for AE. Since it was already described the lower the vesicle size, the higher the skin penetration [66], these rutin-loaded transfersomes are expected to display enhanced skin permeation than the other nanovesicular formulations developed so far. Moreover, the presence of EA in the formulation may further increase the flexibility of the vesicles, contributing even more to the efficient delivery of rutin upon skin application.

3.3. Development of New TransfersomILs

In this section, new TransfersomILs (transfersomes containing ILs) were developed based on the pre-optimized formulation by incorporating the ILs into the nanodelivery systems. Rutin was loaded into these systems at the maximum amount that it is soluble in water, in each water:IL mixture, or in water:IL:IL combinations. Then, the impact of the ILs or of their combinations on the transfersomal properties was assessed.

3.3.1. Physicochemical Characterization

After preparation, the physicochemical properties of all transfersomes were evaluated, namely, the D_h, PDI, ZP, AE, and LC (Table 4).

For the optimized formulation (containing rutin but without ILs), the obtained D_h was slightly smaller than that of the blank transfersomal formulation produced without rutin nor ILs. Interestingly, all transfersomes containing ILs presented a smaller size, with a D_h ranging between 71–83 nm. This indicates that the incorporation of ILs into the nanovesicular systems may be a worthwhile tactic, by allowing to obtain smaller systems that may facilitate skin penetration as previously discussed. Furthermore, for all the formulations, the PDI values were between 0.22 and 0.26, suggesting that the developed TransfersomILs also displayed a uniform size distribution.

Regarding zeta potential values, they ranged between −30 mV in the absence of ILs and −36 to −41 mV in the presence of ILs. These results indicate that the ILs may be present at the vesicles' interface, contributing to the superficial charge of the particles. The observed tendency of ILs to reduce the ZP for values lower than −30 mV may favor the colloidal stability of the vesicles, avoiding particle aggregation during long-term storage [37].

Concerning the AE and LC, it is within these parameters that a higher difference was observed among the impact of ILs on the transfersomes' properties. For instance, the optimized formulation (without IL) and the nanosystem containing only the [Emim][Br] were the formulations that presented the lowest AE and LC. These results make sense

considering the lower solubility of rutin in water and in the water:[Emim][Br] mixture (Table 2), which may lead to a lower incorporation of the phenolic compound within these transfersomes. In contrast, the TransfersomILs that contain the other studied ILs ([Cho][Gly] and [Emim][Gly]) or the IL:IL combinations ([Cho][Gly]:[Emim][Br] and [Cho][Gly]:[Emim][Gly]), they all presented a higher AE and LC. This is also consistent with the higher impact in drug solubility observed for these ILs (Table 2). Nonetheless, between them, the TransfersomILs containing [Cho][Gly] and [Emim][Gly] seem to be the ones that present the most potential in terms of upgrading the physicochemical properties of the developed transfersomes, since they led not only to a higher AE and LC but also to lower values of D_h and ZP.

Table 4. Physicochemical properties of the produced transfersomes in the absence of rutin and IL, in the presence of rutin without IL, and in the presence of rutin with the ILs alone ([Emim][Br], [Cho][Gly], or [Emim][Gly]), or with IL:IL combinations ([Cho][Gly]:[Emim][Br] or [Cho][Gly]:[Emim][Gly]).

