Faculdade de Farmácia, Universidade de Lisboa
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Abstract

Introduction & Aims: Nanotechnology is a new trend in cosmetology and lipid nanoparticles have shown higher degree of biocompatibility and versatility in this field compared to other systems. The aim of this research project was to evaluate the influence of three different systems containing lipid nanoparticles previously well characterized on skin hydration and transepidermal water loss (TEWL).

Methods: Several formulations of lipid nanoparticles based systems (solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) with Dynasan® 114 (D114) or Glycerol monostearate (GMS) and nanoemulsions (NE)) were fully characterized (particle size, polydispersity index and zeta potential), and then incorporated into hydrogel form to study different skin parameters such as skin hydration and TEWL on forearms of six human volunteers.

Results: The capacitance basal values of human volunteers before application of any hydrogel containing lipid nanoparticles were 31,48 (Control), 31,73 (SLN), 30,98 (NE), 30,42 (NLC^{D114}) and 32,07 a.u. (NLC^{GMS}). The TEWL basal values were 7,70 (Control), 7,55 (SLN), 7,72 (NE), 7,50 (NLC^{D114}) and 7,67 $g/h/m^2$ (NLC^{GMS}). One hour after hydrogels` application, the capacitance values measured were 40,07 (Control), 43,20 (SLN), 41,22 (NE), 41,15 (NLC^{D114}) and 44,15 a.u. (NLC^{GMS}). At the same time, the TEWL values obtained were 6,58 (Control), 4,67 (SLN), 4,13 (NE), 3,90 (NLC^{D114}) and 4,22 $g/h/m^2$ (NLC^{GMS}).

Conclusion: The present study showed an increase in skin hydration after exposure to different systems of lipid nanoparticles (even not statistically significant). On the other hand, there was a statistically significant decrease in TEWL after that exposure, compared to the control. In this way, nanolipid systems, i.e. solid lipid nanoparticles, nanostructured lipid carriers and nanoemulsions, are promising systems to improve skin's biophysical parameters in cosmetodermatology.

Keywords: solid lipid nanoparticles, nanostructured lipid carriers, nanoemulsions, transepidermal water loss, skin hydration

Resumo

Introdução & Objetivos: A nanotecnologia é uma nova tendência no ramo da cosmetologia e as nanopartículas lipídicas têm demonstrado um maior grau de biocompatibilidade e versatilidade nesta área, comparando com outros sistemas já bem conhecidos. Este projeto de investigação teve como objetivo principal a avaliação da influência de três sistemas diferentes de nanopartículas lipídicas previamente bem caracterizados ao nível de parâmetros biofísicos da pele, nomeadamente hidratação da pele e perda transepidérmica de água.

Métodos: Diversas formulações de sistemas baseados em nanopartículas lipídicas (nanopartículas lípidicas sólidas (SLN), vetores lipídicos nanoestruturados (NLC) formulados com Dynasan® (D114) ou Monoestearato de glicerol (GMS) e nanoemulsões (NE)) foram rigorosamente caracterizadas (nomeadamente o tamanho das partículas, índice de polidispersão e potencial zeta), sendo depois incorporadas na forma de hidrogel para ser avaliada a influência destes nanosistemas lipídicos em diferentes parâmetros biofísicos da pele, como a hidratação da pele e a perda transepidérmica de água nos antebraços de seis voluntários.

Resultados: Os valores basais de capacitância nos voluntários antes da aplicação de qualquer hidrogel contendo nanopartículas foram 31,48 (controlo), 31,73 (SLN), 30,98 (NE), 30,42 (NLC^{D114}) e 32,07 a.u. (NLC^{GMS}). Os valores basais da perda transepidérmica de água foram 7,70 (controlo), 7,55 (SLN), 7,72 (NE), 7,50 (NLC^{D114}) e 7,67 g/h/m^2 (NLC^{GMS}). Os valores da capacitância, uma hora após a aplicação dos hidrogeles, foram 40,07 (controlo), 43,2 (SLN), 41,22 (NE), 41,15 (NLC^{D114}) e 44,15 a.u. (NLC^{GMS}) e os valores de perda transepidérmica de água medidos após o mesmo período de tempo foram 6,58 (controlo), 4,67 (SLN), 4,13 (NE), 3,90 (NLC^{D114}) e 4,22 g/h/m^2 (NLC^{GMS}).

Conclusão: O presente estudo demonstrou um aumento na hidratação da pele após exposição a diferentes sistemas de nanopartículas lipídicas, mesmo não tendo sido estatisticamente significativo. Por outro lado, houve uma diminuição estatisticamente significativa na perda transepidérmica de água após essa mesma exposição, comparativamente ao valor registado no hidrogel controlo. Desta forma, os nanosistemas lipídicos (nanopartículas lipídicas sólidas, vetores lipídicos nanoestruturados e nanoemulsões) são sistemas promissores ao nível da melhoria dos parâmetros biofísicos da pele em Cosmética e Dermatologia.

Palavras-chave: nanopartículas lipídicas sólidas, vetores lipídicos nanoestruturados, nanoemulsões, perda transepidérmica de água, hidratação da pele

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At the end of a journey that began five years ago, this thesis represents the final step and simultaneously reflects the work developed in my last adventure as a student and one of the best challenges I faced in my life: Erasmus+ Programme.

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Life begins at the end of your comfort zone – N.D.W.

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List of abbreviations

SLN	Solid lipid nanoparticles
NLC	Nanostructured lipid carriers
NLC^{D114}	Nanostructured lipid carriers formulated with Dynasan® 114
NLC^{GMS}	Nanostructured lipid carriers formulated with Glycerol monostearate
NE	Nanoemulsions
D114	Dynasan® 114
GMS	Glycerol monostearate
PdI	Polydispersity index
ZP	Zeta potential
TEWL	Transepidermal water loss
O/W	Oil/Water

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1. Introduction

1.1. Skin – A physiological barrier

Skin is a viscoelastic organ and the largest organ of the human body constituted by two mutually dependent layers, the epidermis, formed by keratinocytes in different stages of differentiation and divided by different layers - *stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, stratum germinativum* - and dermis (as well as the subcutaneous fat tissue). This organ has several essential functions for human survival, such as defensive (as a physical barrier), immunologic, thermoregulatory, metabolic and sensorial (1).

Stratum corneum (SC) is the most external layer of the epidermis with corneocytes molding a cornified envelope embedded in lipid bilayers, responsible for mechanical resistance and involved in water permeability and exchange of substances with the external environment, and thus contributing for skin barrier function. The last step of keratinocyte differentiation is characterized by the constant replacement of corneocytes leading to a renewable skin process (2). This outermost layer of the skin is also able to prevent invasion of microbes and penetration of chemicals/radiation as well as to protect the body from excessive water loss, sustaining the homeostasis. However, epidermis is not totally impermeable to substances applied on its surface (3).

Some substances can pass through skin surface, more specifically compounds with low molecular mass (approx. 600 Da), lipophilic and uncharged. Taking into account these particularities, it is possible to obtain formulations with controlled drug release, allowing specific pharmacologic effects and avoiding toxicological side effects (4). In fact, dermal delivery has been highlighted among different routes of drug delivery for local and systemic action, and it has been studied for delivery of several types of carriers, including nanoparticles. Noteworthy, skin should be in good conditions regarding topical parameters such as skin hydration, transepidermal water loss (TEWL) and transepidermal flux of carbon dioxide, oxygen and ions (5).

1.2. Nanotechnology in cosmetics – an overview

Nanotechnology is an innovative science involved in the design, synthesis, characterization, and application of structures, materials, devices and systems at the nanometer scale, in the size range from 1 to 1000 nanometers (6). In this field, it is possible to control the macroscopic chemical and physical properties of individual molecules and interacting groups. In the last years, it has been observed an increasing interest in nanoscience and nanotechnology in several fields, including cosmetic and pharmaceutical products (7).

Therapy with nanocarriers has been developed since this drug delivery system allows controlling the drug release, and thus leading to an improvement of pharmacokinetic properties of drugs. For example, lipid nanoparticles have been studied for cancer therapy, bacterial infections and dermatological disorders, due to their own characteristics as resistance to chemical degradation, easy penetration through biological barriers and co-delivery of different active substances among other advantages (8).

Nanotechnology is also a new trend in cosmetology, since nanoparticles present several advantages compared to other systems, including a higher degree of biocompatibility and versatility. Regarding their safety, no adverse effects of lipid nanoparticles on human skin have been described so far (9). In this way, many cosmetic manufacturers already use nanoscale products to provide an improvement on their effectiveness, such as higher UV protection, deeper skin penetration and, consequently, long-lasting effects (10).

The potential that nanocosmetics brings is multifaceted as already referred, leading to an improvement on the current production and characterization techniques with different safety assessments (9). Concerns over the safety of nanoparticles are raised since their properties (smaller size, chemical composition, surface structure, solubility, shape and aggregation) may cause different risks to human life (direct or occupational risk) and also to the environment. In the last years, a large number of *in vitro* and *in vivo* studies using nanoparticles has been performed to prove their safety taking into account relevant toxicological endpoints, like penetration through physiological barriers, cellular uptake and translocation, cell damage or cytotoxicity, induction of cellular stress and mutagenicity/ genotoxicity (10). These studies have been conducted through different routes of

administration (inhalation, oral and dermal absorption), with special attention concerning the entrance through skin, since there is less information about skin permeation (11).

In order to ensure the safety and efficacy of such nanocosmetics, FDA issued a report prepared by its Nanotechnology Task Force (12). The Task Force report, formed in August 2006, presents an assessment of scientific and regulatory deliberations concerning the security and effectiveness of FDA-regulated products that use nanotechnology materials. In the meantime, this report submits recommendations regarding these considerations and encourages the development of innovative, safe, and effective FDA-regulated nanoproducts (13). European Comission has also a guidance on the safety assessment of nanomaterials in cosmetics (14).

1.3. Lipid nanoparticles

Lipid nanoparticles have been investigated for different pharmaceutical applications (parenteral, peroral, dermal, ocular and pulmonary). Since the last decade, a huge interest in lipid nanoparticles for dermal use (in pharmaceutical and cosmetic products) has been clearly emerged (15). Overall, these studies showed that lipid nanoparticles can be useful for topical delivery of drugs and active compounds. Simultaneously, they offer several advantages in this field, such as the enhancement of chemical stability of actives sensitive to light oxidation and hydrolysis as well as the ability to increase the occlusion skin effect (decreasing transepidermal water loss) and improve skin hydration (16).

These lipid-based nanodelivery systems are innovative carriers since they cover all the advantages of other nanometric carriers (like emulsions, liposomes and polymeric nanoparticles) (17). Different types of lipid nanoformulations have been studied so far, as nanoemulsions, solid lipid nanoparticles and, the newest, nanostructured lipid carriers.

