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Lyophilization of Human Keratinocytes

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Erklärung

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To my parents.

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Chapter I

Aim of the thesis

The use of therapeutic cells constantly increases and keratinocytes and in particular keratinocyte suspensions became a promising option for the treatment of burn trauma and other skin lesions [1]–[3]. However, the use of therapeutic cells requires successful stabilization of cells during transport and storage. Nowadays, cryopreservation is still the gold standard for storage of active cells, coming along with high costs and energy input. (Freeze-) drying of cells could enable higher shelf live stability at ambient temperatures and could therefore facilitate easier transportation and storage conditions. The overall aim of this thesis was to develop a lyophilization process to freeze-dry human keratinocytes in a way that they can be used as therapeutic option to treat burn trauma and other skin lesions. Furthermore the lyophilized product should get applied directly on the wound bed to facilitate handling and therefore to reduce application errors. Since lyophilization of eukaryotic cells is still a challenging field with so far limited success, it was not our aim to obtain living and proliferating cells. We aimed to achieve cells with certain intact proteins and remaining activity to accelerate wound healing and wound closure, even if the cells itself were too damaged to proliferate. In this context analytical techniques were implemented to measure cell membrane integrity, protein content and activity as well as *in vitro* and *in vivo* activity of the lyophilized cells.

As a first step an intense literature research gives an overview about the actual status in the research field of (freeze-) drying cells to stabilize them in the dry state (see **Chapter II**). The most common excipients, the techniques to load in particular trehalose intracellularly as well as technical approaches to enhance cell survival after lyophilization are described. Finally the current success in (freeze-) drying microorganisms, red blood cells, platelets, spermatozoa and eukaryotic cells is presented.

The following chapters describe the approaches to lyophilize HaCaT keratinocytes, a spontaneously immortalized keratinocyte cell line from adult human skin, which was used as model cell line. 10% Hydroxyethylstarch (HES) and 5% Hydroxyectoine (HE) in a buffer system were used as basic components to stabilize cells during lyophilization. As a first step the basic lyophilization process was optimized and parameters such as buffer system, reconstitution and freezing steps, cell density and growth conditions were experimentally defined and described in **Chapter III**.

In **Chapter IV** the organic solvent DMSO as additional lyoprotectant and the resulting physicochemical properties of the lyophilized cake were analyzed. Furthermore the influence of DMSO addition on cellular conditions after lyophilization was investigated and cell membrane integrity, cell recovery as well as protein content and activity of selected proteins were determined. In addition the stability of the lyophilizates with and without DMSO during storage at 2-8°C was investigated and reported in **Chapter V**.

Besides DMSO also (-)-Epigallocatechingallate (EGCG) as stabilizing excipient was examined and its effect on cellular properties after lyophilization were described in **Chapter VI**. To elucidate the mode of action of EGCG, it was compared with chemically related molecules and other antioxidants. Since EGCG is known to rapidly degrade at higher pH values [4], [5], also the stability of EGCG in the present lyophilization formulation was analyzed as well as its influence on growth and migration behaviour of living cells.

In **Chapter VII**, a selected panel of growth factors and cytokines was analyzed and the effect of the lyophilization formulation (10% HES, 5% HE in HEPES buffer), including the addition of EGCG or DMSO was determined and compared to a formulation without excipients and to non freeze-dried cells. Furthermore, we focused on the *in vitro* activity of lyophilized cells and conducted 2D-migration assays to assess the migration induction of lyophilized cells on adherent keratinocytes. However, only limited information can gained from *in vitro* experiments and *in vivo* models still represent the gold standard to determine the activity and effectiveness of a potential treatment. In particular the porcine skin strongly correlates with the human skin and is especially valuable as wound model [6]. We therefore performed a key porcine *in vivo* experiment with artificially wounded pigs, with special attention on the direct application of the lyophilized samples on the wound bed.

A final summary with all results is provided in **Chapter VIII**, which also stresses success and limitations of this work.

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Chapter II

Introduction: Current approaches of preservation of cells during (freeze-) drying

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Abstract

The widespread application of therapeutic cells, such as stem cell transfer, blood transfusions, probiotic cultures or in vitro fertilisation requires a successful stabilization of cells for the duration of the transport and storage of the cells. Cryopreservation is currently considered the gold standard for the storage of active cells; however, (freeze-) drying cells could achieve higher shelf life stability at ambient temperatures, facilitating the transportation and storage of therapeutic cells, thus contributing to a higher availability and diversity of therapeutic options.

During (freeze-) drying, cells must withstand a wide range of potentially damaging processes: Upon freezing, ice crystal formation can rupture sensitive cellular structures and the concentration of extracellular components can lead to high osmotic stress. With the water removal during drying, the cell membrane undergoes a phase transition which is often linked to fusion or leakage of cell components. The rehydration step is also critical and may complete cell death. To prevent these damaging processes, a wide range of protecting excipients has emerged, which can be classified, according to their chemical affiliation, into sugars, macromolecules, polyols, antioxidants and (especially for drying sperm) chelating agents. Sugars and polyols form hydrogen bonds with membranes and proteins and thus enable water replacement in the dry state. Macromolecules seem to inhibit ice formation and promote vitrification. Antioxidants are believed to reduce ROS formation during freezing and chelating agents can complex divalent cations that catalyse unwanted chemical reactions. As many excipients cannot easily permeate the cell membrane, researchers have established various techniques to introduce especially trehalose intracellularly, such as incubation, ATP induced pore formation, pore formation with hemolysin H5, or transfection with trehalose synthesis genes.

This review aims to summarize the main damaging mechanisms during (freeze-) drying and to introduce the most common excipients with further details on their stabilizing properties and process approaches for the intracellular loading of excipients. Additionally, we would like to briefly explain recently discovered advantages of drying microorganisms, sperm, *platelets*, red blood cells, and eukaryotic cells, paying particular attention to the drying technique and residual moisture content.

II.1 Introduction

The use of therapeutic cells is widely spread and has many application fields, such as stem cell transfer, wound healing or reproduction medicine [1]. These manifold applications, together with the increasing demand of regenerative medicine and blood transfusions, require the constant supply of active cells [2], [3]. However, a common application of therapeutic cells requires the means to sustain sufficient stability during transport and storage. This stability is currently achieved through cryopreservation at subzero temperatures. At temperatures below -130°C, all water is solidified and no movement within the product can facilitate chemical reactions [4]. However, maintaining cells at such low temperatures results in high storage and transportation costs and is, in addition, liable to potential risks such as transient warming events or cross-contamination [3]–[5]. Drying and in particular freeze-drying as a preservation method could overcome the latter issues and offer increased sample stability at higher temperatures, easy transportation, and controlled and (in the case of freeze-drying) fast reconstitution times due to a defined porous structure of the matrix [6]. In the context of this review, we will discuss the previous successes that have already been achieved with (freeze-) drying cells, with subdivision into microorganisms, red blood cells, platelets, spermatozoa and eukaryotic cells. We will further discuss the most common excipients as well as (bio-) technological aspects to load excipients intracellularly with the aim of increased stabilization efficacy. In general, the goal of any kind of drying is to enable long term storage. Due to the time that is required for long term stability studies and the fact that many researches did not even reach the goal of stability after the drying process itself, a major part of the results discussed in this review were obtained directly after drying. Therefore, mainly cell damage caused by freezing and/or drying is discussed. If storage stability studies were conducted, we particularly emphasize that the results were obtained after longer periods of storage.

II.1.1 Damages during drying

II.1.1.1 Freeze-drying

Freeze-drying is a particularly gentle drying method and is divided into three different steps, each of which poses the risk of potential cellular damage: freezing, primary drying and secondary drying. After all solutes are frozen, vacuum is applied to remove frozen water by sublimation with its onset below the triple point of water (primary drying). Subsequently, unfrozen water is removed during secondary drying at elevated shelf temperatures. The initial product can be retrieved by adding the amount of removed water, which is called rehydration or reconstitution [1], [7].

The freezing of living cells is described in detail by P. Mazur. He explains the damaging processes of intra- and extracellular freezing and describes equations that can predict the kinetics

of water loss and the likelihood of intracellular freezing [4]. In short, cell damage can be caused by either intra- or extracellular ice crystal formation, which is mainly dependent on the applied cooling rate [8], [9]. Typically, nucleation and ice formation occurs first in the external medium; the plasma membrane acts as a barrier and prevents the intracellular growth of ice crystals, which results in intracellular water remaining unfrozen and supercooled. As the crystals continue to grow, the concentration of extracellular solutes increases and therefore creates a hypertonic solution. This osmotic imbalance is compensated by elevated water efflux, which leads to cellular shrinkage, also referred to as "freeze-dehydration" [4], [13], [14]. In this concentrated, brine solution, cell organelles and proteins are densely packed, which can lead to protein degradation due to unwanted interactions and chemical reactions.

The formation of intracellular ice and sharp ice crystals can rupture cell organelles and membranes, which usually leads to cell death. The kinetics of nucleation and ice formation are mainly influenced by the applied cooling rate and determine whether the formation of intra- or extracellular ice is favored [4]. At higher cooling rates there is insufficient time for water to move down the chemical gradient from the more dilute intracellular space to the concentrated extracellular medium. As a result, a higher amount of supercooled water remains intracellularly, which crystallizes upon further cooling. However, solutes vitrificate more easily and cells are exposed to the concentrated hypertonic solution for a shorter time [8], [9]. At slower cooling rates, water has sufficient time to exit cells, thus leading to a more pronounced osmotic stress and more shrinkage. Furthermore, P. Mazur states that a major part of slow freezing injury is due to the reduction of surrounding liquid that decreases with more extracellular frozen water. It is therefore important to choose an optimized freezing rate, in order to find the best balance between intracellular ice formation and osmotic stress followed by shrinkage [4].

Once cells are frozen, water is removed by sublimation, which is especially critical for the cell membrane and poses the risk of fusion and phase transition [10]. Under normal circumstances, the phospholipid head groups of the cell membrane are hydrated by water molecules that form hydrogen bonds and separate the head groups from each other. The distance between the phospholipids is maintained, the membrane is flexible and has a phase transition temperature (Tm) below ambient temperatures. Maintained above this temperature, the cell membrane is in a liquid crystalline phase, whereas if the temperature falls below Tm, the membrane converts to a gel like phase [10]–[13]. During drying, water removal leads to a loss of the hydration shell around the head groups and results in denser packing. This distance reduction enables higher van der Waals forces and promotes increasing interaction between the phospholipids. As a consequence, the phase transition temperature Tm at which the lipid chains melt increases and the membrane undergoes a phase transition from a liquid crystalline to a gel like phase [12]. For example, Tm of hydrated POPC is below ambient temperatures (-7°C) and gets elevated to 57°C

in the dry state. Thus, at RT the dry membrane is then in a gel like phase [14], [15]. Figure II-1 schematically illustrates the effect of dehydration on phospholipid membranes.

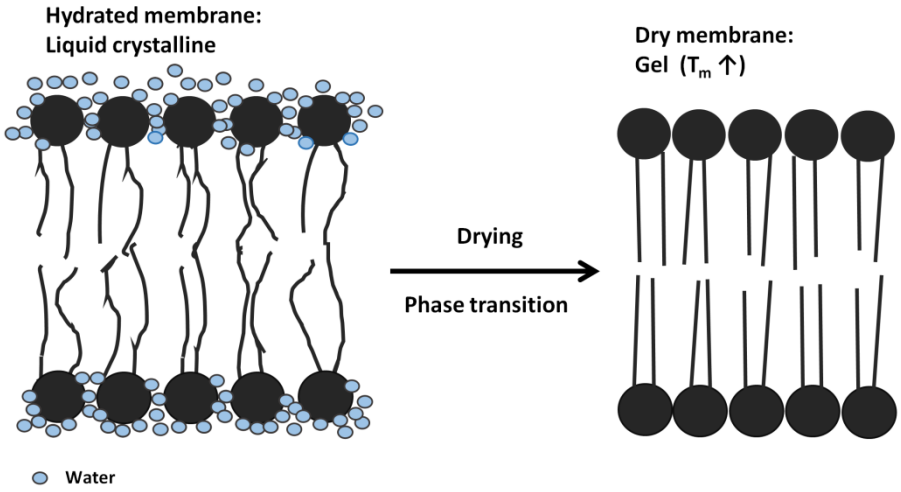

Figure II-1. Cartoon illustrating the effect of dehydration on phospholipid bilayers.