Formulation	Rutin (mg/mL)	IL (%)	D_h (nm)	PDI	ZP (mV)	AE (%)	LC (%)
Water	0	0	111 ± 5	0.22 ± 0.01	-	-	-
Water:[Emim][Br]	0.21	0	102 ± 3	0.26 ± 0.01	−31 ± 3	86.3 ± 2.1	0.43 ± 0.01
Water:[Cho][Gly]	0.22	0.2	83 ± 4 *	0.24 ± 0.02	−36 ± 2	82.1 ± 5.2	0.43 ± 0.01
Water:[Emim][Gly]	1.50	0.2	73 ± 2 **	0.25 ± 0.01	−41 ± 4 *	98.1 ± 0.1 **	3.68 ± 0.01 ***
Water: [Cho][Gly]:[Emim][Br]	1.60	0.2	71 ± 1 **	0.24 ± 0.01	−39 ± 5 *	98.7 ± 0.1 **	3.70 ± 0.02 ***
Water: [Cho][Gly]:[Emim][Gly]	0.79	0.1:0.1	72 ± 1 **	0.24 ± 0.01	−38 ± 3 *	93.6 ± 0.2 *	2.20 ± 0.01 ***
	0.92	0.1:0.1	73 ± 1 **	0.24 ± 0.01	−36 ± 3	97.9 ± 0.1 **	1.76 ± 0.01 ***

IL, ionic liquid; D_h, hydrodynamic diameter; PDI, polydispersity index; ZP, zeta potential; AE, association efficiency; LC, loading capacity. $n = 3$, mean ± SD, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Overall, these results highlight the relevance of developing TransfersomILs, since the presence of ILs within the nanovesicular systems allows a higher drug loading, while leading to a lower particle size, which is an asset for cutaneous applications. Moreover, ILs may also promote the colloidal stability of the vesicles, counteracting one of the main disadvantages of this type of nanosystems.

3.3.2. In Vitro Release of Rutin

Following the physicochemical characterization of the formulations, the in vitro release of rutin from the transfersomes in the absence of ILs (optimized formulation) and in the presence of each IL ([Emim][Br], [Cho][Gly], or [Emim][Gly]) or IL:IL combinations ([Cho][Gly]:[Emim][Br] or [Cho][Gly]:[Emim][Gly]) was also evaluated. The release profiles and the total amount of rutin released in each case are presented in Figure 3.

The release profiles indicate that all transfersomes, in the absence or presence of ILs, were able to release rutin in a controlled manner as previously described for rutin-loaded liposomes [62]. The results showed, once again, a very similar behavior for both the optimized formulation (without IL) and the TransfersomILs containing the [Emim][Br] alone (Figure 3A,B). Both systems led to the lowest total amount of rutin released over 15 h, even though the system allowed the release of 100% of the loaded rutin. On the other hand, the total amount of released rutin from the other TransfersomILs (Figure 3C–F) was higher compared to the optimized formulation and consistent with the solubility studies (Table 2) and the AE and LC results (Table 4). Despite the fact that the later TransfersomILs were not able to release more than 50–60% of the loaded rutin, the inclusion of [Cho][Gly], [Emim][Gly], or their combination into the nanosystem led to an increase of 5–6-fold of the total amount of rutin release in 15 h.

These results point out again the relevance of developing nanovesicular systems based on ILs and the relevance of choosing the appropriate IL or combination of ILs to be incorporated. For instance, in terms of drug release, the new TransfersomILs obtained from [Cho][Gly] or [Emim][Gly] were once more the most promising nanosystems.