1.3.1. Nanoemulsions (NE)

An emulsion is composed by two immiscible phases (water and oil phases) and a surfactant (steric stabilizer) on the interface between them. This surfactant decreases surface tension, stabilizing the emulsion during the emulsification process, and its nature determines the external phase of the emulsion (18). Several types of surfactants, like ionic surfactants and non-ionic surfactants, can be used to stabilize the oil-in-water (O/W) emulsions.

When the surfactant cannot reduce surface tension, the addition of a co-surfactant can play an important role, decreasing the percentage of the surfactant needed to stabilize emulsions. This additive effect of surfactant and co-surfactant is important to avoid potential toxic risks associated with the use of a high amount of the individual surfactant. Although studies have already been developed in this area, there is still limited information regarding the influence of a mixture of both emulsifiers on the reduction of the amount of the surfactant needed (19).

In the last decades, progresses in nanotechnology have led to the development of nanoemulsions, metastable dispersions of droplets (with a diameter from 50 to 1000 nm) of one liquid within another (20). These emulsions can be O/W or W/O, and sometimes they are confused with microemulsions. Microemulsions can be formed by a spontaneous process with low energy since they are thermodynamically stable. However, these systems do not present kinetic stability, and thus a huge concentration of surfactant in formulation would be needed compared to emulsions, which may increase their irritation potential. Nevertheless, nanoemulsions are produced by mechanical shear (high-energy methods) with less concentration of surfactants. Nanoemulsions are also metastable instead of thermodynamically stable (21).

Although microparticles (10^{-6}) are bigger than nanoparticles (10^{-9}), in this case is different as droplets in microemulsions are smaller than in nanoemulsions due to historical development of these formulations (i.e. the term 'microemulsion' was firstly published, and just after three decades the term 'nanoemulsion' appeared) **(Figure 1)** (22).

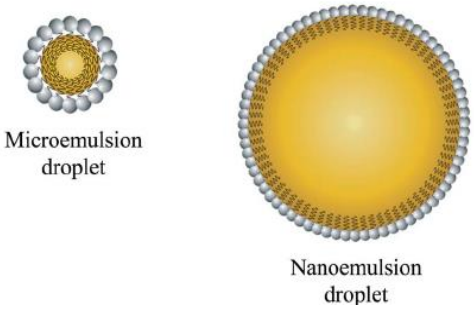

Figure 1 – Microemulsion vs Nanoemulsion (20)

In this work, nanoemulsions were formulated with liquid lipid Miglyol® 812, a mixture of medium-chain triglycerides (Caprylic/Capric Triglyceride) with excellent emolliency and good user properties (23). This compound is able to form reservoir-type drug delivery systems in the liquid oil core, where some drugs insoluble in water can be dissolved with an increased payload (24).

1.3.2. Solid lipid nanoparticles (SLN)

Solid lipid nanoparticles (SLN) were developed to combine the advantages of three other systems: emulsions, liposomes and solid particles. SLN are produced by replacing the liquid lipid (oil) of an O/W emulsion by a solid lipid or a mixture of solid lipids at 37ºC (15).

These systems are produced with excipients generally recognized as safe (GRAS) status for oral and dermal delivery, an important advantage concerning toxicity related with previous formulations among other several benefits (25).

Incorporation of active substances in SLN can protect them from degradation, increasing drug stability (26), and consequently, allowing a target strategy (27). In addition, SLN allow a controlled drug release, since biphasic release profiles were observed in several studies (an initial burst drug release followed by a prolonged release) (28). SLN also incorporate lipophilic and hydrophilic drugs with no need of organic solvents (29). Despite these advantages, SLN have shown several limitations, as drug expulsion during storage, reduced particle concentration and drug loading and high water content of SLN dispersions.

1.3.3. Nanostructured lipid carriers (NLC)

The disadvantages of SLN led to the development of a new generation of nanosystems: nanostructured lipid carriers (NLC) (30). In this second generation, particles are produced not only with solid lipids but also with liquid lipids (oils). To produce these blends of lipids, solid lipids are mixed with liquid lipids (ratio from 70:30 up to 99.9:0.1) (26). There is a melting point depression in NLC, compared with SLN, but the matrix is also solid at body temperature (15). One problem verified with SLN is that during storage, a ‘perfect crystal’ is formed (a matrix totally ordered in conditions of low energy that leads to drug expulsion). In NLC preparation, the use of different molecules gives rise to an imperfect matrix that accommodates the drug in its imperfections. To solve this question, there are three types of NLC (26).

In type I, the highly imperfect type, exists a blend of low liquid lipid (oil) concentration with solid lipid with the formation of a solid particle characterized by an extremely disordered matrix. Type II, known as the multiple type, is processed with a higher liquid lipid concentration, compared to type I, and this increase of lipid concentration leads to phase

separation of both lipids and then, during cooling-down step, to the precipitation of oily nanocompartment and its incorporation into the solid matrix. In this way, this addition of higher oil content prevents drug expulsion. In the amorphous type, type III, as the designation means, an amorphous state is maintained through the control of blend of lipids instead of a perfect crystal with an ordered matrix **(Figure 2)** (30).

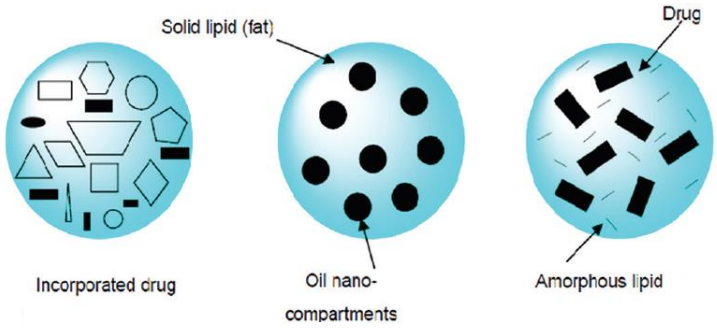

Figure 2 – Different type of NLC. From left to right: Highly imperfect type (I); multiple type (II); amorphous type (III)(28)

1.4. Production and incorporation of lipid nanoparticles into hydrogel

1.4.1. High-energy mechanism

Lipid nanoparticles can be produced through different methods usually with one mutual step: the formation of a nanoemulsion. Regarding SLN, after this step, a successive solidification of the dispersed lipid phase occurs. In this process, the critical stage is nanoemulsion preparation, since it is essential to obtain particles nanosized with a slight polydispersity index (measure of the width of molecular weight distributions) (17).

The solution to avoid high polydispersity index and particle sizes outside the nanoscale, is based on the manipulation of the formed nanoemulsions with strong mechanical forces: high shear homogenization, ultrasonication, high pressure homogenization or microfluidization and membrane emulsification.

High shear homogenization (HSH) and ultrasonication (US) are both common dispersing techniques and easy to handle. In the HSH, the rotor-stator homogenizer is used to break big droplets into small ones since product goes to the center of the stator and is subjected to an intense shear through the gap of the rotor stator, producing a final homogenous mixture. This gap is adjustable for different shear levels and flow rates, depending on the product in cause. In the US exists a succession of mechanical depressions and compression of the system, which causes an implosion sufficiently strong to increase the interfacial area of the droplets (20). Though, some problems, regarding dispersion quality,

were observed when these techniques were used, such as the presence of microparticles and metal contamination in case of HSH and US, respectively. An emulsifying agent (surfactant) is a good solution when the problem is due to the average particle size, since the surfactant allows the formation of nanoemulsion under simple high shear mixing. Another option could be the simultaneous use of different methods to obtain better results, such as two short cycles of HSH and US (to decrease processing intervals and obstacles associated to long preparation times) (31) or different methods used in different steps (for example, one technique to get macroemulsion as a pre-emulsion, and then another one to form nanoscale droplets) (20).

In the nineties, Muller applied a new technique, high pressure homogenization (HPH), which is recognized nowadays as the main and most efficient technique to produce lipid nanoparticles. This method is characterized by different steps in order to increase efficiency and valuable results, and it is subdivided into hot and cold homogenization with dissolution, solubilization or dispersion of the active in the melted lipid (26). Hot homogenization is the most frequently applied technique since temperature sensitive compounds can be processed by this way (exposure time is not so long). However, this technique is not able to incorporate hydrophilic substances as well as extremely temperature sensitive compounds, and to solve this limitation, cold homogenization was developed (32). Hot homogenization involves the dissolution of the drug in the lipid and afterward this lipid should be melted at a specific temperature (5-10ºC above its melting point) (33). After this optimization, this drug dissolved in melted lipid is dispersed in an aqueous surfactant solution at the same temperature to form a pre-emulsion. This pre-emulsion is homogenized with a piston-gap homogenizer and then the lipid re-crystallizes when this O/W nanoemulsion is cooled down (to room temperature). Overall, this procedure leads to lipid nanoparticles production at the end (31). Usually hot homogenization is referred in association with high pressure homogenization since this type of homogenization is on the cutting edge. However, to improve the effectiveness of techniques as HSH and US, the principle of hot homogenization can be applied in these techniques if a pre-emulsion is obtained as described before, and then the homogenization process continues under hot temperature (26, 28). This association can be useful when is not possible to use high pressure homogenization, for example, in the laboratory scale.

This work was performed following the hot homogenization principle since it was produced a pre-emulsion subsequently subjected to high shear homogenization process to obtain lipid nanoparticles.

1.4.2. Particle size, polydispersity index (PdI) and zeta potential analysis

Some properties of particles can play an important role regarding the efficacy of dermal products based on nano-scale particles, such as particle size, polydispersity index (PdI) and zeta potential.

Particle size influences skin penetration since smaller particles are more able to pass through skin. Thus, nanoparticles have suitable topical and dermal delivery (10). In this way, it is essential to measure particle size distributions to understand how this physical property can affect the performance of products with nanoparticles. In addition, it is also important to measure the width of molecular weight distributions (MWD), known as polydispersity index (PdI), which should be at a narrow range (35). Photon correlation spectroscopy is the method used to measure particle size and PdI by detecting the Brownian motion of the particles subjected to dynamic light scattering (DLS). The oscillations intensity of dispersed light arising from Brownian motion is analyzed (smaller particles have faster diffusion than larger particles), and the particle size is obtained using the Stokes-Einstein equation (16). To produce high quality data, it is important to dilute all samples (3) and control the measurement temperature since different temperatures can influence the speed of Brownian motion (36).

On another hand, it is important to measure zeta potential of the particles since this property is also related with physical stability. This stability is due to an electric charge on the particle surface that can repel other particles and avoid particle aggregation/flocculation. This electric charge depends on the medium in which the particles are dispersed and some alterations can decrease the stability. Therefore, it is important to control some formulation parameters such as surfactants, pH and the type/concentration of ions. To keep particle stability, zeta potential values usually should be more negative than -30 mV or more positive than +30 mV (37). Zeta potential measurement is based on electrophoretic mobility of particles, since particles with an electric charge will migrate to an electrode in the presence of an electric field. There is a higher migration speed with stronger field and higher zeta potential (in absolute values) (36).