This phase transition occurs unevenly throughout the membrane and can be accompanied by leakage of intracellular cell components [10], [13].

Another consequence of the water removal is the greater possibility of interaction for the phospholipids as a result of the reduced distance between them. This can lead to fusion of parts of the membrane, which is most likely also associated with leakage [10], [15]. Beside the cell membrane, intracellular structures and proteins are also impaired during drying, since the loss of intracellular water also affects hydrogen bonds between water, proteins and other molecules. Formerly separated structures are forced to interact, which then enables reactions and aggregation processes [10]–[12], [16].

Although rehydration is not part of the freeze-drying cycle itself, it involves various situations that can complete the death of already affected cells [7], [12]. When the rehydration medium is added, surrounding excipients dissolve, while the cell membrane represents a barrier and excess water cannot immediately penetrate intracellularly. The cells are exposed to a highly osmotic situation, which can be decisive for the survival of the already affected cells. Furthermore, as described above, the lateral spacing between the phospholipid head groups decreased during drying and the membrane converted to a gel like phase. Addition of water enables the phospholipids to return to their initial, space separated condition and Tm decreases again to lower temperatures. As a consequence, membranes undergo a “reversed” phase transition from the gel like to the liquid crystalline phase and excess water enables leakage of intracellular components to the extracellular space [12].

II.1.1.2 Other drying techniques

Besides freeze-drying, also alternative drying methods were used to desiccate cells. Most of them include drying through evaporation, either under an air flow, or in an airtight closed box in the presence of a desiccant, but also spray-drying was applied [17]–[20]. During air-drying, the culture medium was either aspirated before drying and cells were dried without further excipients or cells were resuspended in a certain drying buffer. Slight vacuum (around 70 Torr) can be applied to enhance drying speed and to achieve lower residual moisture contents (vacuum-drying, VD) [21], [22]. To improve the uniformity of residual water throughout the sample, Li et al. introduced a spin-drying technique which led to an even residual moisture distribution as well as fast drying times [23]. In contrast to freeze-drying no expensive equipment or special knowledge is needed for air-drying. Furthermore, freezing damage can be avoided and regulation of desired residual moisture contents is very easy, but only relatively high residual moisture contents can be achieved. On the other hand, cells without freezing have longer exposure times to the resulting osmotic gradient and no immobilization of cellular structures is granted. As a consequence, microbiological and chemical reactions as well as ROS formation are not inhibited [24], [25].

II.2 Excipients

Various excipients of different chemical structures have been established to stabilize cells and proteins during freezing and drying.

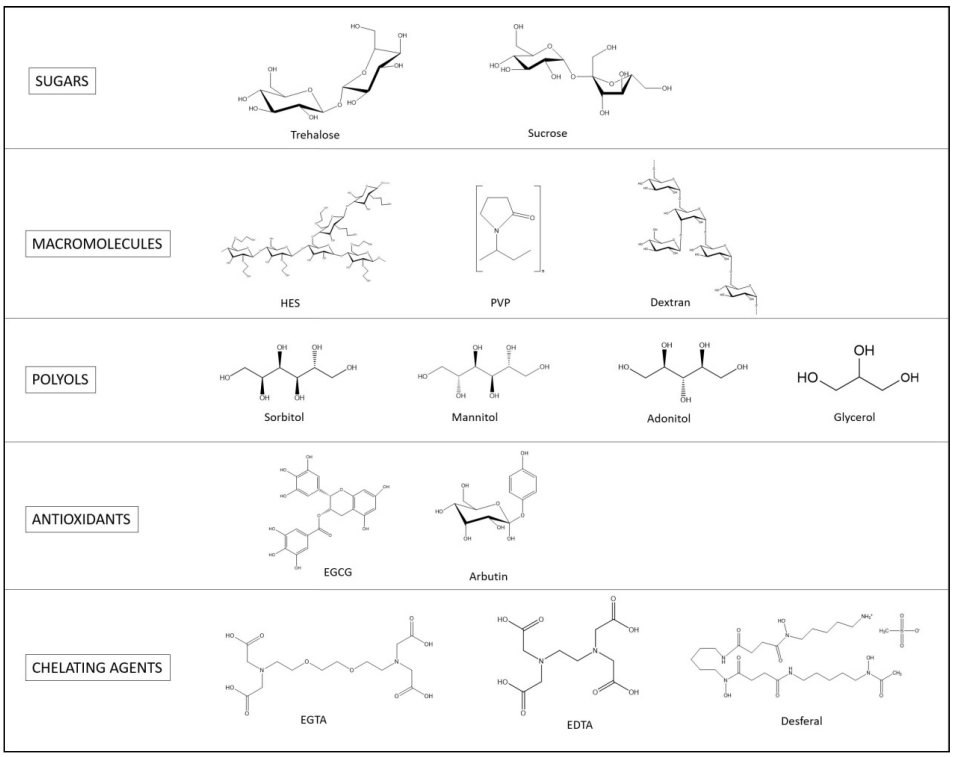

Figure II-2. Chemical structure of introduced excipients.

Given that freezing and drying are fundamental different stress factors on proteins and cells, distinct protection strategies must be applied, respectively [15]. Consequently, excipients can be classified into lyoprotectants and cryoprotectants (CPA). Lyoprotectants are molecules, that provide more stability during both freezing and drying, whereas cryoprotectants enhance stability during freezing/thawing and provide only little stability during the drying step [26]. In this work we want to summarize the most common and promising excipients, divided into sugars, macromolecules, polyols, antioxidants and chelating agents that were used to stabilize cells during (freeze-) drying (see Figure II-2).

II.2.1 Toxicity of stabilizing excipients

Using stabilizing excipients, their toxicity has of course to be taken into consideration. Toxicity of CPAs can be classified into specific toxicity towards the to be protected cells (such as the polymerization of the actin cytoskeleton of stallion sperm by glycerol in concentrations above 1.5), or non specific toxicity that is commonly assessed for all excipients. The specific toxicity of CPAs has been widely discussed, whereas for lyoprotectants there is only limited information in this direction. The concentration of CPAs is a crucial factor, since it must be high enough to assure stabilization, but also correlates with higher potential toxicity [27].

Small, membrane impermeable molecules (e.g. trehalose) can lead to a hypertonic formulation, inducing osmotic stress and eventually cell damage. During freezing and extracellular ice formation, upconcentration of the applied excipients can lead to even higher and eventually toxic concentrations. Also during air-drying, cells are exposed to an osmotic active solution, that further concentrates with proceeding drying time, which is even more critical, as cells are maintained at room temperature. Satpathy et al. reported increasing %-hemolysis, when Red Blood Cells (RBC) were incubated with high amounts of trehalose before lyophilization [28].

In case of (-)-Epigallocatechingallate (EGCG), protective and toxic effects are closely related, as they are based on the same mechanism. Natan et al. described that EGCG stabilizes cells during drying by cell membrane interactions, which in return are also made responsible for proliferation inhibition after reconstitution [5].

In general, the formation of hydrogen bonds is said to be essential for vitrification and water replacement, however, strong hydrogen bonding might on the other hand lead to a disruption of the hydration shell of macromolecules and proteins. Fahy et al. concluded that, as a general rule, the toxicity for CPAs is reduced, if less water molecules are bound to the CPA, thus still assuring vitrification and inhibition of water-water interactions [29], [30].

II.2.2 Sugars

II.2.2.1 Trehalose

Anyone working on the drying of cells inevitably encounters the non reducing disaccharide trehalose, which is also found in many anhydrobiotic organisms. Trehalose plays an important role for the stabilization of biomaterials in a dry state, even though its mechanism is not fully understood yet [31], [32]. The role of trehalose is also described by Crowe et al. [32]. Briefly, they discussed if trehalose must be considered as special compared to other sugars and also gave a short overview about the applied techniques to introduce trehalose intracellularly.

As previously described, an increase of the phase transition temperature Tm during drying is associated with leakage during drying and especially reconstitution. Trehalose can counteract this unwanted event by lowering Tm by approximately 10°C below the fully hydrated membrane and as much as 80°C for the dried bilayer [10], [33]. This means that, even in a dry state, the cell membrane remains in the liquid crystalline phase without undergoing the critical phase transition during drying and rehydration [10], [31], [34]. The so-called water-replacement-hypothesis suggests an interaction of trehalose with the polar head groups of the phospholipids. Due to its small size and various hydroxyl groups, trehalose has the ability to replace water and instead build hydrogen bonds with polar residues. The membrane remains in a “hydrated” state even in the absence of water, which prevents membranes from dense packing and increased interactions (see Figure II-3).

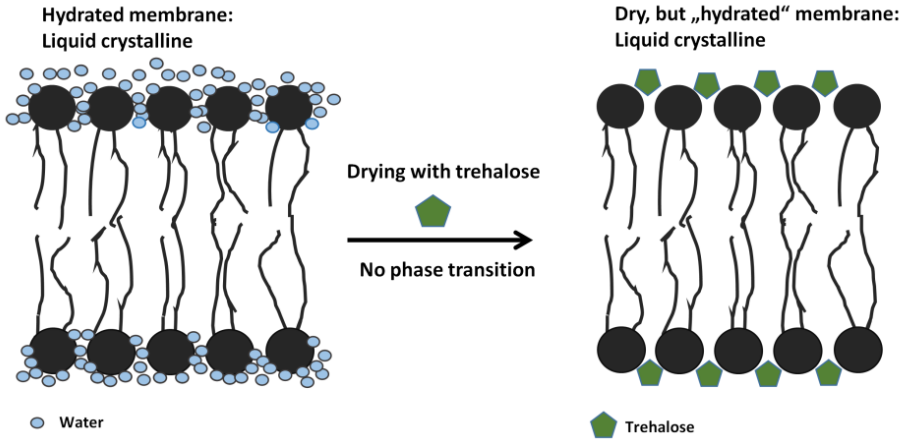

Figure II-3. Cartoon illustrating the stabilizing effects of trehalose when added to a phopholipid bilayer during drying.

Additionally, trehalose is known to vitrificate and to form amorphous glasses. During water removal and solute upconcentration, a viscous liquid forms, thus reducing fluctual movements and ice formation within the sample. Membranes have less interactions, cannot coalescence and as a consequence fusion and leakage are reduced [10], [15], [35].

The glass transition temperature of dry amorphous substances is referred as Tg and is the temperature below which the physical properties change from a glassy to a rubbery phase. This must not be confused with Tg', which is the glass transition temperature of the maximally freeze concentrated solution during freezing. However, despite trehalose also other sugars are able to build amorphous glasses and to form hydrogen bonds to replace water in the dry state. Consequently, Crowe et al. was very right to ask, why trehalose is such predominant in comparison with other sugars [32], [36]. This is partly attributed to the glass transition temperature Tg of trehalose, which is much higher than for other sugars and Koster et al. even proposed that the Tg of sugars must exceed Tm in order to depress Tm in the dry state [37]. The high Tg of trehalose is no exceptional phenomenon, as the Tg of trehalose is just at the end of a row of increasing Tg values for different sugars [38]. Furthermore, amorphous trehalose can diminish the influence of small amounts of adsorbed water vapor, by forming a crystalline dihydrate, without influencing amorphous trehalose parts. This makes products that were dried with trehalose more resistant to suboptimal storage conditions or higher water contents [32]. Crowe et al. therefore concluded that trehalose can be compared with other sugars, but has beneficial properties at suboptimal conditions, facilitates higher storage temperatures and tolerates higher water contents [1], [15], [39].