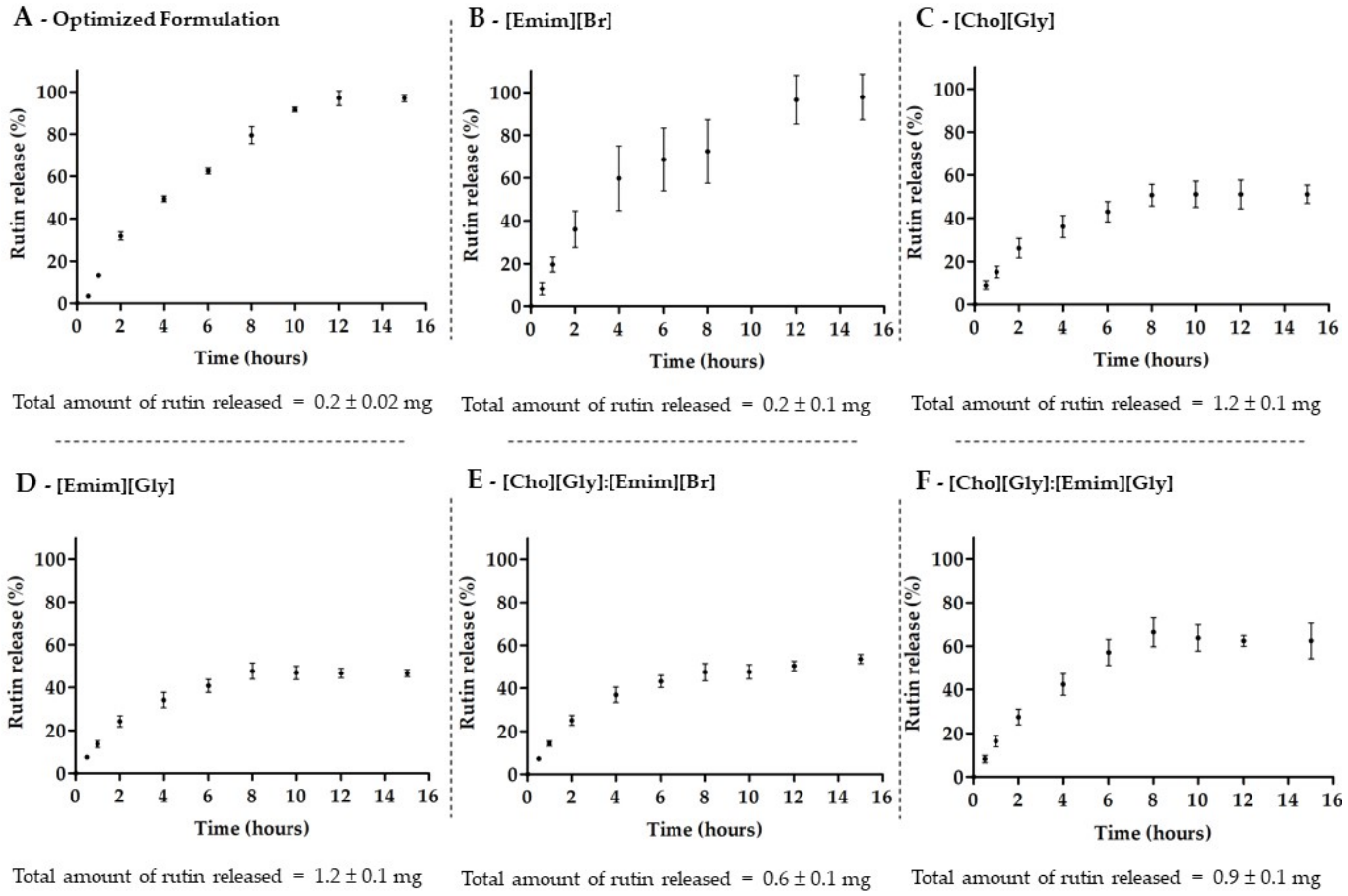

Figure 3. Release profile of rutin from transfersomes, in the absence of ILs (**A**), in the presence of each IL, [Emim][Br] (**B**), [Cho][Gly] (**C**), or [Emim][Gly] (**D**), or of their combinations, [Cho][Gly]:[Emim][Br] (**E**), or [Cho][Gly]:[Emim][Gly] (**F**), during 15 h in phosphate-buffered saline at pH 7.4 (mean ± SD, $n = 3$).

3.3.3. Preliminary Stability Studies

Since poor storage stability is one of the drawbacks that transfersomes may have, stability studies were also performed within the scope of this study to preliminarily assess if the ILs could alter the storage stability of the developed transfersomes.

All the prepared formulations containing rutin and in the absence (optimized formulation) or in the presence of the ILs, or of their combinations, were stored in refrigerated conditions over a period of 90 days. Then, the D_h and PDI of the formulations were evaluated at different time points (15, 30, 45, 60, and 90 days) to assess the impact of the ILs on both parameters, over that period (Figure 4).