1.4.3. Nanolipidgel

After the optimization and production of nanoparticles formulations, these dispersions should be incorporated into a suitable dermal carrier with semi-solid consistency for topical application. Due to adverse systemic effects of oral and parenteral formulations, topical treatment has been extensively studied and used, as it offers many benefits: a) first-pass metabolism is avoided; b) it is usually well accepted by patients since it is convenient, easy to use and suitable for self-administration; c) its efficiency is achieved with a lower daily dose; d) it prevents local fluctuations on the concentration of the active substance; e) active substance is selectively delivered at the target site; f) it has fewer risks associated with oral or intravenous administration, such as interactions or infections (38).

Among many topical formulations, the hydrogel is a good choice to incorporate nanoparticles due to all advantages associated with this topical delivery system (39). Hydrogels are constituted by a system of polymer chains with the ability to absorb huge amounts of water due to their hydrophilic properties, with cross-linked compounds that protect them from the dissolution. The water inside the hydrogel allows the free distribution of some particles and the polymer works as a matrix to hold water. Gel is a system that is considered neither liquid nor solid, exhibiting a semi-solid consistency (40).

When nanoparticles are embedded in a semi-solid form, using a gelling agent, interactions between the constituents of the final formulation could lead to changes in the physicochemical properties of nanolipid preparations. These modifications can be assessed using rheological analysis, particle size and zeta potential measurement. Some studies were performed to understand the influence of different gel-forming polymers used for hydrogel preparation. According to the results, the performance of these systems is highly dependent on the structure of these polymers and some polymers as Hydroxyethylcellulose 4000 (HEC) and Carbopol® 934 preserve physical stability of nanoparticles (41).

1.5. Effectiveness testing – skin's biophysical parameters measurement

In order to provide an adequate barrier effect, skin needs to be in good conditions as was previously mentioned, including skin hydration conditions, TEWL and pH. Some dermal products are designed to improve these skin's biophysical parameters (42). Nowadays, noninvasive techniques have been developed to measure these parameters with high sensitivity and maintaining the skin barrier intact (5).

However, only two parameters (skin hydration and TEWL) will be discussed according to the aim of this work. It is important to take into account that different factors (age, sex, and anatomical site) and different environmental conditions (temperature, relative humidity) can influence these values. Therefore, these measurements should be performed at controlled conditions.

1.5.1. Skin hydration measurement

The water content of epidermis and dermis layers defines the skin hydration. *Stratum corneum* (SC) is able to hold water, due to the presence of corneocytes (with hygroscopic compounds inside mentioned as natural moisturizing factors, NMF) and intercellular lipids bilayer matrix organized to prevent TEWL, and acting as a barrier. SC is characterized by the presence of free water, involved in diffusion processes between skin and the environment, and bound water, associated with NMF present in the epidermis. Lack of water in the skin leads to defective hydration, responsible for dry and flaky skin surface (sometimes related with some dermatological disorders). Moisturizers play an important role on skin hydration which is fundamental to protect and maintain a healthy skin (43,44).

Skin hydration analysis is related with capacitance measurement of a dielectric medium, detected by corneometry with high sensitivity. As the skin is a dielectric medium, variations in hydration show up through changes in the dielectric constant. The corneometer measures the change in the dielectric constant, changing the capacitance (defined as the ability to store energy in an electric field) of a precision capacitor. In a final step, these changes regarding water content are converted to arbitrary units of hydration (16,45).

1.5.2. Transepidermal water loss measurement

Transepidermal water loss (TEWL) is the loss of water from the *stratum corneum*, affecting the level of epidermis moisture. During the normal skin metabolism, some water evaporates from the skin continuously in a passive way depending on the relative humidity of the environment, temperature, season and skin hydration. If the natural barrier function of the skin is damaged, the water loss will increase, so TEWL is a sensitive indicator of the defective skin barrier function (46). In this way, it is important to measure for all cosmetic and dermatological products the influence of this parameter.

In order to measure TEWL under *in vivo* conditions, three techniques can be performed: closed chamber, ventilated chamber and open chamber. In the closed chamber method, a capsule is applied on the skin surface and then an electric hygrosensor measures the water that evaporates and goes into the capsule. Ventilated chamber method is based on the passage of a gas chamber (with gas and a pre-determined water content) along the skin and the posterior measurement of the amount of water captured by the gas in the chamber. Then, this collected water is analyzed by an incorporated hygrometer in the chamber. Despite being a method that provides a continuous measurement of TEWL, incorrect results may occur in case the gas inside the chamber becomes dehydrated, leading to an increase of water evaporation from skin (5). Finally, the most approached method for determining TEWL is the open chamber method based on the diffusion principle in an open chamber. There are several instruments to measure TEWL by open chamber, but it will be discussed only the apparatus used in this experiment: tewameter. This instrument has an open chamber that measures the density gradient of the water evaporation from the skin by two pairs of sensors (temperature and relative humidity) inside the cylinder (head of probe). This head of probe minimizes the influence of air turbulence inside the probe. One pair is higher than the other one, and the moisture at two different places is measured to determine the TEWL. During this measurement, a microprocessor analyses the values expressing the evaporation rate in $g/h/m^2$ (47).

2. Aim of the work

The aim of this research project was to evaluate the influence of three well characterized different lipid nanosystems (solid lipid nanoparticles, nanostructured lipid carriers and nanoemulsions) on skin hydration and transepidermal water loss on human volunteers.

3. Materials and Methods

3.1. Materials

Lipids

- ➔ Dynasan® 114 (Sasol, Germany): Trimyristin, m.p. 55-58ºC (48)
- ➔ Glycerol monostearate 35-50 (Lex, Slovenia): Monoester of glycerin and stearic acid, m.p. 56-59ºC (49)
- ➔ Miglyol® 812 (Sasol, Germany): Caprylic/Capric Triglyceride

Emulsifiers/Steric stabilizers

- ➔ **Surfactant** – Phospholipon® 80 (Phospholipid GmbH, Germany)
- ➔ **Co-surfactant** – Lutrol® F68: Poloxamer 188 (BASF, Germany)
- ➔ **Co-surfactant** – Tween® 80: Polysorbate 80 (Sigma-Aldrich Chemie GmbH, France)

Ingredients of hydrogel

- ➔ Hydroxyethylcellulose 4000 (Merck, Germany)
- ➔ Glycerol 85% (Caesar&Loretz, Germany)
- ➔ Sodium methylparahydroxybenzoate (Lex, Slovenia)

Reagents/Solvents

- ➔ Purified water (Faculty of Pharmacy, University of Ljubljana)

3.2. Equipments

- ➔ Dual range Analytical Balance® AG245 (Mettler Toledo, Switzerland)
- ➔ Precision Balance® XP4002S (Mettler Toledo, Switzerland)
- ➔ Magnetic hotplate stirrer® RH basic 2 IKAMAG (IKA, Brasil)
- ➔ GFL multi-station water bath® TYP 1041 (GFL, Germany)
- ➔ Hotplate® EKP 3582 (Clatronic International GmbH, Germany)
- ➔ Ultra-Turrax® T25D rotor-stator homogenizer (IKA, Germany)
- ➔ PCS naprava Zetasizer Nano ZS® (Malvern Instruments, United Kingdom)
- ➔ Probe Heater® PR 100 (Courage & Khazaka GmbH, Germany)
- ➔ Tewameter® TM 300 (Courage & Khazaka GmbH, Germany)
- ➔ Corneometer® CM 825 (Courage & Khazaka GmbH, Germany)

3.3. Preparation of lipid nanoparticles

(Local: Faculty of Pharmacy, University of Ljubljana)

3.3.1. SLN

- ➔ To prepare SLN, the following ingredients were used: solid lipid (Dynasan® 114, D114, or Glycerol monostearate ,GMS), surfactant (Phospholipon® 80), co-surfactant (Lutrol® F68 or Tween® 80) and purified water;
- ➔ 2.5 g co-surfactant was solubilized in boiled purified water (qs 100 g) under stirring (Magnetic hotplate stirrer® RH basic 2 IKAMAG);
- ➔ Lipid phase (solid lipid and emulsifier) and water phase (co-surfactant solution) were heated at 80ºC in the GFL multi-station water bath® TYP 1041;
- ➔ When solid lipid was melted, water phase was added to lipid phase;
- ➔ This mixture was homogenized by Ultra-Turrax® T25D rotor-stator homogenizer as shown in **Figure 3** at different times (5, 8 or 10 min) and shear rates (15,000; 17,000 or 20,000 rpm). During this homogenization, the water temperature was maintained at 80 ± 3ºC;
- ➔ When the O/W emulsion obtained cooled down to room temperature, SLN were obtained.

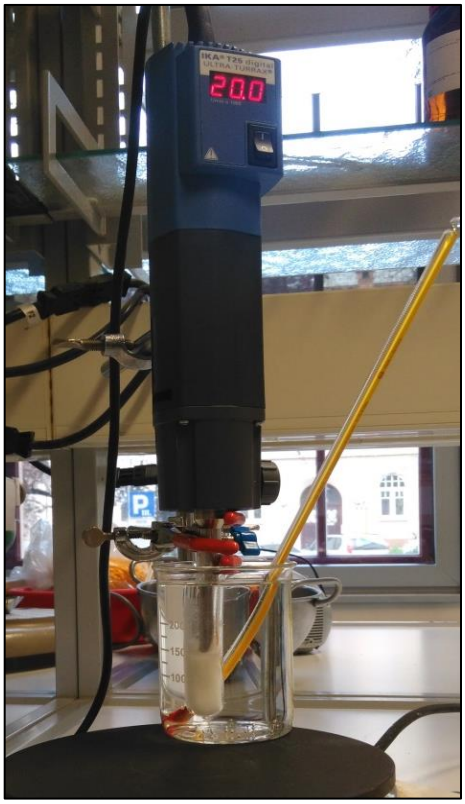

Figure 3 – Homogenization of the mixture

3.3.2. NLC

- ➔ To prepare NLC, the following ingredients were used: solid lipid (Dynasan® 114 or Glycerol monostearate) and surfactant (Phospholipon® 80), co-surfactant (Lutrol® F68) and purified water;
- ➔ NLC were produced as described for SLN. However, all formulations were homogenized at the same time (10 min) and shear rate (20,000 rpm). These conditions were previously set according to better results obtained during SLN preparation;
- ➔ When the O/W emulsion obtained cooled down to room temperature, NLC were obtained.

3.3.3. NE

- ➔ To prepare NLC the following ingredients were used: liquid lipid (Miglyol® 812) and surfactant (Phospholipon® 80), co-surfactant (Lutrol® F68) and purified water;
- ➔ NLC were produced as described for SLN and NLC (homogenization at 20,000 rpm for 10 min);
- ➔ When the O/W emulsion obtained cooled down to room temperature, NE were produced.