However, trehalose was found to be more efficient, if it is present both inside and outside the cell. Because sugars do not easily penetrate membranes, many mechanisms were established to introduce excipients and especially trehalose into cells, which will be further discussed in a later section.

II.2.2.2 Other sugars

Even though trehalose is by far the most prominent sugar, its high costs can be a potential limitation in its industrial use [40]. Therefore, the stabilizing properties of other sugars, in particular disaccharides (lactose, sucrose or maltose), monosaccharides (glucose, galactose, ribose, fructose, myo-inositol or mannose) or even trisaccharides (raffinose) were also studied. In general, disaccharides showed better results than mono- or trisaccarides, but only sucrose, a non-reducing disaccharide, composed of glucose and fructose, resulted in promising results. There is growing evidence that sucrose can provide a similarly efficient protection as trehalose, but – owing to its weaker tendency to interact with cell membranes and stronger tendency to crystallize – higher concentrations of sucrose are required [1], [13], [40]. Like trehalose, other sugars are supposed to depress Tm by water replacement at the phospholipid head groups and to inhibit fusion by vitrification. Leslie et al. stated that both sucrose and trehalose lower bacteria membrane phase transition in a similar way, allowing them to be rehydrated above Tm and to avoid the phase transition during rehydration. However, the use of trehalose resulted in higher viabilities for both tested bacteria strains [41]. Costa et al. compared various sugars (trehalose,

sucrose, lactose, glucose, fructose) and reported 100% viability (CFU/ml) for cells that were freeze dried with sucrose, after optimization of the reconstitution process and initial cell concentration. However, they also used a higher sucrose concentration compared to the other applied sugars [40]. Trehalose is also the most dominant sugar for drying RBCs, *platelets* and eukaryotic cells, although Li et al. showed 61.9% recovery of mononuclear cells after drying them in a combination of sucrose, mannitol, PVP and fetal bovine serum [32], [13].

II.2.3 Macromolecules

Many efforts have been made to discover the mode of action of macromolecules to stabilize cells in a dry state. Impeded by their size, polymers cannot penetrate cell membranes and are not involved in intracellular protein stabilization. Additionally, macromolecules are sterically hindered from direct interaction with the head groups of membranes and it can be concluded that polymers have no effect on the phase transition temperature Tm or the associated leakage of cell components [42], [43]. However, macromolecules are known to have a relatively high Tg, increasing with their molecular weight, which can be beneficial in a twofold manner: On the one hand, faster drying times can be obtained, as the high Tg' of polymers enables drying at higher shelf temperatures [44], [45]. On the other hand, the ability to prevent crystallization and to form amorphous glasses minimizes cell rupture during freezing and reduces fusion and leakage during drying and reconstitution [42]. Moreover, several macromolecules, including polysaccharides, strongly absorb water and lead to a relatively high viscosity, which further reduces water efflux and osmotic stress [43]. However, it is widely accepted that polymers cannot be used solely as excipients and must be combined with other molecules. As additional excipients, they may reduce the required concentrations of other excipients, thereby avoiding toxic concentrations. Crowe et al. stated that for a sufficient stabilization of membranes, both inhibition of fusion and Tm depression are necessary; these can be achieved by combining polymers with other molecules such as sugars [42], [10]. Goodrich et al. reported already in 1992 that macromolecules in combination with sugars can successfully stabilize RBCs. They further specified that the polymer should be amphipatic, with hydrophobic and hydrophilic properties to be most effective for stabilization [46]–[48].

II.2.3.1 Hydroxyethyl starch (HES)

HES is known for its very low toxicity and application as plasma expander for the treatment of hypovolemia [49], [50]. HES is a complex carbohydrate composed of α(1→4) linked glucose with partly substituted hydroxyl groups. It can be categorized by its molecular weight, its degree of molar substitution (number of hydroxyethyl groups per mol glucose) and its substitution pattern (Ratio C2 substitution/C6 substitution). It is important to note that HES qualities differ from manufacturer to manufacturer, which has to be taken into account if different HES batches are

used [51], [43]. HES is widely used to stabilize cells during (freeze-) drying, though it is not expected to permeate cell membranes as described above and therefore has no effects on Tm or intracellular structures [42]. As a large polysaccharide, HES is supposed to have a relatively high Tg, but the determination of exact values for Tg and Tg' of HES or HES-mixtures is difficult. HES shows a broad glass transition with a small specific heat capacity, leading to values from 44°C to 98°C as described in the literature [42], [52], [53]. Differences are probably due to different molecular weights and extrapolation difficulties, but if HES derivatives with higher molecular weight are chosen, a high Tg can be assumed. Goodrich et al. used HES in combination with glucose to successfully stabilize RBCs during freeze-drying [46], [47]. Also eukaryotic cells, such as hematopoetic stem and progenitor cells, were lyophilized with HES, BSA and intracellular trehalose, which was followed by intact clonogenic activity and colony formation [54].

II.2.3.2 Other polymers

The most commonly applied macromolecule is HES, but also other polymers can be used to enhance cell condition after (freeze-) drying cells. Dextran (MW 40 kDa) was used in combination with the antioxidant (-)-Epigallocatechingallate (EGCG) or trehalose to freeze-dry mononuclear cells. With this dextran/EGCG combination, a viability of 25% and a recovery rate of 62% after freeze-drying were obtained. Viability was defined as the percentage of living cells, measured with live/dead staining of fluorescent dyes (Syto-13/PI). In general, recovery is calculated as the cell concentration or CFU/ml after freeze-drying, divided by the cell concentration before freeze-drying (multiplied by the factor 100), regardless if cells were dead or alive [5]. Following the theory of Crowe and Goodrich that a polymer can only show its protecting characteristics in combination with a sugar, a combination of dextran, EGCG and trehalose could have resulted in even higher survival rates. Zhang et al. reached 40% viability (measured with trypan blue exclusion) of freeze-dried MSCs with 30% PVP 40 and trehalose. They also assumed that PVP 40 inhibits crystallization and stabilizes an amorphous phase [6]. Li et al. also used PVP (MW 10000-70000 kDa) in combination with sucrose, mannitol and FBS to freeze dry MNCs and achieved recovery rates of 62% [55].

Even though the effects of macromolecules have not been studied systematically, they are widely used and it can be concluded that they have some positive influence on cell survival when applied in combination with sugars or other excipients. Additionally, macromolecules improve the physico-chemical properties of lyophilizates after and during freeze-drying. With a high Tg', they allow for more aggressive and time saving freeze-drying cycles; furthermore, the resulting high Tg makes storage at higher temperatures possible.

II.2.4 Polyols

Polyols are widely applied to cryopreserve cells. Glycerol especially has become a mundane cryoprotectant; however, the sugar alcohols sorbitol and mannitol were also in the focus of research. It remains to be evaluated whether they also exhibit adequate properties to stabilize cells in a dry state.

II.2.4.1 Glycerol

Glycerol is a trihydric alcohol with a structure of propane substituted at positions 1, 2 and 3 by hydroxyl groups. It is a viscous, water miscible liquid with a remarkably low toxicity (LD_{50} human: 1428 mg/kg) [57]. Glycerol can act as a hydrogen bond acceptor or as a donor. Due to its small size, it can also replace water by forming hydrogen bonds with intracellular structures and the head groups of the cell membrane [56]. Dashnau et al. described the behaviour of water and glycerol with molecular dynamics simulations and showed a strong interaction, driven by hydrogen bonds [57]. Because of these water binding properties, glycerol can suppress ice nucleation and finally leads to more unfrozen water and a change in vitrification and glass formation [29]. Additionally, it was shown that glycerol can penetrate cell membranes either by facilitated or passive diffusion and can also access and stabilize intracellular components [58]–[60]. Due to this intracellular presence, glycerol is able to minimize cellular shrinkage by balancing the increasing extracellular osmotic pressure that results from the concentration of extracellular solutes [61].

Mittal et al. combined extracellular trehalose with the membrane permeable glycerol to investigate the desiccation tolerance of adult stem cells during air-drying. Even if they did not particular address the question of the effect of glycerol, they could achieve results with 40% of cells still showing intact cell membranes [62]. After freeze-drying and reconstitution of bacteria, glycerol led to increased survival rates compared to water or maltodextrin, but still resulting in lower absolute numbers than trehalose or non-fat skim milk as drying excipients [63]. Bircher et al. compared the effects of inulin, sucrose and glycerol on 6 different bacteria strains and reported enhanced viability (MPN method, optical density) after cryoconservation with glycerol, whereas enhanced viability after freeze-drying could be only observed for inulin and sucrose. They even found a decreased viability of fresh bacteria after incubation in glycerol and attributed this to its relatively high osmotic pressure [61]. Abadias et al. could also not confirm any positive effect of glycerol on stabilization of *Candida sake* during lyophilization [64].

These findings suggest that the protective attributes of glycerol during cryopreservation cannot be easily transferred to (freeze-) drying cells and that glycerol is only a good cryoprotectant, but no lyoprotectant. Moreover, glycerol is not solid at RT and has a very low Tg (-93°C), which negatively influences the physico-chemical properties of a freeze-dried product due to a

pronounced Tg depression [65]. Several researchers reported that glycerol inhibits the formation of dry lyophilized cakes and obtained only insufficiently dried products with the addition of higher amounts of glycerol [64], [66]. Thus, if applied, its concentrations must be kept as low as possible and it should be combined with another excipient, that counterbalances this Tg depressing properties.

II.2.4.2 Sorbitol, Mannitol and Adonitol

The two sugar alcohols sorbitol and mannitol only vary in the configuration of the hydroxyl group of the carbon atom C2, but this leads to some more differences in their behavior during freeze-drying. Both sorbitol and mannitol form amorphous glasses during freezing, but throughout warming above the glass transition temperature Tg', amorphous mannitol transforms into a more stable crystalline form. These mannitol crystals can rupture cell membranes and other cell organelles, so mannitol needs to be maintained in its amorphous state, which then again complicates storage [65]. With their many hydroxyl groups, sugar alcohols are able to form hydrogen bonds and can replace water in the dry state, yet they seem less effective in stabilizing membranes and proteins than sugars. One explanation for this could be reduced flexibility with increasing number of carbon atoms in the backbone, which could lead to reduced opportunities for interaction with the cell membrane and its phospholipid head groups [67]–[69]. Besides that, Shen et al. hypothesized that the permeability of mannitol or sorbitol through cell membranes is reduced compared to glycerol, which may inhibit interaction with the inner side of membrane head groups and other intracellular structures [70]. Nevertheless, some researchers reported success in the stabilization of cells with sorbitol. In 1997, Linders et al. found that sorbitol enhances the dehydration resistance of *Lb. plantarum* in a more significant manner than trehalose [71], [72]. Furthermore, in 2009 Carvalho et al. proposed a protective effect of sorbitol during long term storage (1 to 8 months at 20°C) of lyophilized bacteria strains, even if no higher survival rate was observed directly after lyophilization [73]. However, also lack of success of sorbitol and mannitol to stabilize cells during drying was reported. As already mentioned, Abadias et al. tested the protective properties of various excipients, but neither mannitol, nor sorbitol could reach similar effects to those of sugars [64]. Similar findings were made during freeze-drying of malolactic bacteria, where sugars also appeared to be superior to polyols. This lack of activity was explained by the crystallization of mannitol and the associated formation of ice and mannitol crystals [68].