When considering the vesicles' diameter, it was possible to observe from our results (Figure 4A) that D_h was sensitive to the storage conditions. Indeed, an increase in D_h was observed for all of the formulations within the 90 days. Nonetheless, it was interesting to note that all formulations containing ILs presented a less pronounced increase in diameter over time. This result is in line with the obtained ZP data (Table 4), as the inclusion of ILs caused an increase in superficial charge, resulting in a less pronounced aggregation phenomena. Among TransfersomILs, [Cho][Gly] was the IL displaying the lowest stabilizing properties in terms of the vesicles' size. In fact, all TransfersomILs containing an IL derived from the imidazole cation allowed the maintenance of the D_h below 300 nm, which is suitable for a cutaneous application [37]. Moreover, these results indicate that the presence of ILs derived from the imidazole cation may be quite relevant to improving the storage stability of the developed TransfersomILs. This may be due to the higher ability of

imidazole cations to intercalate within the lipid bilayer due to enhanced lipophilic characteristics in comparison with choline cations, mainly provided by the side chain present in the imidazole cation.

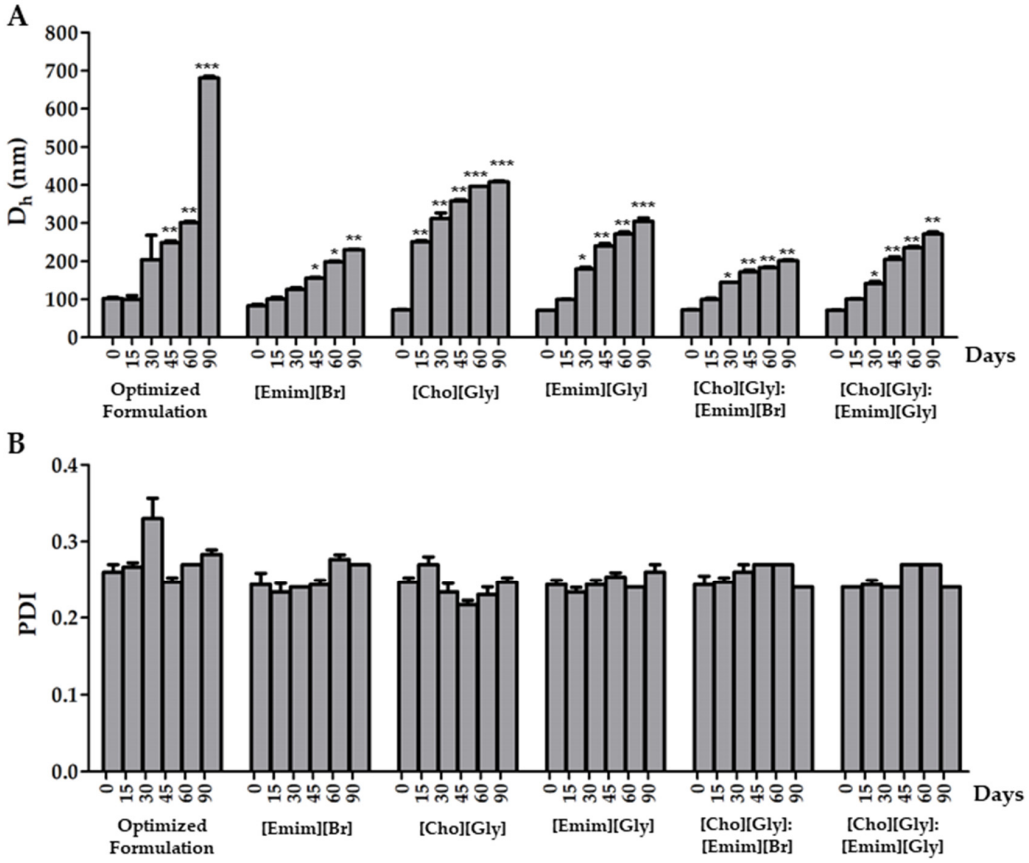

Figure 4. Variation of the hydrodynamic diameter (D_h) (**A**) and the polydispersity index (PDI) (**B**) of rutin-loaded transfersomes in the absence (optimized formulation) or in the presence of each IL, or of their combinations, upon refrigerated (5 ± 2 °C) storage for 90 days. Mean ± SD, $n = 3$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

In terms of PDI, the results were mostly below 0.3 and with no statistically significant differences between all of them (Figure 4B). This shows that for the developed transfersomes, the uniformity of size distribution was not affected upon storage within the studied period of time.

Bearing all the data from this study in mind, when considering the physicochemical properties (i.e., D_h, PDI, ZP, AE, and LC), of the newly developed TransfersomILs, the formulations containing [Cho][Gly], [Emim][Gly] or the [Cho][Gly]:[Emim][Br] and [Cho][Gly]:[Emim][Gly] combinations all led to improvements when compared to the optimized formulation without IL. Nonetheless, for the