The final composition of each nanolipid system is represented on **Table 1**.

Table 1 – Composition (expressed in percentage) of each nanolipid system

	SLN	NLC^{D114}	NLC^{GMS}	NE
Dynasan® 114	3.5	2.8	-----	-----
GMS	-----	-----	2.8	-----
Miglyol® 812	-----	0.7	0.7	3.5
Lutrol® F68	1.5	1.5	1.5	1.5
Phospholipon® 80	1	1	1	1
Purified water	qs 100	qs 100	qs 100	qs 100

3.4. Evaluation of the physical parameters of nanosystems

After the production of these nanosystems, particle size, PdI and zeta potential of the individual dispersions were measured on the Zetasizer Nano ZS®, to evaluate the main physical parameters of the lipid formulations. The samples were previously diluted with purified water.

3.4.1. Particle size and polydispersity index

These parameters were evaluated under the following conditions:

- ✓ Dispersant: Water (Temperature: 25 ° C, Viscosity: 0.8872 mPa.s, RI: 1.330)
- ✓ Temperature (T): 25 ° C
- ✓ Equilibration time: 30 s
- ✓ Cell type: DTS0012 - Disposable sizing cuvette
- ✓ Measurement angle: 173 ° Backscatter (NIBS default)

3.4.2. Zeta potential

This parameter was evaluated under the following conditions:

- ✓ Dispersant: Water (Temperature: 25 ° C, Viscosity: 0.8872 mPa.s, RI: 1.330, Dielectric constant: 78.5)
- ✓ F(Ka) selection: Model Smoluchowski
- ✓ Temperature (T): 25 ° C
- ✓ Equilibration time: 30 s
- ✓ Cell type: DTS1060C – Clear disposable zeta cell

3.5. Preparation of hydrogels

- ➔ To prepare hydrogels (21 g), the following ingredients were used: hydroxyethylcellulose 4000, glycerol 85%, sodium benzoate (preservative), nanolipid systems (3 g x 7 samples of each system) and purified water according to **Table 2**;
- ➔ Hydroxyethylcellulose and glycerol were mixed apart in a mortar. Then, this mixture was added to the rest of formulation under stirring;
- ➔ After 24 hours, the hydrogels were conditioned in a centrifuge tube (**Figure 4**).

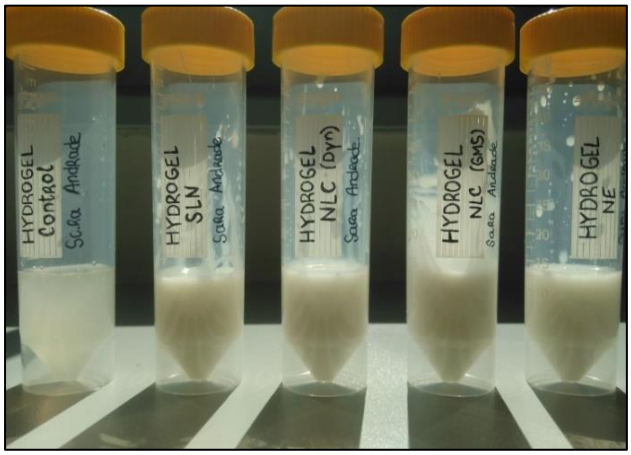

Figure 4 – Hydrogels. From left to right: hydrogel-control, SLN-based hydrogel, NLC^{D114}-based hydrogel, NLC^{GMS}-based hydrogel, NE-based hydrogel

Table 2 – Composition (expressed in percentage) of each hydrogel

	Hydrogel - Control	SLN-based Hydrogel	NE-based Hydrogel	NLC^{D114}-based Hydrogel	NLC^{GMS}-based Hydrogel
Hydroxyethylcellulose	2.5	2.5	2.5	2.5	2.5
Glycerol 85%	5.0	5.0	5.0	5.0	5.0
Sodium benzoate	0.1	0.1	0.1	0.1	0.1
SLN	-----	qs 100	-----	-----	-----
NE	-----	-----	qs 100	-----	-----
NLC^{D114}	-----	-----	-----	qs 100	-----
NLC^{GMS}	-----	-----	-----	-----	qs 100
Purified water	qs 100	-----	-----	-----	-----

3.6. Design of the clinical study

3.6.1. Subjects

The clinical study was performed in 6 healthy volunteers. These participants received and signed an informed consent form, in order to prove that they accepted the test conditions (**Annex 1 (A1)**). All procedures of this study were performed in accordance with the principles of the Declaration of Helsinki and respective revisions.

The criteria for inclusion and exclusion in this study were previously established.

The inclusion criteria of volunteers were as follows: subjects aged between 21 and 27 years old; few or no hair on the volar forearms; absence of dermatological diseases, tattoos, scars and/or history of frequent sunburns; to be present in the Faculty of Pharmacy of the University of Ljubljana on the pre-set day to perform the measurements; to be cooperative, discerning and able to follow instructions and comply with the study requirements.

On the other hand, the exclusion criteria taken into account were as follows: subjects aged less than 18 years old; excessive hairiness on the forearms; presence of dermatological diseases, tattoos, scars and/or history of frequent sunburns on the arms or forearms; the use of other cosmetics products on the forearms that were not defined in the study protocol; known history of allergy and/ or hypersensitivity to the ingredients contained in the composition of the hydrogels tested; pregnant or lactating women; the presence of severe disease in the last 6 months prior to the beginning of the study; the presence of clinically relevant skin diseases or any other physical disorder with cutaneous manifestations; the presence of mental/psychiatric illness or systemic disease in the beginning of the study; the presence of any type of immunological alterations, including autoimmune diseases; the presence of fever for more than 24 hours, for less than 8 days before the beginning of the study; the participation in other cosmetic or clinical studies for less than 2 weeks before the beginning of the study; recent dermo-cosmetic or aesthetic treatments, 2 months prior to the beginning of the study; recent (one month prior to the study) and intense exposure to ultraviolet radiation (sun/tanning beds); the application of any topical medication in the forearms for less than 1 month prior the study initiation; recent vaccinations, 2 weeks prior to the commencement of the study; taking any systemic medication such as corticosteroids or antihistamines for less than 4 weeks, anti-inflammatory or antibiotics for less than 2 weeks and retinoids for less than 3 months before

the beginning of the study; to have strong smoking habits (≥ 20 cigarettes per week over more than 2 years); to be a consumer of drugs.

The participants were also asked not to apply any cream in the test area two days before the measurement and not to smoke, exercise or drink coffee/tea or any other energy drink in the measurement day.

3.6.2. Measurement conditions

The clinical study was carried out in a specific room in the Faculty of Pharmacy, University of Ljubljana. It was attempted to control the temperature (T), to minimize sweat production, and the relative humidity (RH) of the environment in order to perform all measurements under certain room conditions (T: 20°C; RH: 40-60%) to obtain reproducible results.

Accordingly, all volunteers were previously set in a comfortable position and prepared removing the clothing from the arm area to be tested and letting the skin to acclimatize to these environmental conditions for 20 minutes before the measurement.

During this clinical study, non-invasive biophysical measuring methods were used in order to measure the influence of formulated hydrogels in skin hydration and TEWL. All measurements were performed by one of the principal investigators.

3.6.3. Application of the hydrogel

The selected area to be tested was the volar forearm, 3 centimeters below the antecubital fossa, since this region presents low amount of hair follicles and sebaceous glands.

After volunteers` preparation and acclimatization, 5 squares (4 cm^2 of surface area) were drawn in the test area: 3 squares in the left volar forearm and the

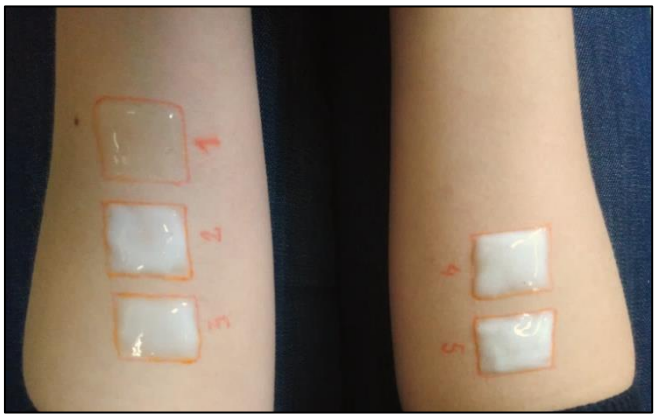

Figure 5 – Tested area: 1) Hydrogel-control; 2) SLN-based hydrogel; 3) NE-based hydrogel; 4) NLC^{D114}-based hydrogel; 5) NLC^{GMS}-based hydrogel

others in the right volar forearm. Hydrogels with and without nanolipid systems (0.5 ml) where applied as shown in **Figure 5**.

The study was designed as single blind. Therefore, the hydrogels were assigned by a number, so that the volunteers were unaware of the type of system under test in each square.

Two measurements were performed in each square for both parameters (skin hydration and TEWL). A first measurement was performed before hydrogels` application, to obtain the basal values. Then, hydrogels were applied on the corresponding areas. After 30 minutes the remnants of hydrogels were removed, and second measurements were performed after 60 minutes, measuring TEWL followed by skin hydration. Measurements were carried out in the counterclockwise direction.

3.6.4. Skin hydration measurement

Skin hydration was measured using a Corneometer® CM 825. The probe was placed perpendicularly to the skin area to be measured. The spring inside the probe head ensures constant pressure (1.0 N ± 10 %) on the skin enabling exact and reproducible measurement (uncertainty degree: ± 3%) (45). This spring covers an area of 49 mm^2 and assesses the epidermal water content from 20 to 30 µm (50).

Measurements were performed three times in each area. The display shows immediately the measured values in arbitrary units (arbitrary Corneometer® units), and it is necessary to wait about 5 seconds between each measurement. Between different squares, the probe head was cleaned softly with dry paper.

To evaluate the level of skin hydration, the table below **(Table 3)** was used as reference (this table was described in the data sheet of the corneometer used in this experiment, Corneometer ® CM 825) (51).

Table 3 – Interpretation of measured values of skin hydration with Corneometer ® CM 825

Skin hydration – arbitrary units	Interpretation
<30	Very dry
30-40	Dry
>40	Sufficiently moisturized

3.6.5. TEWL measurement

TEWL measurements were carried out with Tewameter® TM 300. The water evaporated from the skin is measured indirectly by two pairs of sensors (T and RH), and it is analyzed by a microprocessor. The results obtained are expressed in $g/h/m^2$.

The probe was sited tightly on the skin surface, for approximately 90 seconds. During this time, the volunteer could not move the arm to ensure reliable results. Between each measurement, the probe was cleaned softly with wet paper (with deionized water).

The sensors in the probe usually have room temperature, however they should reach skin temperature (32ºC) since the amount of evaporating water measured with the probe is particularly low. Thus, the probe head is constantly warmed up to around 32 ºC in Probe Heater® PR 100 in order to get very quickly accurate and stable results before and between different measurements.