Adonitol, also known as ribitol, is a 5 carbon sugar alcohol and can be originated by reduction from the sugar ribose. In a polymerized form, ribitol is part of the cell wall of many gram positive bacteria, forming a part of the wall teichoic acids [74]. In 1983, De Valdez et al. tested many polyols (adonitol, dulcitol, glycerol, mannitol and sorbitol) on 12 different lactic acid bacteria (LAB) strains, with little or no effect for all polyols except adonitol, followed by glycerol.

Throughout all strains a very high survival rate, reaching up to 100%, could be achieved with the addition of adonitol to 0.75 M non-fat skim milk (NFSM). They explained the low effect of sorbitol and mannitol by the metabolization of LAB, whereas adonitol represented no substrate. They hypothesized further that the esteric structure of adonitols hydroxyl groups can better interact with cellular structures than other polyols [75]. Abadias et al. confirmed some effect (maximum ~ 25% viability, determined by CFU/ml) of adonitol during freeze-drying of *C. Sake*, but higher viabilities were achieved with sugars than with polyols [64].

II.2.5 Antioxidants

to the excipients mentioned above, a wide range of small molecules such as antioxidants were used in lower concentrations to prevent damages resulting from oxidative stress. Osmotic or cold shock during freezing, but also desiccation of cells can lead to the formation of reactive oxygen species (ROS). ROS can react with cellular components and initialize oxygen-based damaging reactions such as lipid oxidation or peroxidation of proteins and nucleic acids [76]. This may induce disruption of membranes, DNA single and double strand breaks, impaired cellular signalling and finally apoptosis. A shift from redox homeostasis towards ROS formation is called oxidative stress. To avoid the latter, antioxidants are widely applied in cryoconservation of particularly reproductive cells. However, until now, antioxidants are implemented only to a very little extent to stabilize cells during (freeze-) drying [77].

Arbutin is a naturally occurring β-D-glucopyranoside derivative of hydroquinone and was found in high concentrations in dry leaves of *Myrothamnus flabellifolia*. Applied during vacuum-drying of mesenchymal stem cells (MSCs), an increase in viability (as measured by propidium iodide exclusion) was noted; additionally, the ability of MSCs to differentiate down the osteogenic pathway could be preserved. Its strong antioxidative properties and the inhibition of ROS formation might have contributed to these stabilizing effects. Furthermore, the authors could show that arbutin induces the expression of HSP70, a heat shock protein that can function as protein stabilizer and is known to inhibit apoptosis. Considering the amphiphilic properties of arbutin, it is also possible that arbutin interacts with cell membranes and decreases the membrane phase transition Tm in the dry state. Interestingly, the effect of Arbutin was not universally applicable to all cell types and toxic effects were recorded for other cell lines, such as 193H and murine B cells [22].

Another used excipient of vegetable origin is (-)-Epigallocatechingallate (EGCG), a polyphenol naturally found in green tea with a wide range of physiological attributes, such as antioxidative, antibacterial, anticarcinogenic or antiviral effects [5], [78]. Natan et al. added 0.945 mg/ml EGCG to a formulation containing 0.1 M trehalose and reached up to 91% survival (live/dead fluorescent staining with Syto-13/PI) of MNCs which were still able to form colonies. They hypothesized that

EGCG on the one hand reduces ROS formation and on the other hand stabilizes cell membranes by interaction with its polar head groups. They further argued that EGCG was shown to permeate cell membranes in a concentration-dependant manner, thus reaching intracellular components also. However, proliferation and differentiation inhibition of EGCG was also reported, probably caused by its anticarcinogenic and antiviral properties and the authors therefore suggested removing excess EGCG after reconstitution [5].

Sitaula et al. investigated the protective properties of catalase and the chelator Desferal® in combination with trehalose or sucrose for freeze-drying of bovine sperm [79]. The enzyme catalase is ubiquitous in nature and is a known antioxidant that can decomposit H_2O_2 into water and oxygen [80]. Desferal® is the marketing name of desferrioxamine, a metal chelator that is used originally to treat hemochromatosis and iron poisoning [81]. The authors hypothesized that the combination of a chelating agent and an antioxidant will reduce the formation of ROS by two complementary ways: a chelator to complex free metal ions, thus reducing formation of hydroxyl radicals, and an antioxidant to prevent potential formation of peroxides. However, only Desferal® was able to significantly improve membrane integrity when dried with intra- and extracellular trehalose, whereas catalase could not influence drying results [79]. They concluded that the formation of hydrogen peroxides is not responsible for oxidative stress during drying in bovine sperm, whereas Desferal® could have reduced the production of hydroxyl radicals by complexion of iron (Fe^{3+}), thus minimizing the osmotic stress and lipid peroxidation. The following section will discuss another possible mechanism of chelating agents, which might also have contributed to the success of Desferal® compared to catalase when freeze-drying sperm.

To summarize, only few antioxidants have been successfully introduced as protectants during (freeze-) drying and their performance cannot be explained only by inhibition of ROS formation: otherwise, the implementation of other antioxidants would also have been successful. Many researchers therefore assumed an additional membrane stabilizing effect by direct interaction with the phospholipid head groups.

II.2.6 Chelating agents

It was found during (freeze-) drying of sperm that membrane damage leads to an altered intracellular ionic homeostasis, which facilitates the release of endonucleases that can be activated by Ca^{2+} and Mg^{2+}. Chelators were applied to complex these Ca^{2+} ions, in order to avoid unwanted endonuclease activity and to enhance DNA integrity [82]. Especially EDTA and EGTA became established as common excipients in the field of spermatozoa preservation [83]. Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) is structurally similar to EDTA, but has less toxicity and a higher affinity to Ca^{2+} ions [84]. Comparing EGTA and EDTA, Kaneko and Nakagata proved that small concentration of EDTA (1 mM) led to similar results as high

concentrations of EGTA (50 mM). Higher EDTA concentrations (50 mM) showed adverse effects, probably owing to its higher toxicity [85]. Nakai et al. reported an advantage of EGTA compared to EDTA, measuring the increase of blastocyte development after ICSI of lyophilized boar sperm in porcine oocytes [86]. Chelating agents appear to be an important excipient class for spermatozoa freeze-drying, however – to our knowledge – no experiments have been published yet, regarding their effect on other cell types.

II.3 Technical approaches

II.3.1 Intracellular excipient loading

Extracellular excipients might be sufficient to stabilize cell membranes and to inhibit extracellular ice crystal formation, but intracellular cell organelles and the inner side of membrane head groups can obviously only be stabilized when excipients are available intracellularly. As a consequence, much effort was made to introduce excipients, in particular trehalose, intracellularly. Table II-1 summarizes the applied techniques to load trehalose intracellularly prior to freeze-drying cells.

II.3.1.1 Endocytotic uptake

Without any further technical requirements, smaller amounts of trehalose can be introduced intracellularly simply by incubation with external trehalose. Different temperatures, concentrations and durations were investigated to find optimum loading conditions [28], [90], [100]. Satpathy et al. reached 75 mM intracellular trehalose after incubation of RBCs with 1 M extracellular trehalose for 24 hours. However cellular viability gets affected with longer incubation times and higher osmolality, therefore a compromise between internal trehalose concentration and cellular impairment must be found [28]. Wolkers et al. used lower extracellular concentrations (0 – 100 mM) and could subsequently achieve lower intracellular concentrations of maximally 20 mM [90]. Throughout literature, best uptake was described for incubation at elevated temperatures, thereby giving a hint on the underlying uptake mechanism [28], [100]. A widely accepted theory explains uptake by fluid phase endocytosis, encouraged by the fact that trehalose uptake is concentration dependent and highest when incubated at elevated temperatures. At temperatures above Tm, plasma membranes are in a liquid crystalline phase with increased mobility, which can facilitate transport through membranes. Additionally, the use of benzyl alcohol as membrane fluidizer increased trehalose uptake in a concentration-dependent manner, supporting the assumption that the physical state of the membrane plays a role in trehalose uptake [28]. It was also shown that several endocytosis inhibitors, such as nocodazole and colchicine could inhibit trehalose uptake. Nocodazole and colchicines block the depolymerisation of microtubules, which are involved in a clathrin-mediated endocytosis, suggesting that this endocytosis mechanism is involved in trehalose uptake [100].

Cells	Loading techniques	Extracellular trehalose	Intracellular trehalose	References
Platelets	Incubation	50 mmol/l 4 h, 37°C	Not mentioned	Fan et al., 2009 , [87]
Platelets	Incubation	40 mmol/l 4 h, 37°C	Not mentioned	Tang et al., 2006, [88]
Red blood cells	Incubation	800 mM 7 h, 37°C	Not mentioned	Török et al., 2005, [89]
Platelets	Incubation	0-80 mM 30 – 250 min, 5-35°C	~ 20 mM	Wolkers et al., 2001, [90]
Platelets	Incubation	45 mM 4 h, 37°C	Not mentioned	Wolkers et al., 2003, [91]
Red blood cells	Incubation	0 - 1000 mM, 7 h, 37°C	75 mM	Satpathy et al.,2004, [28]
MSC	Incubation	70 – 100 mM 24 h, 37°C	Not mentioned	Jamil et al., 2005, [22]
MSC	Incubation	10 – 200 mmol/l 24 h, 37°C	16 mmol/l	Zhang et al., 2010, [6]
Human embryonic kidney cells	Incubation	100 mM 24 h, 37°C	~ 40 mM	Ma et al., 2005, [21]
Human corneal epithelial cells	Incubation	2 – 200 mM 15 min	Not mentioned	Matsuo et al., 2001, [92]
NIH 3T3 murine fibroblasts	H5 Pore formation	0.2 M 45 min	Equilibrium concentration	Acker et al., 2002, [17]
NIH 3T3 murine fibroblasts	H5 Pore formation	0 – 0.5 M 1 h	Equilibrium concentration	Chen at al., 2001, [20]
Mouse spermatozoa	H5 Pore formation	0.5 M	Not mentioned	Mc Ginnis et al., 2005, [93]
Human foreskin fibroblasts	Thermal/osmotic shock	50 mM	5.1 nM/10^6 cells	Puhlev et al., 2001, [94]
Mouse embryonic fibroblasts	Freezing induced uptake	250 mM	Not mentioned	Zhang et al., 2017, [95]
HSC	P2X7/P2Z receptor (ATP)	200 mM	Not mentioned	Buchanan et al., 2010, [54]
J774.A1 macrophage cells	P2X7/P2Z receptor (ATP)	225 - 500 mM	~ 130 mM	Elliot et al., 2006, [18]
Bovine spermatozoa	P2X7/P2Z receptor (ATP)	0.2 M	9.27 µg/ml	Sitaula et al., 2009 and 2010, [79], [96]
Mouse fibroblasts ($LMTK^-$)	Trehalose synthesis genes	-	~ 80 mM	Garcia de Castro and Tunnacliffe, 2000, [97]
Human primary foreskin fibroblast (F12)	Trehalose synthesis genes	-	1.0–1.5 nM/10^6 cells	Guo et al., 2000, [98]
Chinese Hamster Ovary Cells	Trehalose transporter genes	400 mM 4 h	23.45 ± 2.67 mM	Chakraborty et al., 2012, [99]
HepG2 cells	Trehalose transporter genes	50 mM 24 h	0.0217 pmol/cell	Li et al., 2010, [23]

Table II-1. Applied trehalose loading techniques prior to freeze-drying cells.

II.3.1.2 Gene transfection

Another smart method is the implementation of trehalose biosynthetic enzymes into cells by gene transfection. Already in 1999, Guo et al. expressed two genes from E.coli (otsA and otsB), which encode trehalose biosynthetic enzymes in human fibroblasts, which resulted in a concentration of 1.0 – 1.5 nM (0.3 – 0.5 pg/cell) trehalose per 10^6 cells [98]. In the year 2000, Garcia de Castro obtained an intracellular trehalose concentration of 40 pg/cell (80 mM) in a mouse cell line [97]. Upon drying of these genetically improved cells, Guo et al showed cellular survival for up to five days, whereas Garcia de Castro only reported increased osmotolerance but no survival of any cells. This is especially interesting as the internal trehalose concentrations differ in a remarkable manner, with the lower concentration being more successful.