IL:IL combinations, the observed improvement was less pronounced than that obtained for [Cho][Gly] or [Emim][Gly] alone. Thus, the costs associated with using two ILs may not justify using these combinations. Finally, although both formulations containing either [Cho][Gly] or [Emim][Gly] showed promising results, the preliminary stability studies seem to suggest that opting for the [Emim][Gly] may be an even superior strategy to produce upgraded transfersomes based on ILs.

The ability of ILs to upgrade skin delivery systems has been shown in other formulations such as emulsions [2,67], microemulsions [68,69], bacterial nanocellulose membranes [70], and polymeric nanoparticles [28]. In comparison with these formulations, the developed TransfersomILs may represent a further breakthrough for transdermal delivery, since (a) transfersomal systems are highly deformable and may thus stand out by

presenting remarkable skin penetration abilities, and (b) the presence of ILs improve the physicochemical properties and the colloidal stability of the nanovesicular systems.

4. Conclusions

In the present work, new TransfersomILs (transfersomes containing ILs) to load rutin were developed and their physicochemical properties were evaluated to ultimately assess the impact of ILs' incorporation on the nanosystem's performance.

The impact of the studied ILs, namely, [Emim][Br], [Cho][Gly], [Emim][Gly] and of the studied combinations [Cho][Gly]:[Emim][Br] and [Cho][Gly]:[Emim][Gly] on the cell viability of HaCaT cells and on the solubility of rutin was initially assessed. The results showed that, at the studied concentrations, all the ILs and IL:IL combinations were equally safe to be included in the developed transfersomes. In addition, apart from [Emim][Br] alone, they all led to an increase in drug solubility.

Then, the optimization of a transfersomal system was performed using a Box–Behnken factorial design (BBD). This quality-by-design approach was essential for reducing the development costs of the formulation and was found to be a robust predictive model as shown by the similarity observed between the predicted data and the corresponding obtained experimental properties. Overall, BBD proved to be a valuable strategy to develop rutin-loaded transfersomes with suitable physicochemical properties for cutaneous applications.