To evaluate TEWL, the table below **(Table 4)** was used as reference (this table was described in the data sheet of the tewameter used in this experiment, Tewameter ® TM 300) (52).

Table 4 – Interpretation of measured values of transepidermal water loss with Tewameter ® TM 300

TEWL – $g/h/m^2$	Interpretation
0-10	Very healthy condition
10-15	Healthy condition
15-25	Normal condition
25-30	Strained skin
>30	Critical condition

3.6.6. Statistical analysis

The results were reported as mean + standard deviation (SD) of at least three samples. The results of all these experiments were statistically analyzed using SigmaPlot 11.0 software®. The differences were considered statistically significant when $p < 0.05$.

4. Results and Discussion

4.1. Pre-experimental work: the influence of different production parameters on the particle size, polydispersity index and zeta potential

The first step of this experimental work was to study the influence of different dynamic parameters (time and stirring rate of homogenization) as well as the effect of lipids and co-surfactants (type and concentration) on the preparation of lipid nanoparticle systems. In order to characterize the best formulations, the particle size (mean size), polydispersity index and zeta potential were measured.

Regarding particle size, these particles should exhibit nanosize (from 1 to 1000 nm) and be as small as possible, since an increase in the particle size will lead to lower physical stability (16). It is important to obtain the optimal nanoparticle size range. In fact, several studies, that have been developed in this field, showed the best clinical results with nanoparticles at the size range of approximately 10–250 nm (53).

Measurement of PdI of nanoparticles is essential to obtain their size distribution. PdI is dimensionless and scaled, so dispersity values range from 0 to 1. Samples are not suitable for DLS technique if they have a very large size distribution, i.e. PdI values higher than 0.7 (54). The higher the PdI value, the less monodispersed nanoparticle system is (55).

Nanoparticles should have zeta potential (ZP) more negative than -30 mV or more positive than +30mV to be physically stable (16). Charged particles, with high zeta potential modulus, repeal each other and prevent particle aggregation, allowing storage stability of colloidal systems (56). In this case, the zeta potential of the developed systems (NE, SLN and NLC) was negative due to the anionic nature of the surfactant components (Phospholipon® 80 has phosphatidylcholine with negatively charged phospholipids) (57).

These preliminary formulation studies were performed before the experimental design in order to select the appropriate lipid, co-surfactant and homogenization conditions. These conditions were firstly set during SLN preparation.

i. Type of lipid and co-surfactant (SLN formulations)

The influence of lipid (Dynasan® 114, D114 or Glycerol monostearate, GMS) and co-surfactant (Lutrol® F68 or Tween® 80) on physical parameters of SLN is presented in **Table 5**. The surfactant was the same for tested formulations (Phospholipon® 80) which were prepared at the same conditions.

Table 5 – Composition and parameters of SLN formulated with different lipids and steric stabilizers and prepared at same conditions (results expressed as mean ± standard deviation, n= 3)

SLN	Solid Lipid	Steric stabilizer	Homogenization rate (rpm)	Homogenization time (min)	% of lipid	% of steric stabilizer	Results		
							Particle size (d.nm)	PdI	Zeta Potential (mV)
1.1	D114	Lutrol® F68	20,000	10	2.5	0.5	121.1	0.496	-35.8±0.9
1.2	GMS	Lutrol® F68	20,000	10	2.5	0.5	105.5	0.362	-37.2±1.2
2.1	D114	Tween® 80	20,000	10	2.5	0.5	204.9	0.592	-31.6±0.1
2.2	GMS	Tween® 80	20,000	10	2.5	0.5	229.0	0.554	-34.0±0.8

According to these results, the effect of Dynasan® 114 on the particle size was similar to GMS, being the major difference obtained when using different steric stabilizers. SLN formulated with Lutrol® F68 showed improved parameters, compared to SLN formulated with Tween® 80, since those particles were smaller and less polydispersed. As zeta potential was higher than -30 mV (absolute values) in all samples, a predictable physical stability should be observed.

In other reported studies, SLN produced with GMS (the lipid with the lowest molecular weight) showed the smallest particle size (58,59). Nevertheless, it would be necessary to develop further studies to prove some correlation between molecular weight of the solid lipids and particle size (59). Regarding the effect of co-surfactant, our results are in accordance with another experimental work where formulations prepared with Lutrol® F68 produced smaller particles than those with Tween® 80 (19).

Thus, both solid lipids and Lutrol® F68 were used on the preparation of nanosystems for following studies.

ii. Homogenization time and shear rate (SLN formulations)

It is crucial to optimize the homogenization conditions, i.e. shear rate, temperature and time of homogenization to obtain a stable emulsion with nanoparticles, avoiding the formation of microparticles.

Regarding temperature, the lipid is heated at approx. 5-10ºC above its melting point for the hot pressure homogenization (HPH) process (33). Since high-shear homogenization (HSH) was used in this experimental work, instead of HPH, a higher recommended temperature, at least 10ºC above lipid melting point, was used (60). In that way, the lipids were heated at 20ºC above their melting point to increase the effectiveness of this method. In fact, higher temperature is beneficial for emulsification process due to reduction of surface tension (61).

The influence of homogenization time and shear rate on physical parameters of SLN is presented in **Table 6.**

Table 6 – Composition and parameters of two SLN formulations prepared at different homogenization conditions (results expressed as mean ± standard deviation, n= 3)

SLN	Solid Lipid	Steric stabilizer	Homogenization rate (rpm)	Homogenization time (min)	% of lipid	% of steric stabilizer	Results		
							Particle size (d.nm)	PdI	Zeta Potential (mV)
3.1	D114	Lutrol® F68	20,000	5	2.5	0.5	259.7	0.790	-30.4±1.2
3.2	D114	Lutrol® F68	20,000	8	2.5	0.5	208.4	0.582	-38.4±0.1
3.3	D114	Lutrol® F68	20,000	10	2.5	0.5	110.9	0.248	-34.0±0.8
3.4	GMS	Lutrol® F68	20,000	5	2.5	0.5	220.5	0.575	-31.8±1.2
3.5	GMS	Lutrol® F68	20,000	8	2.5	0.5	201.4	0.523	-34.8±0.9
3.6	GMS	Lutrol® F68	20,000	10	2.5	0.5	178.5	0.345	-35.0±1.0
4.1	D114	Lutrol® F68	15,000	10	2.5	0.5	218.8	0.667	-36.5±1.0
4.2	D114	Lutrol® F68	17,000	10	2.5	0.5	163.5	0.491	-35.1±0.3
4.3	D114	Lutrol® F68	20,000	10	2.5	0.5	110.9	0.359	-39.4±1.1
4.4	GMS	Lutrol® F68	15,000	10	2.5	0.5	233.4	0.452	-30.7±0.7
4.5	GMS	Lutrol® F68	17,000	10	2.5	0.5	215.8	0.416	-31.4±1.0
4.6	GMS	Lutrol® F68	20,000	10	2.5	0.5	101.0	0.286	-30.2±1.8

According to this table, the average SLN diameter and PdI were reduced with increasing homogenization time (from 5 to 10 min) and shear rate (from 15,000 to 20,000 rpm), as theoretically expected. In addition, all formulations presented acceptable zeta potential values.

It was previously observed that smaller particles were obtained by increasing the shear rate. However, above 20,000 rpm (25,000 rpm) the average particle diameter did not significantly changed. On the other hand, the shear rates below 15,000 rpm were not sufficient for the formation of suitable SLNs, since large particles were visible in the dispersion. If homogenization extends for more than 10 minutes, particles may become unstable due to high energy input, leading to the formation of microparticles (60).

Therefore, better results were obtained for 10 minutes and 20,000 rpm, which were the values selected to produce all lipid formulations.

iii. Percentage of solid lipid and steric stabilizer (SLN formulations)

To investigate the influence of solid lipid and steric stabilizer concentrations, a study was performed with 3.5% and 2.5% of solid lipid and 1.5% and 0.5% of steric stabilizer (**Table 7**).

To maintain a low PdI, it is essential to optimize both type and percentage of steric stabilizer, since these emulsifiers play an important role in stabilizing emulsions and preventing aggregation of the droplets (19). Accordingly, the percentage of selected emulsifier (Lutrol® F68) was studied. This percentage was not higher than 1.5% since high concentration of steric stabilizer (≥2%) could decrease its emulsifying effect and contribute to toxic effects. On the other hand, if this percentage is lower than 0.5%, nanoparticles may not be effectively obtained and particle agglomeration may also occur (58).

Lipid content should not be too high (up to 5%) to produce small particles since the homogenization process is less effective for a more viscous formulation, and thus microparticles may be formed (62). Notwithstanding, the amount of lipid cannot be less than 2.5%, otherwise the consequent low viscosity of hydrogel-based nanoparticles will compromise the topical application (27).

Accordingly, it is quite perceptible that it is essential to balance all possible phenomena to optimize the composition of both steric stabilizer and lipid in formulation study. This study was performed with two different percentages of each parameter since the main goal was to understand whether a proportional correlation would be observed between them.

Table 7 – Composition and parameters of SLN formulated with different solid lipid and steric stabilizer concentrations and prepared at same conditions (results expressed as mean ± standard deviation, n= 3)

SLN	Solid Lipid	Steric stabilizer	Homogenization rate (rpm)	Homogenization time (min)	% of lipid	% of steric stabilizer	Results		
							Particle size (d.nm)	PdI	Zeta Potential (mV)
5.1	D114	Lutrol® F68	20,000	10	3.5	1.5	214.6	0.431	-31.0±1.1
5.2	D114	Lutrol® F68	20,000	10	3.5	0.5	291.2	0.761	-31.2±0.6
5.3	D114	Lutrol® F68	20,000	10	2.5	1.5	265.5	0.792	-33.6±0.7
5.4	D114	Lutrol® F68	20,000	10	2.5	0.5	174.4	0.590	-34.1±1.0
5.5	GMS	Lutrol®F68	20,000	10	3.5	1.5	190.7	0.401	-32.1±0.9
5.6	GMS	Lutrol® F68	20,000	10	3.5	0.5	289.9	0.762	-33.4±0.6
5.7	GMS	Lutrol® F68	20,000	10	2.5	1.5	280.7	0.753	-32.4±1.0
5.8	GMS	Lutrol® F68	20,000	10	2.5	0.5	210.4	0.421	-31.0±0.9

Table 7 shows that best results (regarding particle size and PdI) were obtained with the formulations 5.1 (3.5% of D114 with 1.5% of Lutrol® F68), 5.4 (2.5% of D114 with 0.5% of Lutrol® F68), 5.5 (3.5% of GMS with 1.5% of Lutrol® F68), and 5.8 (2.5% of GMS with 0.5% of Lutrol® F68). Zeta potential presented good values in all formulations.