With the recent discovery of late embryogenesis abundant proteins (LEA) in desiccation tolerant organisms, these became the focus of intense research. LEAs were first discovered in plants, especially during the embryogenic development of plant seeds; however they were also found in bacteria, fungi and animals [101]. Marunde et al. expressed two LEA proteins (AfLEA1.1/3) in *Drosophila melanogaster* cells and reported an enhanced desiccation tolerance and higher mitochondrial functionality [102]. Trehalose uptake and LEA synthesis were combined when human hepatoma cells were transfected with genes encoding for two LEA proteins and for a trehalose transporter. Cells showed almost 100% membrane integrity after spin-drying to a water content below 0.12 g H_2O/g dry weight and subsequent staining with Syto13 and Ethidiumbromide [23]. However, the constant supply of transfection material such as adenovectors and a significant toxicity, especially after multiple infections, led to a limited application of the implementation of trehalose transporter or protein synthesis genes [98], [103].

II.3.1.3 Self-assembling pore H5

Self-assembling porating molecules have been found by an exotoxin of *Staphylococcus aureus*, which is responsible for the poration of cell membranes followed by cell lysis. By site-directed mutagenesis of this α-toxin, Russo et al. designed a molecule, referred to as H5, which forms "*uniform 2-nm transmembrane, water-filled pores specially designed with a metal-actuated switch*", which permits passage of molecules up to 1000 Da. The opening of the pores can be reversed and controlled by the addition of Zn^{2+}. They proved cellular viability throughout the entire treatment and that cells were able to attach and showed normal proliferation rates [104]. With this method, high internal trehalose concentrations can be achieved, as an equilibrium between external and internal trehalose is proposed and no transport restrictions need to be overcome. Eroglu et al. used H5 to introduce trehalose intracellularly (external trehalose concentrations up to 0.4 M), without loss of cellular function [105]. Chen et al. reported successful preservation of plasma membrane integrity of fibroblasts over a storage period of several weeks, when they were air-dried after a treatment with H5 and trehalose. However, it must be mentioned that the water content after drying was 15 wt% and at lower water contents, the rate of membrane intact cells drastically decreased [20]. Yet, with the use of H5 an easy method was introduced to load trehalose and other excipients intracellular, which may lead to promising results in the future.

II.3.1.4 $P2X_7$/P2Z receptor

The $P2X_7$/P2Z receptor presents another technique to load trehalose intracellularly. This plasma membrane receptor is expressed by a variety of cells and is activated by fully dissociated extracellular ATP^{4-}. Stimulating the receptor with higher ATP doses induces a pore formation, enabling nonselective permeation for lower molecular weight molecules (< 900 kDa). The addition of magnesium can complex excess ATP and pore formation can be reversed [106], [107].

Elliot et al. reported up to 50 mM intracellular trehalose after ATP poration of mouse macrophages, with subsequent significant improvement of desiccation tolerance [18]. Moreover, Buchanan et al. proved clonogenic differentiation from stem cells after ATP poration and trehalose loading after freeze-drying [54]. The $P2X_7$/P2Z was also used to load trehalose and other sugars intracellularly in the freeze-drying of sperm, to enhance membrane integrity and even sperm motility [79], [96]. However, the use of ATP poration has some drawbacks, as not every cell line expresses the required receptor and ATP concentration and incubation time must be carefully chosen to reduce cell toxicity.

II.3.1.5 Required internal trehalose

Many techniques to load trehalose intracellularly have been established, but the question about the exact amount of intracellular trehalose needed to sufficiently stabilize cells in the dry state is still a point of discussion. What is the minimum number of trehalose molecules to effectively replace water at cell membranes and proteins and additionally to promote vitrification? Assuming that anhydrobiotic organisms produce and accumulate trehalose in an adequate amount to withstand desiccation, their internal trehalose concentration (20 – 400 mM, [108]) might give a clue as to the optimum trehalose concentration. Chen et al. reported – based on their results – an optimal trehalose concentration of 10^9 molecules per cell [20]. Table II-1 summarizes the obtained intracellular trehalose concentrations after trehalose loading. It is apparent that huge discrepancies in internal trehalose concentrations are found, very much depending on the loading technique. Highest concentrations of up to 0.5 M were achieved by poration of cells with H5, being based on the assumption of an equilibrium between extra and intracellular trehalose, which was confirmed by GC-MS analysis [109]. Trehalose incubation and uptake by fluid phase endocytosis could only lead to low concentrations of internal trehalose, whereas uptake via $P2X_7$/P2Z receptor was more efficient, always considering the harmful effects of high external trehalose and ATP treatments. Additionally, the exact determination of the intracellular trehalose concentration requires the knowledge of the cell volume where trehalose can be distributed. Some researchers used the overall isotonic cell volume when calculating the trehalose concentration, whereas others only used the osmotically active isotonic volume. Others still use the trehalose concentrations per cell number [109]. Different trehalose detection methods were also applied, such as the anthrone reaction, HPLC or GC-MS analysis. Because of this variety of approaches, results are difficult to compare and a minimum or required trehalose concentration is hard to predict; it is uncontroversial, however, that higher trehalose concentrations should be aimed for, if the additional osmotic stress is considered. However, Tunnacliffe et al. assumed that intracellular trehalose is necessary, but not sufficient to achieve desiccation tolerance of cells. They speculated that to obtain a successful stabilization of cells in the dry state during storage, also additional

adaptions are necessary, which can be learned by truly anhydrobiotic organisms, such as the use of heat shock proteins, chaperons or a down regulation of cellular metabolism [108].

II.3.2 Freezing rate

The cooling rate and therefore freezing behavior is a critical step during freeze-drying. As explained earlier, a balance between intracellular ice crystal formation and excess osmotic stress and shrinkage needs to be found, which is dependent on the applied cooling rate. As also summarized in Table II-2-5, no universal freezing method has been established and one may get the impression that many researches did not give much attention to the kinetics during freezing. The majority of samples were frozen by immersion in liquid nitrogen or with the use of precooled shelfes, resulting in – depending on freezing volume and shelf temperatures – rapid freezing rates. Especially for microorganisms and sperm, these were the predominant freezing techniques; Abadias et al., however, examined different freezing rates for yeast cells. Freezing of *C. sake* in 10% skim milk by immersion in liquid nitrogen was disadvantageous compared to medium cooling rates (freezing at -20°C over night and drying at -45°C), which resulted in 30% viable cells, determined by CFU/ml before and directly after (freeze-) drying and reconstitution [64]. Zhao et al. reported higher survival rates for two bacteria strains after freezing 24 hours at shelfs precooled to -65°C compared to shelfs precooled to -20°C prior to freeze-drying [68]. However, freezing to -20°C probably does not result in temperatures below Tg' and to our understanding it must be considered that at this temperature, a potential movement within the frozen sample is still possible, which could have also contributed to cellular damage.

In general, the higher the cellular complexity, the more attention was given to freezing procedures and more intermediate cooling rates (1-5 K/min) were favoured. Natan et al. showed best results for freeze-drying mononuclear cells with an intermediate freezing rate of 5.1°C/min, which is in accordance with commonly applied freezing rates. Additionally, they reported a correlation of optimal freezing rates, determined by freeze thaw experiments with freeze-drying experiments, whereas Rindler et al. concluded that freeze thaw results are not a sufficient model to predict cooling rates for freeze-drying [5], [110]. Nevertheless, when the question of an optimal cooling rate for freeze-drying cells was addressed directly, in most cases intermediate cooling rates were found to be superior to rapid freezing. Apparently, intracellular ice formation is more critical to cells than longer exposure times to a hypertonic solution. Therefore rapid freezing in LN or in a -80°C freezer should be avoided and if no controlled freezing device is available, a stepwise freezing protocol should be considered.

II.3.3 Drying and residual moisture content

As previously described, drying is the most critical part during (freeze-) drying and many excipients have been investigated to replace water and to stabilize cellular structures in a dry state.

Despite optimizing the excipient composition, different drying techniques, such as freeze-drying, spray-drying, air-drying or spin-drying have been established, all leading to differences in residual moisture/water content.

In general, lyophilization results in very low residual moisture contents. During the freezing step, ice crystals build a defined porous structure with a large surface area that allows water removal during primary and secondary drying to very low moisture contents. To increase storage stability, residual moisture contents below 1% are desired, however, for special purposes, higher moisture contents may be favoured [111]. During air-drying at ambient temperatures, water cannot be removed as effectively as during freeze-drying, thus leading to higher residual moisture contents. The received practice is therefore to designate remaining water in gram water per gram dry weight (g H_2O/g dw), instead of percent residual moisture. For air-dried samples, moisture contents of 0.1 – 10 g H_2O/g dw are described in literature, which must be considered as water reduction rather than drying. To our understanding, drying to such high water contents will not result in a "dry" product that can be stored for longer time periods, and in most cases these samples are rehydrated and analysed immediately after drying. Many researchers stated a correlation of cell survival with residual moisture content, with higher viabilities for higher remaining water content [20], [54], [102]. Together with the discrepancies in residual moisture content, this makes it very difficult to compare results for dried cells. For example, Li et al. showed 98% membrane integrity for human hepatoma cells, spin-dried to a water content below 0.12 g H_2O/g dw, whereas Natan et al. freeze dried mononuclear cells to a residual moisture content of 4.69 ± 0.07% and reported a membrane integrity of 88%–91% [5], [23]. Big differences exist also within air-dried samples: Acker et al. reported their main results for samples with water contents of 10%, whereas Elliot et al. described results for samples with water contents between 0.5 and 2.5 g H_2O/g dw. [17], [18]. It is therefore necessary to carefully examine the drying technique and especially the remaining water content before comparing results and drying efficacies.

II.3.4 Rehydration

Though rehydration is not particularly part of the (freeze-) drying process itself, its influence on cellular survival cannot be neglected. Parameters such as the rehydration medium, temperature, osmolality or volume can significantly influence damages during rehydration. As described earlier, cells must withstand high osmotic stress during rehydration, until the cells are completely hydrated. In principle, reconstitution with the exact volume that is removed during drying would be sufficient to restore the original condition. However, since it may take some time until the dried solids completely dissolve, a concentration gradient can arise: depending on the osmolality of the rehydration medium, a hypotonic environment can be created until the dried excipients are

fully dissolved. On the one hand, larger amounts of reconstitution volume can enable faster dissolution of the dry solids. On the other hand, high volume of hypotonic rehydration medium (such as water) can be detrimental, since it creates a hypotonic extracellular solution after full reconstitution [12]. As a consequence, rehydration media of higher osmolality, e.g. plasma or buffered solutions are widely used [90], [40]. Costa et al. tested various rehydration media, such as 10% non fat skim milk (NFSM), PTM medium (consisting of 1.5% peptone, 1% tryptone, 0.5% meat extract), 10% sucrose, PBS or water and found highest viabilities for the most complex media, NFSM and PTM. Apart from the prevention of osmotic shock, they hypothesized that complex media might have the ability to repair affected cells [40].

Furthermore, during hydration, the phospholipids return to their initial state and the cell membrane converts back from the gel like to the liquid crystalline phase, undergoing a reversed phase transition. Intracellular components may exit the cells, as excess water enables material transport. However, rehydration at elevated temperatures (e.g. 35-38°C) seems to be beneficial for reconstitution. If Tm is only increased to moderate temperatures during drying, rehydration above Tm can avoid the reversed phase transition of the membranes from gel to liquid [87], [90].