Following this, the studied ILs and their combinations were incorporated into the optimized transfersomal formulation. The new TransfersomILs systems showed upgraded physicochemical properties, namely, reduced diameter and increased association efficiency, loading capacity, and total amount of rutin release. Moreover, the presence of ILs also led to an improved colloidal stability, reducing the aggregation phenomena observed during storage. It is noteworthy that, although the majority of the studied ILs improved the overall properties of the nanosystems and they all presented similar cytotoxicity, [Cho][Gly] and [Emim][Gly] were found to be the most promising materials to further enhance the vesicles' performance. They both led to systems with smaller particle size, improved colloidal stability, and higher association efficiency and loading capacity. The only distinguishing factor seems to be their performance in the preliminary stability studies, where the [Emim][Gly] stood out. Hence, this work revealed ionic liquids as a bridge to design new and refined TransfersomILs systems.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nano12010007/s1, Table S1: Description of the rutin-loaded transfersomes produced by Box–Behnken factorial design. Both the factors used to prepare the transfersomes and the obtained responses are presented; Table S2: Results from the regression analyses performed for hydrodynamic diameter (D_h), polydispersity index (PDI), association efficiency (AE), and loading capacity (LC) using the two-way interaction model (linear vs. quadratic) considering the effect of lipid concentration (X_1), EA:lipid ratio (X_2), and sonication time (X_3).

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Supplementary Material

TransfersomILs: From Ionic Liquids to a New Class of Nanovesicular Systems

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Table S1. Description of the rutin-loaded transfersomes produced for Box-Behnken factorial design. Both the factors used to prepare the transfersomes and the obtained responses are presented.

Sample	X_1	X_2	X_3	D_h	PDI	AE	LC
1	4	5:95	15	107 ± 1	0.25 ± 0.01	79.4 ± 1.4	0.40 ± 0.01
2	8	5:95	15	116 ± 1	0.27 ± 0.01	91.3 ± 0.4	0.23 ± 0.01
3	4	15:85	15	112 ± 1	0.27 ± 0.01	85.3 ± 0.4	0.43 ± 0.01
4	8	15:85	15	114 ± 3	0.27 ± 0.01	88.5 ± 0.9	0.22 ± 0.01
5	4	10:90	10	109 ± 1	0.27 ± 0.01	74.4 ± 0.5	0.37 ± 0.01
6	8	10:90	10	115 ± 2	0.26 ± 0.01	85.5 ± 0.4	0.21 ± 0.01
7	4	10:90	20	113 ± 1	0.28 ± 0.01	87.1 ± 0.4	0.44 ± 0.01
8	8	10:90	20	115 ± 3	0.28 ± 0.01	86.9 ± 0.8	0.23 ± 0.02
9	6	5:95	10	104 ± 2	0.26 ± 0.01	77.8 ± 1.9	0.26 ± 0.01
10	6	15:85	10	110 ± 2	0.26 ± 0.01	83.9 ± 1.0	0.28 ± 0.01
11	6	5:95	20	110 ± 1	0.26 ± 0.01	85.4 ± 0.1	0.28 ± 0.01
12	6	15:85	20	116 ± 2	0.27 ± 0.01	84.4 ± 0.9	0.28 ± 0.01
13	6	10:90	15	112 ± 1	0.27 ± 0.01	89.2 ± 0.7	0.30 ± 0.01
14	6	10:90	15	114 ± 1	0.28 ± 0.01	87.2 ± 0.5	0.29 ± 0.01
15	6	10:90	15	116 ± 3	0.28 ± 0.01	88.6 ± 0.7	0.30 ± 0.01

X_1, lipid concentration (% w/v); X_2, EA:lipid ratio (w/w); X_3, sonication time (min); D_h, hydrodynamic diameter (nm); PDI, polydispersity index; AE, association efficiency (%); LC, loading capacity (%).

Table S2. Results from the regression analyses performed for hydrodynamic diameter (D_h), polydispersity index (PDI), association efficiency (AE), and loading capacity (LC) using the two-way interaction model (linear vs quadratic) considering the effect of lipid concentration (X_1), EA:lipid ratio (X_2), and sonication time (X_3).