These values suggest that to obtain smaller particles with narrower PdI, the amount of solid lipid and steric stabilizer should be proportional, i.e. if a higher percentage of lipid is used (3.5%), then a higher percentage of steric stabilizer should be used as well (1.5%), and vice versa, a lower content of steric stabilizer (0.5%) should be used with a lower lipid amount (2.5).

iv. Percentage of solid lipid (NLC formulations)

To study the influence of solid (s) lipid concentration in NLC, two solid lipids (Dynasan® 114 and GMS) were used at different concentrations (2.8% and 2.1%, respectively). On the contrary, the liquid (l) lipid, Miglyol® 812, was maintained at the same concentration (0.7%) (**Table 8**).

Regarding steric stabilizer in this formulation, 1.5% was the selected amount (**Table 8**), taking into account the results from the last experiment (iii) (**Table 7**).

Since it was already studied the influence of liquid lipid percentage in NLC formulations (using the same solid lipid content) reported in literature (63), here we proposed to study the influence of only solid liquid percentage in NLC and how different total lipid concentration and ratio solid/liquid lipid influences the particle size and zeta potential.

Table 8 – Composition and parameters of NLC formulated with different solid lipid type and concentration and prepared at same conditions (results expressed as mean ± standard deviation, n= 3).

NLC	Solid lipid	Liquid lipid	Steric stabilizer	Homogenization rate (rpm)	Homogenization time (min)	% of lipid (s/l)	% of steric stabilizer	Results		
								Particle size (d.nm)	PdI	Zeta Potential (mV)
6.1	D114	Miglyol® 812	Lutrol® F68	20,000	10	2.8 / 0.7	1.5	271.3	0.601	-31.4±1.2
6.2	D114	Miglyol® 812	Lutrol® F68	20,000	10	2.1 / 0.7	1.5	294.5	0.705	-36.2±0.7
6.3	GMS	Miglyol® 812	Lutrol® F68	20,000	10	2.8 / 0.7	1.5	128.1	0.448	-38.5±0.5
6.4	GMS	Miglyol® 812	Lutrol® F68	20,000	10	2.1 / 0.7	1.5	138.7	0.301	-37.6±1.3

Smaller particle size and lower PdI values were obtained at higher solid lipid concentration (samples 6.1 and 6.3), especially in NLC formulated with GMS. Some literature reports suggest that the optimum solid / liquid lipid ratio to produce NLC varies from 70:30 to 99.9:0.1. Thus, samples 6.1 and 6.3 which presented a ratio of 70:30 are in accordance with these reports (15).

v. Percentage of liquid lipid (NE formulations)

To produce an emulsion, it is important to consider the optimum liquid lipid and steric stabilizer ratio, which lead to a low surface tension and spontaneous droplet formation. It has been reported that the surface tension between oil and water phases has a huge influence on droplet size of the dispersed phase (even more than the oil viscosity). In this way, the concentration of the liquid lipid and the liquid lipid/co-surfactant ratio are critical to optimize droplet size through the influence of a lower interfacial tension (23,64).

Accordingly, the effect of the percentage of liquid lipid on nanoemulsions (NE) formulations was also evaluated (**Table 9**), using the same percentage of steric stabilizer used before in the design of NLC formulations (1.5%), and their ratio as well.

Table 9 – Composition and parameters of NE formulated with different liquid lipid concentration and prepared at same conditions (results expressed as mean ± standard deviation, n= 3)

NE	Liquid lipid	Steric stabilizer	Homogenization rate (rpm)	Homogenization time (min)	% of lipid	% of steric stabilizer	Results		
							Droplet size (d.nm)	PdI	Zeta Potential (mV)
7.1	Miglyol® 812	Lutrol® F68	20,000	10	3.5	1.5	181.0	0.341	-34.6±0.5
7.2	Miglyol® 812	Lutrol® F68	20,000	10	2.5	1.5	185.0	0.391	-35.0±1.2

These results show that the use of both liquid lipid concentrations presented quite similar and favorable results (nanoparticles with an acceptable PdI and zeta potential).

Regarding liquid lipid content, the percentages chosen were used to compare with the results of another previous formulation with 3.5% of lipid and 1.5% of steric stabilizer (63). Accordingly, 3.5% of liquid lipid was a good choice to produce a nanoemulsion with suitable medium droplet size, being in accordance with the results here presented.

Finally, it is important to consider the liquid lipid/co-surfactant ratio. Several studies proved that the use of Miglyol® 812/co-surfactant at 7:3 or 6:4 ratios presented similar results, exhibiting an optimal droplet size (65). Since samples 7.1 and 7.2 with similar ratios showed good results, it can be concluded that those ratios can decrease the interfacial tension, and consequently, optimize the droplet size.

4.2. Final lipid nanoformulations

After these preliminary studies, the final formulation of each system was defined according to **Table 1**, taking into account all parameters optimized in the pre-experimental work.

i. Mean particle size measurement

To evaluate the mean particle size of each formulation, 7 samples (3 g) of each final system were prepared and characterized (**Table 10**).

Table 10 – Size parameters of lipid formulations (SLN, NLC and NE)

SLN		NLC^{D114}		NLC^{GMS}		NE	
Sample	**Size (nm)**	**Sample**	**Size (nm)**	**Sample**	**Size (nm)**	**Sample**	**Size (nm)**
8.1	205.2	9.1	174.8	10.1	141.5	11.1	225
8.2	201.5	9.2	183.6	10.2	162	11.2	214.2
8.3	244.9	9.3	194.3	10.3	157.9	11.3	216
8.4	216.5	9.4	202.5	10.4	131.2	11.4	196.5
8.5	214.5	9.5	209.4	10.5	178.7	11.5	188.7
8.6	229.6	9.6	173.4	10.6	151.2	11.6	180.2
8.7	198.4	9.7	211.2	10.7	141.9	11.7	201.4
Average	215.8	Average	192.7	Average	152.1	Average	203.1
SD	16.6	SD	15.8	SD	15.8	SD	16.1
RSD %	7.7	RSD %	8.1	RSD %	10.4	RSD %	7.9

Overall, an average particle size of around 200 nm was obtained as expected. Among all formulations developed, NLC presented significantly smaller particles compared to NE and SLN (with the worst mean size). These outcomes confirm the results of other experiments performed before in which particle size was optimized in different carriers (66–68).

Regarding NLC formulated with different solid lipids, NLC with GMS presented the smallest particles, which coincides with the results from other experiments involving SLN (58,59). As mentioned before (in topic 4.1.i.), this could be related with the lowest molecular weight of GMS compared to D114.

ii. Evaluation of the physical stability

The selection of suitable lipids and steric stabilizers, as well as their ratio, is an important prerequisite for the production of physically stable nanolipids based hydrogels. The physical stability of the final lipid nanoformulations was performed at room temperature for 30 days. Particle size, PdI and zeta potential were measured on the 1st, 15th and 30th days (**Table 11)**.

Table 11 – Physical stability of final lipid nanoformulations

Formulation	Time	Particle size (d.nm)	PdI	Zeta Potential (mV)
SLN (5.1)	t=0 days	214.6	0.431	-31.0±1.1
	t=15 days	198.0	0.410	-36.4±1.3
	t=1 month	148.0	0.389	-40.5±1.2
NLC ^{D114} (6.1)	t=0 days	271.3	0.601	-31.4±1.2
	t=15 days	254.5	0.598	-35.7±0.80
	t=1 month	245.9	0.476	-43.3±0.87
NLC ^{GMS} (6.3)	t=0 days	128.1	0.448	-38.5±0.5
	t=15 days	120.6	0.404	-34.0±1.2
	t=1 month	114.7	0.305	-33.2±0.9
NE (7.1)	t=0 days	181.0	0.341	-34.6±0.5
	t=15 days	187.6	0.401	-37.6±1.0
	t=1 month	209.5	0.420	-43.5±0.79

It can be observed that all final formulations showed suitable parameters and good physical stability after that period. Only NE presented a small size increase but not statistically significant.

4.3. The influence of SLN, NLC and NE – based hydrogels on skin hydration and transepidermal water loss

After all preliminary formulation studies, the final lipid nanocarriers were incorporated into hydrogel form to evaluate the influence of these systems on skin hydration and TEWL in human volunteers.

i. Skin hydration measurement

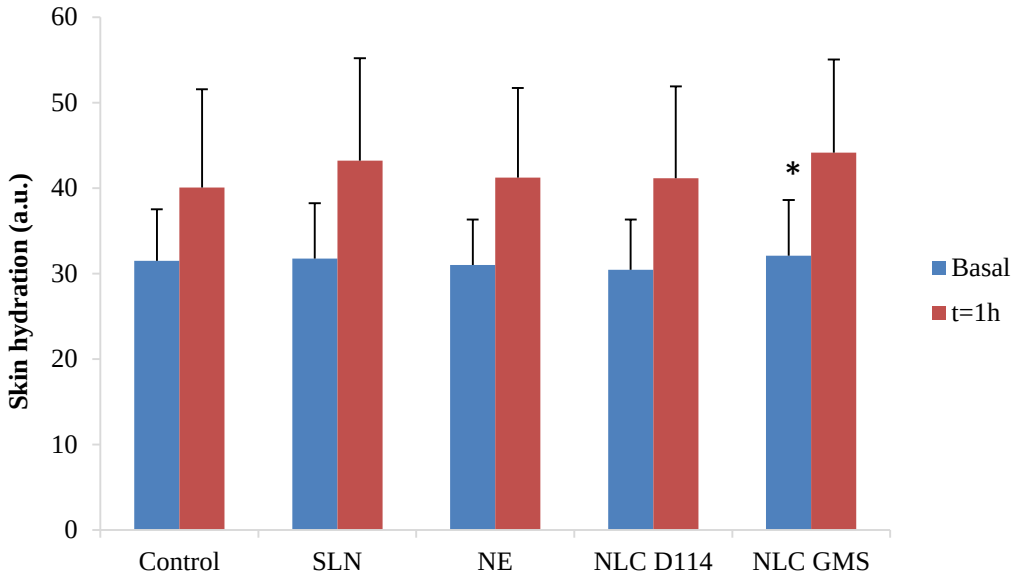

Figure 6 – **Influence of different nanolipid based hydrogels on skin hydration**

To understand the effect of different nanolipid based hydrogels on skin hydration, capacitance values were measured before the application of hydrogel (basal values) and one hour after application of all hydrogels (hydrogel control without nanolipids and the optimized nanolipid based hydrogels).