Considering the results, it still appears difficult to give universal rehydration advice, because rehydration processes depend on cell type, cellular damage (influenced by protection efficacy), solubility and concentration of the dry excipients. Zhao et al. showed even different optimal rehydration parameters for two similar malolactic bacteria strains [68].

Wolkers at al. introduced the method of pre-hydration and incubated freeze dried *platelets* in moisture saturated air at elevated temperatures prior to complete rehydration [90]. By introducing small amounts of water (in form of water vapour) to the dry sample, hydrogen bonds between the cell membrane can form and the membrane can return to a liquid crystalline phase without free water present that enables leakage and transport of intracellular components. The exact amount of water and rehydration duration needs to be carefully titrated: if the amount of water is too low, the membrane cannot absorb enough water to be rehydrated. Excess water leads to free water that further dissolves excipients, thus forming a highly concentrated solution, enabling mass transport and leakage [7]. Fan et al. reported a moisture content of approximately 5% as sufficient, which can be achieved by incubation of 15 minutes. Longer incubation times may be required for larger sample volumes and Wolkers et al. examined between 60 and 100 minutes as optimal rehydration time [7], [90]. However, it must be mentioned, that these pre-hydration studies were conducted on *platelets* and no success for other cell types have been reported until now.

II.3.5 Cell density

Another aspect is the initial cell density during the drying process. Typically higher cell concentrations are used for bacteria, sperm and *blood cells*, as isolation or cultivation generally produce higher cell numbers than cultivation of eukaryotic cells. The determination of the maximum cell concentration is not only of interest for an optimized survival rate, but also from an economic point of view. Using the highest possible concentration, smaller volumes can be (freeze-) dried, resulting in a reduction of production and storage costs, while still obtaining a sufficient number of active cells [112].

Wolkers et al. findings showed increased rates of aggregation for cell concentrations above 10^8 platelets/ml, followed by a loss of recovery [90]. Zhou et al. successfully freeze-dried higher platelet concentrations (up to to $4 \cdot 10^9$ platelets/ml), but they also observed a decrease in recovery with higher concentrations [113]. A U-shaped viability curve was also observed for bacteria and MNCs, with a maximum at medium concentrations and a decrease for excessively high concentrations [5], [114]. Even if no exact maximum cell concentration has been discovered, there is evidence that a certain concentration limit exists, above which the survival rates go down. Besides increased aggregation tendencies, a higher cell concentration leads to more cell-cell interactions, which could facilitate fusion and increased mechanical tension between individual cells. Costa et al. showed that the optimum cell concentration is also dependent on the applied excipients. For sucrose, a concentration of 10^{10} CFU/ml was optimal, whereas for NFSK a cell concentration of 10^8 CFU/ml achieved the best results [40]. This indicates that a certain excipient concentration and molecule/cell ratio is required, which can be exceeded by too high initial cell concentrations.

II.4 Current approaches

We aimed to give a short overview of the current approaches of (freeze-) drying microorganisms, spermatozoa, red blood cells, platelets and eukaryotic cells and summarized several publications, however, we make no claims of completeness.

II.4.1 Microorganisms

Prokaryotic cells, such as bacteria, differ substantially from the more complex eukaryotic cells. As the name already suggests, in contrast to prokaryotes, eukaryotic cells have a membrane-bounded true nucleus. The genome size of prokaryotes is between 600 kb and 9.5 Mb, whereas it ranges from 3 – 140000 Mb for eukaryotes. This follows the fact that prokaryotes have limited DNA synthesis rates and that a smaller genome facilitates faster reproduction rates. Usually, prokaryotes have no cytoskeleton, are not compartmentalized, and are in general much smaller in size, even though giant bacteria cells are known to exist [115], [116]. These fundamental differences make bacteria less complex and contribute to a facilitated stabilization in the dry state.

Without compartmentalization or a nucleus wall, excipients can more easily access intracellular structures after passing the outer membrane. Additionally, many prokaryotic cells have at least one DNA replicon and show a different gene distribution among various plasmids [116]. It is therefore possible that potential DNA damage during drying can be counterbalanced with other intact plasmids that might carry a genomic copy. Even though yeast belongs to its own classification and is different from bacteria in composition, it is only slightly more genetically complex [117]. For the sake of simplicity, we bundle bacteria and yeast in the section "microorganisms". In the focus of (freeze-) drying, microorganisms were mainly lactic acid bacteria (LAB), as they are widespread in fermented food production, such as dairy products, wine and meat, but also as medication to improve intestinal microflora balance [118], [73]. The stabilization of gut microbes came into play as personalized probiotic therapies came into focus [61]. The analytical parameter to obtain evidence about cellular conditions of microorganisms after (freeze-) drying is usually the colony forming unit (CFU) per ml of a certain number of bacteria or yeast. Some researchers have also investigated the remaining enzymatic activity or membrane integrity. Morgan et al. summarized concepts for drying microorganisms, with special attention on growth phase, growth medium and initial cell concentration. They further outlined the introduction of desiccation tolerance in microbial cells (pH change, stress), rehydration parameters and packaging and storage conditions. In general, microorganisms are dried in high concentrations (greater than 10^8 cells/ml) to obtain a sufficient number of cells to form new colonies. However, different starting concentrations must be considered, if results are compared. Furthermore, Morgan et al. summarized that there is still no generic drying method for all applications and emphasized that desiccation tolerance cannot be achieved only by the addition of protective agents and recommends a treatment prior to drying to improve desiccation tolerance [112]. Table II-2 summarizes some results of drying bacteria and yeast with different excipients and drying methods. Survival rates after drying varied from 20 to 100%, and cells were able to form colonies after rehydration. No intracellular excipient loading was applied, but addition of trehalose or other sugars into the growth medium enabled cells to accumulate them intracellularly. Carvalho et al. described higher cell survival after freeze-drying, when a LB strain was grown in the presence of fructose, lactose or mannose [119]. Intracellular accumulation of the added carbohydrate is only possible if the sugars are not metabolized as carbon source by the microorganisms [112]. Some researchers have also investigated the effect of different drying rates, rehydration parameters or growth conditions. Many microorganisms were dried in NFSK or serum as basis for additional excipients, whereas for other cell types usually less complex media such as buffer systems were used. With the use of complex drying media, other substances such as proteins or fat can also contribute to a successful stabilization; especially proteins were described to alter the glassy structure of sugars in a positive way [112].

Cells	Drying (Freezing rate)	Excipients	Other approaches	Analytical techniques	Results/Functions after Drying	References
Candida sake	FD (various)	Monosaccharides (1-10%) Disaccharides (1-10%) Raffinose (1-10%) Polymers (1-10%) Polyols (1-10%) Nitrogen compounds (1-10%) Skim milk (10%)	Freezing rate	CFU	Freezing at -20°C: highest survival 10% Skim milk + 10% sucrose: 37% cell viability Some protection with adonitol	Abadias et al., 2000, [64]
6 different bacteria strains	FD (directly LN)	Sucrose (5%) Inulin (5%) Glycerol (15%)		Membrane integrity (Sybr Green/PI) Growth rate (OD) Viability (MPN method)	Glycerol: survival of fresh cells ↓ Sucrose and Inulin: cell viability ↑	Bircher et al., 2018, [61]
Lb. bulgaricus *Lb. plantarum* *Lb. rhamnosus* *Enterococcus durans* *Enterococcus faecalis*	VD	Sorbitol (12.5 mg/ml) Monosodiumglutamate (12.5 mg/ml) In skim milk (11%)		CFU	Differences between bacteria strains Sorbitol: storage stability ↑	Carvalho et al., 2003, [120]
See Carvalho 2003, [121]	VD	Sorbitol (12.5 g/l) Monosodiumglutamate (12.5 mg/ml) In skim milk (11%)		CFU	No survival increase for sorbitol or glutamate Sorbitol: long term storage stability ↑	Carvalho et al., 2009, [73]
Lb. bulgaricus	VD	Sorbitol, Glucose, Lactose, Mannose, Fructose (each 10 g/l) In skim milk (11%)	Addition of sugars to growth medium	CFU	Mannose: highest viability Fructose, lactose and mannose: Storage stability ↑	Carvalho et al., 2004, [121]
Lb. bulgaricus	FD (directly -80°C)	Maltodextrin (11%) Glycerol (1 mol/l) Trehalose (5%) Skim milk (11%) Water		K+ and Na+ content ATPase activity Lipid composition Internal pH β-Galactosidase activity Water activity CFU	Skim milk and trehalose: 25% viability General: ATPase activity↓ Changes in fatty acid composition	Castro et al., 1996, [63]
Lb. acidophilus	FD (directly -LN)	Trehalose (2.5 – 30%) Trehalose–borate systems (20% + 0.1/0.3 mol B/mol)		CFU	Trehalose-borate system: storage stability ↑	Conrad et al., 2000, [122]
Pantoea agglomerans	FD (directly -20°C)	Trehalose (5%), Sucrose (10%), Lactose (7%), Glucose (5%), Fructose (5%) NFSM (10%) Dextran (0.33 M) Sodiumglutamate (1 M) Cystine (0.04 M) Water, Phosphate buffer PEG (0.05 M) Glycerol (5 M)	Initial cell density Rehydration medium	CFU	Sucrose as protectant, NFSM as rehydration medium, 10^{10} CFU/ml: 100% viability	Costa et al., 2000, [40]
13 different bacteria strains	FD (directly -60°C)	Adonitol, m-inositol, Glycerol, Sorbitol (each 0.32 M) In NFSM (10%) or water		CFU	Adonitol: 100% survival rate	De Valdez et al., 1983, [75]

Lb. bulgaricus	FD (directly -72°C)	Chitosan-Coated Calcium Alginate Microparticles		CFU	High molecular weight chitosan: highest survival rate	Lee J.S. et al., 2004, [118]
Yeast strain 131	FD (directly -80°C)	None/Water	Rehydration temperature	CFU	Trehalose lowers membrane phase transition Rehydration above 40°C: Survival ↑, leakage ↓	Leslie et al., 1994, [33]
Escherichia coli DH5α *Bacillus thuringiensis*	FD (directly -80°C)	Trehalose (100 mM) Sucrose (100 mM)		CFU	E. coli: 70% viability for 100 mM trehalose B. thuringiensis: 57% viability for 100 mM trehalose Trehalose and sucrose: lowering Tm	Leslie et al., 1995, [41]
Lb. plantarum	Fluidized bed drying	Maltose, Sucrose, Lactose, Trehalose, Glucose, Fructose, Sorbitol		Fermenting activity	Maltose: highest increase in activity No protection by lactose and trehalose	Linders et al., 1997, [71]
Lb. plantarum	AD	Trehalose, Sorbitol , Maltose Each 0.3 g/g cell pellet		Fermenting activity	Highest activity for sorbitol and maltose Sorbitol and maltose: lowering of Tm (dry state)	Linders et al., 1997, [72]
7 different bacteria strains	VD	Trehalose (5%) Activated charcoal (1%) Dextran (5%) Meso-inositol (5%) Gelatin (5%)		CFU	Highest efficacies: Trehalose + charcoal+ dextran, Charcoal + meso-inositol, Meso-inositol + gelatine + charcoal	Malik et al., 1996, [123]
Lb. reuteri	FD (directly -80°C)	Skim milk	Cultivation pH Time in stationary phase	CFU	Cultivation at pH 5, harvested 2.5 h after entering stationary phase: 80% viability	Palmfeldt et al., 2000, [124]
Ps. chlororaphis	FD (directly -80°C)	Lactose (100 g/l), Sucrose (0 – 300 g/l), Trehalose (100 g/l) Glutamate (100 g/l) Ascorbic acid (1 g/l) Skim milk (200 g/l)	Cell concentration Carbon starvation Heat treatment	CFU	Concentration of 1 x 10^9 – 1 x 10^{10} CFU/ml, dried with sucrose: 15-25% survival Carbon starvation enhances freeze-drying tolerance	Palmfeldt et al., 2003, [114]
Lb. Brevis *Oenococcus oeni H-2*	FD (various)	Yeast extract (4.0%) Sodiumglutamate (2.5%) Trehalose, lactose, maltose, sucrose, fructose, glucose (each 10%) Sorbitol (5%), Mannitol (5%) MGY (minimal glycerol) medium Phosphate buffer	Freezing rate Rehydration medium	CFU	Yeast extract + Na-glutamate, rehydration with 10% sucrose and MGY medium, quickly frozen (-65°C): viability↑ Strain specific freeze-drying resistance	Zhao et al., 2005, [68]

Table II-2. Approaches of (freeze-) drying microorganisms.