Factor	D_h		PDI		AE		LC	
	Coef.	***p***	**Coef.**	***p***	**Coef.**	***p***	**Coef.**	***p***
Intercept	**111.8**	**0.00002**	**0.27**	**0.00004**	**84.1**	**0.00001**	**0.3**	**0.00003**
X_1	5	0.079	0.005	0.365	**6.92**	**0.012**	**-0.19**	**0.0005**
X_1^2	-0.63	0.609	0.0008	0.808	0.83	0.261	**-0.03**	**0.01**
X_2	3	0.181	0.008	0.192	1.95	0.126	0.01	0.146
X_2^2	2.38	0.150	0.01	0.069	1.43	0.116	0.007	0.142
X_3	3.33	0.155	0.01	0.113	**6.05**	**0.016**	**0.03**	**0.016**
X_3^2	1.63	0.259	0.003	0.383	**4.03**	**0.017**	**0.02**	**0.04**
X_1X_2	-3.5	0.222	-0.01	0.225	**-4.45**	**0.049**	-0.02	0.074
$X_1X_2^2$	-0.75	0.649	-0.008	0.208	-1.1	0.269	0.003	0.602
$X_1^2X_2$	2.25	0.253	-0.003	0.602	0.45	0.598	$4x10^{-18}$	1
X_1X_3	-2	0.423	0.005	0.478	**-5.65**	**0.031**	**-0.03**	**0.049**
$X_1^2X_3$	2	0.293	-0.005	0.345	-1.5	0.175	-0.02	0.050
X_2X_3	0	1	0.005	0.478	-3.55	0.074	-0.01	0.225

Statistically significant effects ($p < 0.05$) are highlighted in bold.

Chapter 6

Final Remarks

The development of more efficient delivery systems is one of the targets of the pharmaceutical industry. In this context, the interest in sustained release formulations has grown due to the many advantages associated with these particular systems, such as reduced side effects and lower frequency of administration, that may lead to improved patient compliance. Nonetheless, this goal faces consecutive challenges such as possible high production costs, inflexible drug release profiles, poor drug solubility and stability problems of the developed systems. As a consequence, finding new strategies and/or materials to develop innovative and more efficient formulations is part of this journey and in this context, ionic liquids may represent a pertinent approach. Thus, this thesis aimed to investigate the applicability of ILs in the development of innovative sustained delivery systems, specifically IL-polymeric nanoparticles (**Chapter 3**), IL-lipidic implants (**Chapter 4**), and TransfersomILs (**Chapter 5**).

For all the developed systems, the first step was to optimize the preparation procedures, namely, in the attempt to obtain less time-consuming formulation procedures and/or to reduce the production costs. Then, the goal was to prepare new sustained release formulations containing ILs and assess how these materials impacted the developed formulations.

For the IL-polymeric nanoparticle systems and for the lipidic implants, the prepared and incorporated biobased ILs were [Cho][Phe] and [Cho][Glu]. For the TransfersomILs systems, the studied ILs were [Emim][Br], [Cho][Gly], [Emim][Gly] and the ILs combinations [Cho][Gly]:[Emim][Br] and [Cho][Gly]:[Emim][Gly]. All the studied ILs were included in the systems at concentrations at which the cell viability of HaCaT cells was maintained.

For all the developed formulations, the results showed that the ILs led to a considerable improvement in the physicochemical properties and in the performance of the formulations. For instance, the ILs led to the improvement in drug incorporation for all the three types of developed systems, either by allowing a better blend of the components within the formulation (lipidic implants, **Chapter 4**) or by enhancing the drug loading (for both IL-nanosystems, **Chapters 3** and **5**).

Additionally, it was possible to observe several similarities in terms of the impact of the ILs on the performance of the two types of IL-nanosystems (IL-polymeric nanosystems or TransfersomILs, both containing rutin). Namely, the ILs allowed to reduce the particle size, improve the colloidal stability of the systems, and increase the association efficiency (AE).