In **Annex 2 (A2)** it is possible to observe the influence of different nanolipid based hydrogels on skin hydration of each volunteer. **Figure 6** shows this parameter on skin hydration regarding 6 volunteers. Values of skin hydration before the application of hydrogels (basal values) were 31.48 (Control), 31.73 (SLN), 30.98 (NE), 30.42 (NLC^{D114}) and 32.07 a.u. (NLC^{GMS}). One hour after hydrogels` application, skin hydration increased as

expected, and the values measured were 40.07 (Control), 43.20 (SLN), 41.22 (NE), 41.15 (NLC^{D114}) and 44.15 a.u. (NLC^{GMS}). According to control measurements, the hydrogel (without nanoparticles) has a slight moisturizing effect by itself (63,69,70). Since basal values are between 30 and 40 a.u., all volunteers presented a 'Dry' condition regarding skin hydration. After hydrogels` application, all volunteers shown higher capacitance values, above 40 a.u., which means a 'Sufficiently moisturized' skin.

Overall, there was not a statistically significant difference detected by One way Annova for the skin hydration measurement after each formulation exposure (for both time points). Nevertheless, there was a statistically significant difference (*, $p < 0.05$) detected by t-test between the skin hydration measured at basal and 1 hour after NLC^{GMS} exposure. This result may be related to the smallest particles of this formulation compared to others, which may form a coherent film on the skin surface, leading to higher occlusive effect, and therefore, an improved effect on skin hydration (3,16,71). Although NE provided a good moisturizing effect, increasing skin hydration and skin permeation in literature reports (72–74), here NLC presented higher increase of skin hydration compared to NE (15,16,75).

ii. TEWL measurement

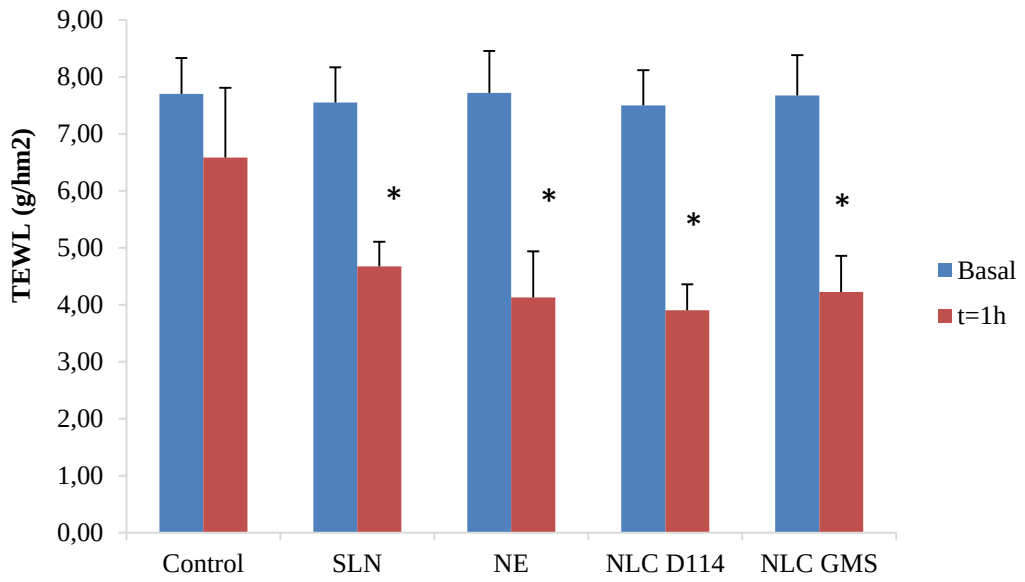

Figure 7 – Influence of different nanolipid based hydrogels on TEWL

The influence of different nanolipid based hydrogels on TEWL was evaluated following the procedure described before.

According to **Figure 7**, basal values of TEWL were 7.70 (Control), 7.55 (SLN), 7.72 (NE), 7.50 (NLC^{D114}) and 7.67 g/h/m^2 (NLC^{GMS}). One hour after hydrogels´ application, this parameter decreased as expected, and the values measured were 6.58 (Control), 4.67 (SLN), 4.13 (NE), 3.90 (NLC^{D114}) and 4.22 g/h/m^2 (NLC^{GMS}). These results are discriminated for each volunteer in **Annex 3 (A3).** Since these values are between 0 and 10 g/h/m^2, all volunteers presented a 'Very healthy condition' regarding TEWL (even before hydrogels ´ application).

As shown in **Figure 7,** there was a statistically significant difference (*, $p < 0.05$) detected by t-test between the TEWL measured at basal and 1 hour for all formulations, except the control group. There was also a statistically significant difference (*, $p < 0.05$) detected by One way Annova Pairwise Multiple Comparison Procedures (Holm-Sidak method) between the TEWL measured 1 hour after each formulation exposure and the control. However, no significant differences were obtained among nanolipid formulations for this parameter. Therefore, it can be concluded that nanolipid systems incorporated into hydrogels can lead to a decrease in TEWL, being in accordance with the preliminary findings of some researchers (76).

Accordingly, a smaller particle size is associated with a decrease in TEWL since it promotes a larger surface area of the particles reducing water evaporation from the skin surface (3,16). Thus, it would be expected a higher reduction in TEWL after the application of NLC-based hydrogel compared to SLN formulation. In addition, NLC usually decreases TEWL more efficiently than NE due to a higher effect on skin hydration as well, leading to an improved barrier function of the *stratum corneum* (16). However, no statistical differences were detected among these formulations in this study, as mentioned before. In order to understand these outcomes, it is important to approach the factors behind skin hydration and TEWL measurements as described below.

iii. Factors behind skin hydration and TEWL measurements

Although the nanolipid systems were optimized before their incorporation in hydrogel form, it should be noticed that some external conditions were not possible to control, which might affect the final results.

Regarding environment conditions, measurements should be ideally obtained at 20°C and 40 to 60% of RH, however, the room temperature and relative humidity varied from 23 to 26°C and 33% to 47% (respectively). This dry surrounding environment may have affected the skin hydration and TEWL (77).

Concerning human error, the position and the pressure used between the probe and the skin surface should be maintained through all measurements to obtain reproducible results. However, it was difficult for the investigator to apply always the same exact pressure. Moreover, the possible presence of remaining hydrogel should be considered as well since it was difficult to select the optimal strength to remove all formulation of the skin of the volunteers. If this strength was too high, it would affect skin properties, and on the other hand, if it was too soft, the hydrogel would not be removed was supposed (50).

It is also important to consider the differences between volunteers, despite the selection of a homogenous group following restrict inclusion and exclusion criteria. In fact, some previous studies reported the influence of personal differences in skin's biophysical parameters (such as age, gender and race) but the results were controversial (78,79). Regarding this work, the volunteers were Caucasian, 21-27 years old, four men and two women. The gender is quite important mainly to the difference between the amount of hair on the volunteers` forearms, which could affect the removal and drying of the hydrogel (79).

Finally, it is essential to focus the number of volunteers and the duration of this clinical study. In future perspective, this study will be conducted on a larger number of volunteers (at least 25 volunteers) (80) and under strict control conditions.

5. Conclusion

According to pre-experimental results, it can be concluded that particle parameters such as particle size, PdI and zeta potential clearly affected the physical stability of these nanosystems. Firstly, the optimal homogenization conditions were achieved at 20,000 rpm for 10 minutes. Besides homogenization parameters, different types of lipids have also influenced the particle size in NLC production. The smallest nanoparticles were obtained with GMS probably due to the lowest molecular weight of this lipid compared to D114. Regarding the co-surfactants used, Lutrol® F68 produced smaller particles than Tween® 80. Moreover, the amount of solid lipid and steric stabilizer should be added at a proportional ratio. At least, considering the liquid lipid/co-surfactant ratio, an optimal droplet size was obtained using Miglyol® 812/ Lutrol® F68 at 7:3 ratio. After formulation selection, all final formulations showed suitable parameters and good physical stability for a month after its production.

Regarding skin hydration and TEWL measurements, the hydrogel-control had a slight moisturizing effect by itself. In fact, none of skin hydration results presented statistical significant differences for each formulation exposure, compared with the control. Nevertheless, a statistically significant difference was observed for basal values 1 hour after NLC^{GMS} exposure. Moreover, all volunteers presented dry skin regarding basal values and a sufficiently moisturized skin after the hydrogels` application. Towards TEWL values, all volunteers presented a very healthy condition. In addition, a statistically significant difference was observed between the basal values and the TEWL values 1 hour after nanolipid-based hydrogels` application for each formulation compared with the control. Accordingly, nanolipid-based hydrogels could decrease TEWL values.

Notwithstanding, some factors might influence the measurement of skin's biophysical parameters such as environmental conditions, the human error and the skin differences between participants. The number of participants and the duration of the study were quite important as well. Thus, the standardization of all these conditions will be taken into account in future studies.

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Annexes

A1. Informed Consent Form

Univerza v Ljubljani
Fakulteta za farmacijo

INFORMED CONSENT FORM

The influence of different nanocarriers on skin's biophysical parameters

Principal Investigators: assist. prof. dr. Pegi Ahlin Grabnar and Sara Andrade
Organization: Faculty of Pharmacy – University of Ljubljana

This Informed Consent Form is for healthy subjects aged 21-27 years, who we are inviting to participate in a research project in the framework of a Master's Thesis of Pharmaceutical Sciences.

The aim of our research project is to evaluate the influence of three well characterized different lipid nanosystems (solid lipid nanoparticles, nanostructured lipid carriers and nanoemulsions) on skin hydration and transepidermal water loss on human volunteers.

Nanotechnology is a new trend in cosmetology, since nanoparticles have shown higher degree of biocompatibility and versatility in this field compared to other systems. Several formulations of lipid nanoparticles were produced to control different parameters referred to dermal preparations such as TEWL and skin hydration. Because of their biocompatible chemical nature, no adverse effects of these compounds on human skin have been described so far.

This research will involve one application of four different nanolipid formulations (one with SLN, two with NLC (different type of lipids) and one with NE) incorporated into hydrogel and one control on your forearms. Therefore, you should come to the Faculty of Pharmacy on the scheduled day. During this day, you will need to spend approximately two hours, twenty minutes to acclimatize the skin and approximately one hour and a half to measure the biophysical parameters.

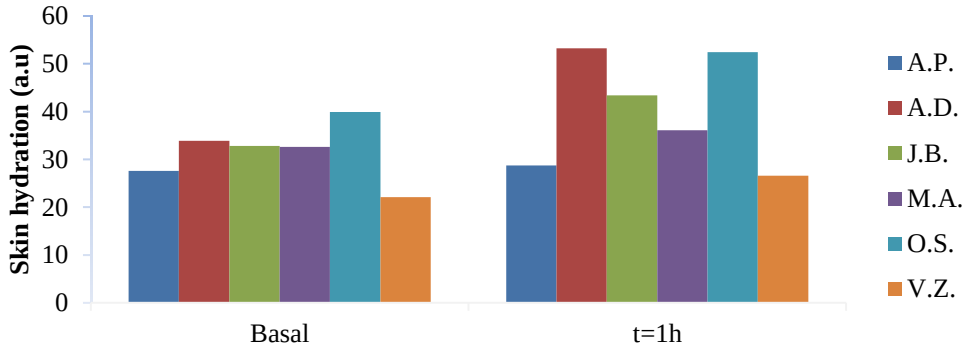
Your participation in this research is entirely voluntary and you may refuse or stop participating at any time that you wish. If you have any questions you may ask us now or later.