II.4.2 Red blood cells and platelets

Red blood cells (RBCs) and platelets can be easily derived from whole blood by centrifugation in a large number. RBCs emerge from hematopoetic stem cells and during differentiation they undergo major structural and morphological changes. Upon erythropoiesis, cells undergo a size reduction with chromatin condensation and reduce their proliferative activity. At their terminal maturation they lose their nucleus and other cell organelles such as the golgi apparatus, mitochondria, endoplasmatic reticulum and ribosomes [126]. Fully differentiated erythrocytes consist mainly of hemoglobin and are only capable of some basic metabolic activities such as glycolysis or methemoglobin reduction [12].

During thrombopoiesis, platelets or thrombocytes also differentiate from hematopoetic stem cells into megakaryocytes which in a later stage of differentiation release a large number of blood platelets [127]. Even if platelets also lack nuclei, they contain a large amount of RNA and approximately 4000 proteins. Their membrane is coated with a glycoprotein rich glycocalix which is important for cell-cell and cell-vessel interactions. Under normal circumstances, platelets circulate in the blood vessels but upon a vascular injury, platelets surface receptors bind to collagen of the extracellular matrix. This leads to platelet activation, followed by an activation of the coagulation cascade and the formation of a blood clot to interrupt the bleeding event [128], [129].

The basic cellular organization of RBCs and platelets and the fact that they can be easily derived by whole blood separation make them an ideal cell model, which can be used to understand damages and protection mechanisms during (freeze-) drying. But also the increasing demand of blood and platelet donations together with their short shelf life, make the preservation of RBCs and platelets a desirable goal.

To evaluate the success of (freeze-) drying of RBCs, usually the percentage of hemolysis or the functionality of basic metabolic enzymes is analysed (see Table II-3). Owed to an absence of nucleus or higher metabolic activity, no transfection with trehalose or LEA synthesis genes was applied, whereas promising results were made via trehalose loading by endocytotic uptake and incubation. Török et al. loaded trehalose by incubation for 7 hours at 37°C and reported survival rates of 55% for freeze-dried RBCs (based on %- hemolysis) with intact metabolic enzyme [89].

Cells	Drying (Freezing rate)	Excipients	Other approaches	Analytical techniques	Results/Functions after Drying	References
RBCs	FD (LN freezing)	Albumin (15%) Glucose (139 mM) In Buffer system	Crosslinking with dimethyl-3,3-dithiobispropion-imidate (DTBP) CO treatment Glucose loading	Microscopy Free Hb concentration Met-Hb formation	Complete recovery after crosslinking (% free Hb) CO treatment prevents oxidative damage	Bakaltcheva et al., 2000, [129]
RBCs	FD (LN freezing)	Glucose, Mannose, Xylose, Fructose, ribose (23%) PVP (10, 24, 40, 360 K) (12.8-18.1%) Inositol-Hexaphosphate (0.009 – 0.9%) Pyrophosphate (0.09 – 4.5%) Dextran (10, 40, 80, 400 K) (18.1 – 3.5%) Hydroxyethyl starch Albumin, Ficoll 400K (3.5%), Fish Gelatin (6.8%) ATP (0.5%), Tripolyphosphate (1%), Trimetaphosphate (0.8%), 2,3-Diphosphoglycerate (0.7%)		Adenin synthesis MetHb Reduction Glycolytic enzyme activities	Synthesis of adenin nucleotides MetHb Reduction Mainly intact enzyme activity	Goodrich et al., 1992, [46], [47]
RBCs	FD (LN freezing)	as described above [47] Amphiphatic polymer (e.g. PVP 10-40K (12-20%)) Carbohydrates (26%) Polyanions (0.1 – 10%)	Rehydration buffers	ATP synthesis MCV, MCH and MCHC values Enzyme activity	Intact blood enzymes Intact membrane structure Best rehydration buffer: 19% polymer, 7-37.5% monosaccharide, rehydration at RT with an equal volume	Goodrich et al., 1989, [48]
Platelets	FD (directly -60°C, 10 K/min)	Trehalose (528.6 mmol/l) BSA (1%)	Pre-hydration/Re-hydration Trehalose incubation	Recovery (hemocytometer) Mean platelet volume Platelet distribution width Aggregation response	Up to 95% recovery Intact Aggregation response Optimum pre-hydration time: 15 min Rehydration solution: platelet poor plasma	Fan et al., 2009, [89]
Platelets	FD („Shell frozen“)	150 parts of “ACD solution” (NIH formula) 20 parts of a 2% Triton solution per liter water		Bleeding events Prothrombin consumption	Difference between single patients Prothrombin consumption not adequate indicator for platelet condition	Klein et al., 1956, [130]
Platelets	FD (not mentioned)	BSA (5%) In buffer system	Fixation with paraformaldehyde	Saline bleeding time Platelet adhesion (visualized by SEM) Antibody binding (flow cytometry)	Morphology intact Ability to adhere to subendothelium Reaction with antibodies Participation in Thrombus formation Restore hemostasis in thrombocytopenic rats	Read et al., 1995, [131]
RBCs	FD (100 K/min)	HES (20%) Maltose (5%)	Drying temperature	Hemolysis	-35°C ST: lowest hemolysis	Rindler et al., 1999, [132]
RBCs	FD (various)	HES (24.5%)	Freezing rate	Hemolysis	Higher cooling rates: best cell survival (4200 K/min)	Rindler et al., 1999, [110]
Platelets	FD (2-5°C/min)	Trehalose (150 mmol/l) HSA (5%)	Trehalose incubation	Intracellular pH	Largely preserved pH regulation	Tang et al., 2006, [90]
RBCs	FD (1.5°C/min)	100 mOsm ADSOL buffer (Glucose, Adenine, Mannitol, NaCl) Trehalose (100 mM) HES (15%), HSA (2.5%) Ascorbic acid	Trehalose incubation	Viability (% hemolysis) ATP and 1,2-DPG level Enzyme activity (catalase and superoxide dismutase) Protein structure (FTIR)	55% survival ATP and 2,3-DPG synthesis Enzyme activity Intact protein structure	Török et al., 2005, [91]
Platelets	FD (2-5°C/min)	Trehalose (30 mM) HSA (1%)	Platelet concentration Trehalose incubation Pre-hydration/Re-hydration	Recovery (Coulter counter) Aggregation response (thrombin, collagen, ADP, ristocetin) Protein structure (FTIR)	85% Recovery Aggregation response Similar Membrane and protein structure	Wolkers et al., 2001, [90]
Platelets	FD (2-5°C/min)	Trehalose (30 – 150 mM) HSA (1-5%)	Trehalose incubation	Recovery (Coulter counter) Aggregation response (thrombin, ristocetin)	90% recovery Intact aggregation response Minor changes in morphology	Wolkers et al., 2003, [92]

				Morphology		
RBCs	FD (1°C/min)	Glucose, Trehalose, Mannose and lactose (each between 15-25%) PVP (5-15%) BSA (5%)		Cell integrity (microscopic) Hematocrit Hemoglobin content SEM	Size reduction after FD 15% PVP, 22,5% Glucose, 5% BSA: best composition (86% intact cells)	Yu et al., 2004, [133]
Platelets	FD (various)	Trehalose (1-20%), Sucrose (20%), Maltose (20%), Lactose (20%), Glucose (20%) BSA (1%)	Cell concentration Freezing rates	Platelet activation (Annexin V) SEM Aggregation properties (thrombin)	93.0 % recovery for 20% trehalose (85% non activated) Optimum freezing rate: 10°C/min Intact morphology Best concentration: $4 \cdot 10^9$ platelets/ml 83.9% aggregation rate	Zhou et al., 2007, [113]

Table II-3. Approaches of (freeze-) drying RBCs and platelets.

To assess the success of drying platelets, the physiological behaviour of platelets towards aggregation agonists such as thrombin or collagen can be assayed. Until now, good recovery rates of up to 90% after freeze-drying have been achieved; basic metabolic pathways could be preserved and intact physiological response on aggregation agonists was reported. Furthermore, researchers tried to stabilize blood cells by fixation of cellular structures prior to drying. Read et al. lyophilized with paraformaldehyde fixed platelets in the presence of albumin and obtained cells with normal morphology which maintained basic hemostatic functionality [131]. A similar approach was taken by Bakaltcheva et al., who freeze-dried RBCs that were treated with carbon monoxide (CO) and subsequently cross-linked with the reversible cross-linking reagent dimethyl-3,3-dithiobispropionimidate (DTBP). The Co-treatment with CO prevented the cells from methemoglobin formation and oxidative damage resulting from DTBP, which drastically reduced the percentage of cell lysis [129]. However, even if the effect of the stabilizing excipients can be reversed, the toxicity and safety of these methods must be considered.

II.4.3 Spermatozoa

The preservation of spermatozoa is of great interest for reproductive medicine, but it is also important for the dairy industry, where it is used to preserve the semen of high-quality breeding bulls. For the sake of completeness, we have briefly summarized the recent advantages in freeze-drying sperm (see Table II-4). More information can be found in this review by Gil et al., in which sample preparation, rehydration and sample evaluation are described in detail. They concluded that the sperm membrane is highly damaged after freeze-drying and that cells lose their motility, membrane integrity and partially also DNA integrity. Since reduced DNA integrity could lead to impaired embryo development, DNA preservation is therefore the major goal of freeze-drying sperm, which is mainly achieved by inhibition of endonucleases and ROS release [134].

At the last stage of spermatogenesis, the nucleus condenses, excess cytoplasm is discarded and the sperm tail is formed. As a result, sperm cells consist almost entirely of their nucleus with condensed chromatin and some nuclear matrix. The sperm head exhibits only low water content, hardly contains cytoplasm and no cell organelles [93], [135], [136]. The sperm tail, which is responsible for the motility, is made up of flexible membrane extensions, called cilia and flagella [136]. Directly after (freeze-) drying, sperm survival can be assessed by three different aspects: sperm motility, membrane integrity and DNA damage. Subsequently, the genetic integrity can be determined by Intracytoplasmic Sperm Injection (ICSI), where a single sperm is directly injected in oocytes. This can be followed by blastocyte development and, after several stages of embryogenesis, by live offspring [134].