In particular for the IL-polymeric nanosystems (**Chapter 3**), even though for all the formulations both ILs [Cho][Phe] and [Cho][Glu] improved the physicochemical properties of the systems, [Cho][Phe] led to the best performance (higher AE). FTIR results indicated that rutin was efficiently encapsulated into the hybrid systems. Also, upon freeze-drying, with or without the classical lyoprotectant trehalose, no particle aggregation was observed. This suggested that ILs may contribute to decrease the stress effect of the lyophilization process on the hybrid nanocarriers and thus unveils another possible functionally of ILs. Moreover, from DSC analysis, it was possible to observe a less marked PLGA endothermic peak, which suggests that the ILs may stabilize the formulations, and SEM results revealed that the presence of ILs did not alter the morphologic characteristics of PLGA nanocarriers. Also, since permeation studies showed no relevant skin permeability and the cytotoxicity studies, performed in HaCaT cells, indicated that the IL-sustained systems were biocompatible, these results suggested that these hybrid IL-polymeric nanosystems may be appropriate for topical application.

Concerning the IL-lipidic implants (**Chapter 4**) since these systems are quite different from the IL-nanosystems, other major impacts were observed. For these systems, different materials were incorporated into the developed implants, namely, the ILs and/or Gelucire® or sucrose. In this study, a total of 54 batches were prepared. The prepared implants showed homogeneous dye content distribution (both with a lipophilic and a hydrophilic dye) and uniform drug content. Considering the incorporated materials, Gelucire® and sucrose did not lead to an appropriate drug release profile of caffeine (hydrophilic) or of salicylic acid or rutin (both lipophilic), contrasting with the results in the presence of the ILs, where a higher and/or more suitable release profile was observed. The ILs also altered the surface properties of the implants by increasing the surface

wrinkling, which along with drug diffusion, seemed to be the factors that led to the observed increase in the drug release, particularly in the presence of [Cho][Glu].

Results up to this point also revealed that the type of IL that gives the better outcomes depends on the type of system in which they are included. This reinforces the importance of studying the applicability of these materials in different formulations to reveal which is more suited for each system.

Returning to the transferomILs systems (**Chapter 5**), performing a Box-Behnken factorial design (BBD) proved to be key to develop systems with physicochemical properties appropriate for cutaneous delivery. In this study, it was also assessed the influence of the IL:IL combinations on the viability of HaCaT cells, as well as, on the solubility of rutin and on the impact of their incorporation into transfersomes. Nonetheless, the results showed that even though these combinations also increased the solubility of rutin and improved the formulations' features, they do not surpass the outcomes obtained when using the [Cho][Gly] or [Emim][Gly] alone. In terms of cytotoxicity, the ILs [Emim][Br], [Cho][Gly] and [Emim][Gly] alone, as well as the IL:IL combinations presented similar results. This study also showed that the [Cho][Gly], [Emim][Gly] as well as the [Cho][Gly]:[Emim][Br] and the [Cho][Gly]:[Emim][Gly] combinations, all generated a higher total amount of rutin released. The ILs alone and the studied IL:IL combinations also allowed to reduce the aggregation phenomena during storage. Even though [Cho][Gly] and [Emim][Gly] led to the better outcomes in terms of upgrading the performance of the transfersomes, [Emim][Gly] stood out in the preliminary stability studies. Finally, in contrast with the IL-polymeric nanosystems, the transfersomILs may be more suited for drug delivery across the skin, due to their higher elasticity and deformability.

In conclusion, herein the multifunctionality of ILs proved to be a pertinent strategy to attain different innovative sustained release formulations and overcome some of the drawbacks associated with these systems. Namely, this PhD research showed that ILs may contribute to refine the formulation procedures, increase drug loading, allow to obtain more suitable drug

release profiles, stabilize formulations upon different conditions, alter surface characteristics, and improve several other characteristics of the developed systems. Thus, ILs were successfully disclosed as multitalented materials to upgrade sustained drug delivery systems.

The obtained results reinforce the relevance of continuing to study the applicability of ILs in drug delivery, particularly in sustained delivery systems since these formulations are less explored when compared to the incorporation of ILs in other types of delivery systems.