After reading the foregoing information and explanations, I consent voluntarily to participate as a participant in this research.

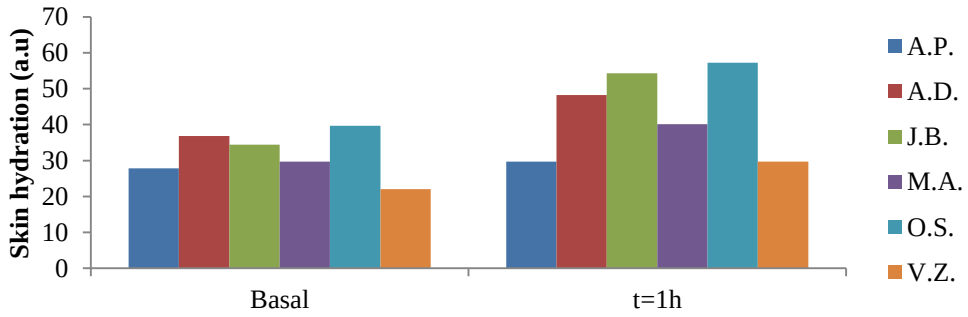
Signature of Participant: Date:

A2. Results of skin hydration in six volunteers

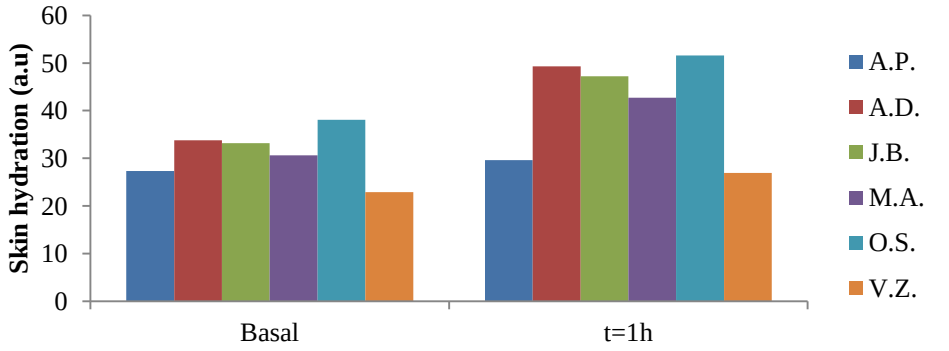
Control



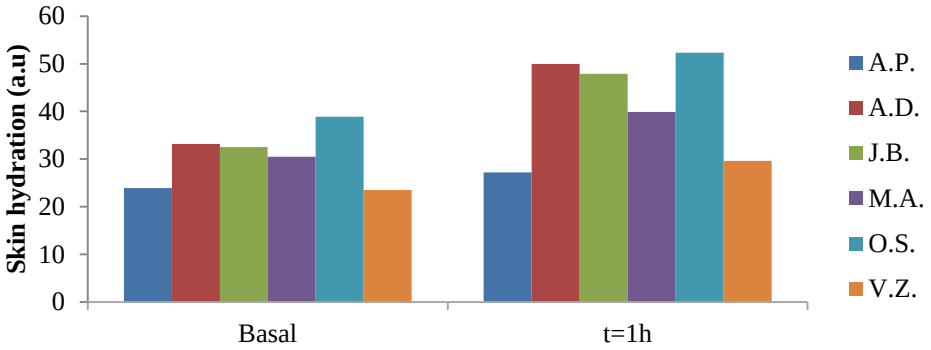
SLN



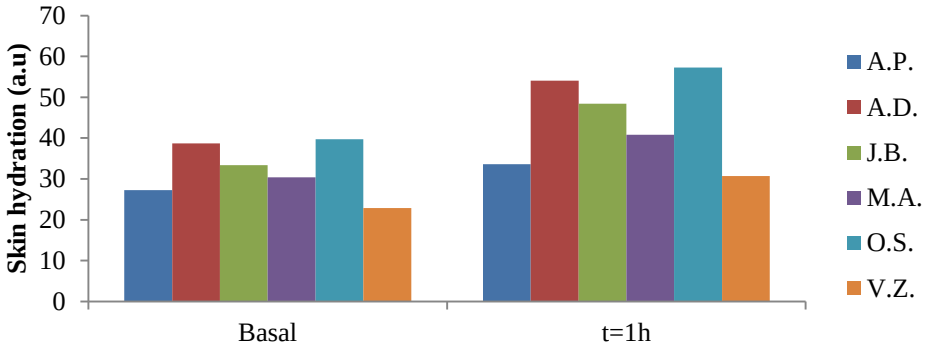
NE



NLC^{D114}

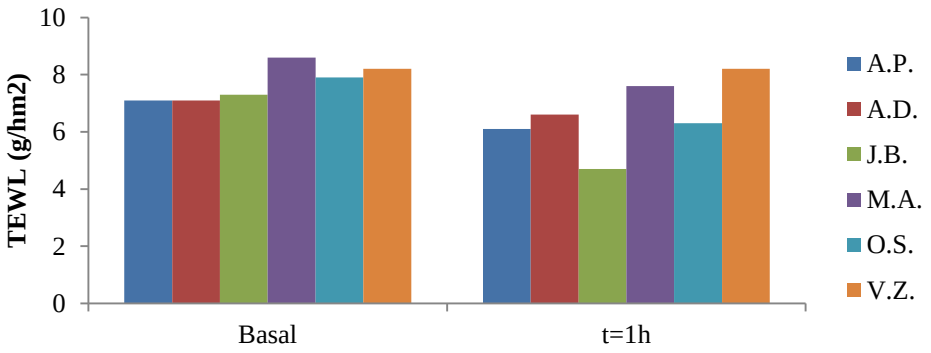


NLC^{GMS}

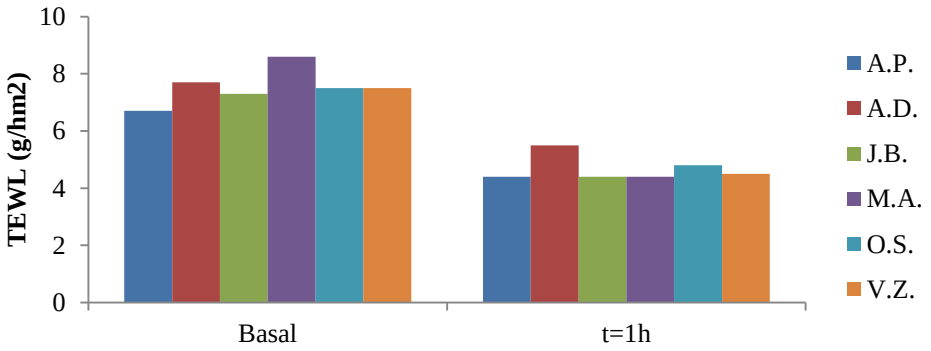


A3. Results of TEWL in six volunteers

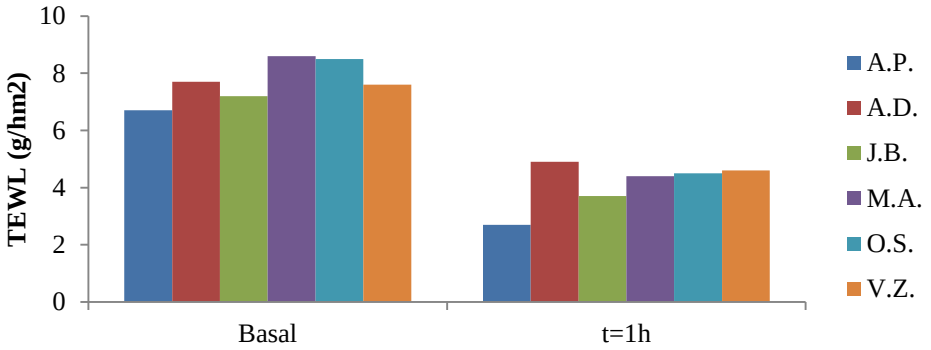
Control



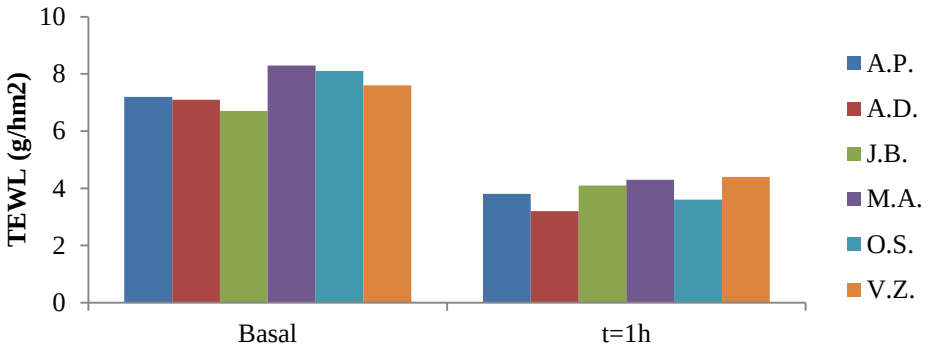
SLN



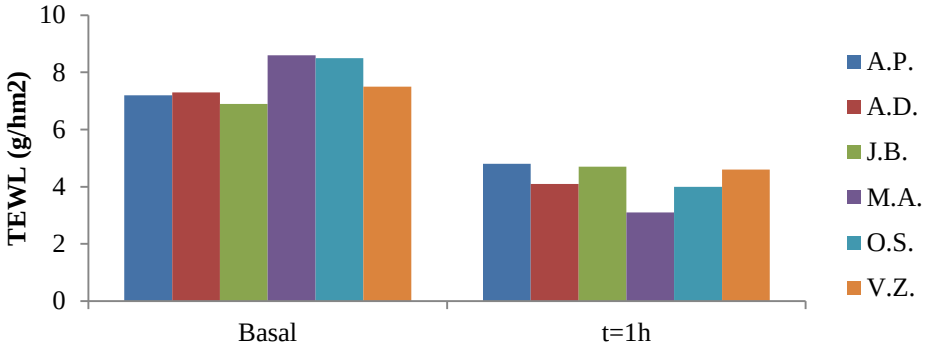
NE



NLC^{D114}



NLC^{GMS}



NOTE:

In a pre-experimental work, three different measurements were performed: 1st) before application of the hydrogel; 2nd) 30 min after the application of all hydrogels and 3rd) 1 h after the application of all hydrogels. After 30 min, it was possible to observe a great increase on skin hydration that slightly decreased after 60 min. However, these results were discarded since they might measure the hydration of the remaining gel that had not been yet absorbed (whereas hydrogel was removed 30 min after hydrogels` application, immediately before the second measurement).