Cells	Drying (Freezing rate)	Excipients	Other approaches	Analytical techniques	Results/Functions after Drying	References
Gel free semen from stallions	FD (directly -80°C)	FBS (10%) in DMEM/F-12		DNA fragmentation index Blastocyte development	Blastocyte development Pregnancy resulting in one living foal	Choi et al., 2011, [138]
Bovine spermatozoa	FD (0.3 – 6°C/min)	Egg yolk concentrate (23%) Glycerol (7.5%)	Drying temperature	Motility (microscopic) Viability (Eosin Nigrosin staining)	No effect of drying temperatures Big loss in motility with lower water content	Bialy and Smith, 1957, [139]
Hamster, rabbit, human, spermatozoa	FD (directly -80°C)	None, FD in distilled water		Embryonic development	Pronucleus development No motility	Hoshi et al., 1994, [137]
Mouse epididymal spermatozoa	FD (directly LN)	EDTA (1, 50 mM)		Chromosome normality Embryo development	1 mM EDTA: 73% normal chromosomes (22% for 50 mM)	Kaneko et al., 2006, [85]
Bull spermatozoa	FD (directly LN)	10% FBS Glutamine (1 M) Nucleoside and Aminoacid mix		Morphology	29.5% blastocyte development Karyotypically normal blastocytes Physical integrity	Keskintepe et al., 2002, [140]
Mouse and human spermatozoa	FD (directly LN)	EGTA (50 mM) TRIS-HCl (100 mM)	Incubation with freezing solution	Chromosome analysis Embryo development (mouse)	85-95%: normal chromosomes Embryo development	Kusakabe et al., 2001, [141]
Mouse spermatozoa	FD (directly LN)	EGTA (50 mM) TRIS-HCl (100 mM) NaCl (50 mM)		Chromosome analysis Viability (SYBR 14/PI)	70-90% Viability Embryo development by ICSI	Kusakabe et al., 2008, [141]
Mouse spermatozoa	AD	EGTA (50 mM) Trehalose (1 M)	H5 Pore formation	Blastocyte development	Live offspring Trehalose: higher blastocyte development Trehalose: higher water content	McGinnis et al., 2005, [93]
Boar spermatozoa	FD (directly -40°C)	EDTA (10 – 50 mM) EGTA (50 mM) Sorbitol (0.117 M)		DNA fragmentation	50 mM EGTA: highest blastocyte formation EDTA and EGTA (50 mM): DNA damage ↓	Nakai et al., 2007, [86]
Fresh bovine semen	AD	Sucrose (0.2 M) Trehalose (0.2 M) Desferal (1 mM) Catalase (0.05 mg/ml)	$P2X_7$/P2Z receptor (ATP) [18]	Motility Membrane integrity (Hoechst/PI)	Intracellular sugars or desferal: membrane integrity ↑ No increase in motility	Sitaula et al., 2009, [79]
Fresh bovine semen	AD	Sorbitol (200 mM) Trehalose (100 mM)	$P2X_7$/P2Z receptor (ATP) [18]	Motility	Sorbitol + trehalose: sperm motility ↑	Sitaula et al., 2010, [96]
Mouse spermatozoa	FD (directly -50°C)	CZB medium + 10% FBS DMEM + 10% FBS		Viability Microscopy	No motile spermatozoa 100% dead stained Activation and fertilization of oocytes Offspring developed from freeze dried sperm	Wakayama et Yanagimachi, 1998, [142]
Mouse spermatozoa	FD ("rapid" freezing)	none		Chromosomal analysis	68% normal karyoplates Fetus development Live offspring	Ward et al., 2003, [143]

Table II-4. Approaches of (freeze-) drying spermatozoa.

Sperm is known to be very resistant to physical and chemical stresses and is capable of inducing embryonic development even after harsh treatment. Hoshi et al. showed already in 1994 that sperm that had been freeze dried without protective agents could develop the pronuclear stage if injected directly in oocytes [137]. This stability of genetic material can be attributed to DNA association with the protein protamin that contains high levels of arginine, serine and cysteine. During sperm maturation, cysteines are oxidized and form disulphide bonds between neighbouring protamines, thus creating strong DNA-protamine complexes.

Since sperm consists mainly of DNA, the conservation of genetic material is the dominant goal. As described in an earlier section, chelating agents might positively influence DNA stability and EGTA and EDTA are common excipients to preserve spermatozoa during drying [144]. However, during (freeze-) drying, sperm motility and membrane integrity are highly affected and extensive DNA damage is frequent. Yet, live offspring of mice have been successfully produced after ICSI of sperm freeze dried without any protection [143]. To our opinion, optimising the freezing procedure for spermatozoa is also a chance to enhance desiccation tolerance, as until now only rapid freezing directly at -80 or LN was performed.

II.4.4 Eukaryotic cells

Compared to previously described cell types, eukaryotic cells exhibit the most complex cellular structure with a well controlled interplay of single cell organelles and proteins. Even small loss of functionality can lead to complete breakdown of all cellular processes. Without intact cell membranes, no viable cells can exist, as compartmentalization and organisation of fundamental cellular processes cannot be maintained. Therefore the cell membrane plays a key role in preservation of eukaryotic cells during drying and in many research papers, an intact cell membrane is referred as “viable cells” or “viability”. This led to an establishment of various assays with membrane impermeable dyes, such as propidium iodide (PI), ethidiumhomodimer-1 (EH) or trypan blue to assess membrane integrity after (freeze-) drying. For further characterisation many researchers measured the basic metabolic activity of cells with substances like Calcein-Acetoxymethylester (CAM) or Alamar Blue. Table II-5 depicts the different approaches that have been taken to (freeze-) dry eukaryotic cells.

Almost all researchers used trehalose as excipient and higher viabilities were achieved, when trehalose was introduced intracellularly by different loading techniques or gene transfection. One complicating factor here is the compartmentalization of the eukaryotic cells. If the outer cell membrane is overcome, trehalose may still not be able to access mitochondria and other organelles with a further membrane [109].

Cells	Drying (Freezing rate)	RM content	Excipients	Other approaches	Analytical techniques	Results/Functions after Drying	References
NIH 3T3 fibroblasts	AD	5 – 40%	No further excipients, drying in wash buffer (0.2 M trehalose, hyper- or isotonic)	H5 Pore formation Trehalose incubation	Membrane integrity (Syto-13/EB) Cell adhesion and proliferation	Isotonic trehalose solution: membrane integrity ↑ Cell growth at higher moisture content	Acker et al., 2002, [17]
HSC and HPC	FD (2.5 K/min)	1.9 – 39.5 g H_2O/100 g solid	Trehalose (6.8%) HES (2%) HSA (5%)	$P2X_7$/P2Z receptor (ATP) [18] Lyophilization cycle	Clonogenic activity	Colony formation Cell damage in secondary drying	Buchanan et al., 2010, [54]
Chinese hamster ovary cell	AD	0.5 – 8 g H_2O/g dw	Trehalose (200 mM) Glucose (5 mM)	Transfection: Trehalose transporter genes (TRET1)	Membrane integrity (trypan blue, Syto-13/PI) CFU	Trehalose transporter: 170 % increase in membrane integrity 400 % increase in growth (after 7 days)	Chakraborty et al., 2012, [99]
NIH 3T3 fibroblasts	AD	0 – 2.5 g H_2O/g dw	No further excipients, but growth and drying in 0 – 0.4 M trehalose	H5 Pore formation	Membrane integrity (Syto-13/EB)	Optimum trehalose concentration: 10^9 molecules/cell Higher storage temperatures: membrane integrity ↓	Chen et al., 2001, [20]
Somatic cells (pig fetal fibroblast cells)	FD (directly LN)	-	FBS (10%) DMSO (7%) EGTA (50 mM)		Membrane integrity (FDA and trypan blue) DNA damage	No membrane integrity Complete embryonic development Reduced blastocyte formation	Das et al., 2010, [83]
J774.A1 macrophage cells	AD	0.75-3.5 g H2O/g dw	Trehalose (200 – 250 mM)	$P2X_7$/P2Z receptor (ATP) [18]	Membrane integrity (trypan blue)	Trehalose loading by ATP poration: drying tolerance ↑	Elliott et al., 2006, [18]
Mouse fibroblasts ($LMTK^-$)	AD	Not mentioned	Trehalose (1 M)	Transfection: Trehalose synthesis genes Trehalose incubation	Membrane integrity (trypan blue, PI/CAM)	Osmotolerance ↑ No viable cells or colony formation	Garcia de Castro et Tunnacliffe, 2001, [97]
Human mesenchymal stem cells	AD	-	No further excipients, removal of culture medium	Growth in trehalose (50 mM) and glycerol (3%)	Viability (trypan blue) Morphology	Trehalose: Viability ↑ Inconsistency between batches Spindle shaped morphology Intact adhesive capabilities	Gordon et al., 2001, [19]
Human primary foreskin fibroblast (F12)	AD	Detected by FTIR (not quantified)	No further excipients, removal of culture medium	Transfection: Trehalose synthesis genes	Membrane integrity (CAM/EH)	Rehydration directly after drying: High degree of viability	Guo at al., 2000, [98]
Human mesenchymal stem cells	VD	0.2 – 12 g H_2O/g dw	BSA (5.7%) Trehalose (150 mM) Arbutin (70 mM)	Trehalose/Arbutin Incubation	Membrane integrity (PI) Metabolic activity (Alamar blue) Osteogenic differentiation (von Kossa staining) Calcium concentration	Trehalose: membrane integrity↑ (below 2 g H_2O/g dw↑ (no difference for arbutin)) Arbutin: metabolic activity ↑, osteogenic differentiation ↑ (0.37 g H_2O/g dw)	Jamil et al., 2005, [22]
Mononuclear cells Whole human cord blood	FD (1-3 K/min)	6.5 ± 0.87%	PVP 10000-70000 MW (40%) Sucrose (20%) Mannitol (10%) FBS (10%)		Recovery (hemocytometer) Morphology	Sucrose, Mannitol, PVP, FBS: 61.87% recovery Karyon change Decrease of cytoplasm	Li et al., 2005, [55]
Human hepatoma cells (HepG2)	SD	below 0.12 g H2O/g dw	No further excipients, but growth and drying in 50 mM Trehalose	Transfection: LEA synthesis genes Transfection: Trehalose transporter genes (TRET1)	Membrane integrity (Syto-13/EB)	Preloading with trehalose + LEA expression: 98.3% membrane integrity Proliferation after rehydration	Li et al., 2012, [23]

Peripheral blood lymphocytes Cumulus Oocyte Complexes	FD (5.1 K/min)	-	FBS (10-50%) Trehalose (0.1 M)		DNA integrity (Comet assay) Membrane integrity (PI)	Extensive DNA damage Trehalose: 50% of nuclear integrity Embryonic development following injection into enucleated oocytes	Loi et al., 2008, [144]
Human embryonic kidney cells 293H	VD/AD	0.1 – 10 g H_2O/g dw	Trehalose (150 mM) BSA (5.7%)	Transfection: stress protein p26 Physical shape/structure of samples	Metabolic activity (Alamar blue) Colony formation Viability (trypan blue)	Cells suspended in rounded droplets more likely to survive than in thin film Transfected cells: viability ↑, metabolic activity and colony formation ↑	Ma et al., 2005, [21]
Drosophila melanogaster	AD	0 – 7 g H_2O/g dw	Trehalose (200 mM)	Transfection: LEA synthesis genes	Membrane integrity (trypan blue)	Cells expressing LEA: desiccation tolerance ↑	Marunde et al., 2013, [102]
Human corneal epithelial cells	AD	Not mentioned	No further excipients, removal of culture medium	Incubation with freezing solution (Trehalose (2-200 mM), Maltose (2-200 mM), PBS, sodium hyaluronate eye drops (Hyalein), artificial tear eye drops (Mytear)	Membrane integrity (Syto 10/EH)	Trehalose (50 – 200 mM): 88.1 – 79.1% survival rate	Matsuo et al., 2001, [92]
Human adipose-derived stem cells	AD	9 - 12.9%	Trehalose (50 mM) Glycerol (384 mM)	Drying speed Storage conditions Cellular passages	Membrane integrity (SYBR-14/PI)	Slow drying and vacuum storage: 37% viability Earlier passages: Membrane integrity ↑	Mittal et al., 2008, [62]
MNCs	FD (various)	4.69 ± 0.07%	Trehalose (0.1 M) HSA (12.5%) EGCG (0.189 mg/ml – 1.89 mg/ml) Dextran 40	Freezing rates Cell concentration	Membrane integrity (Syto-13/PI) Clonogenic activity (CFU)	0.945 mg/ml EGCG, 0.1 M Trehalose: 88% - 91% membrane integrity Increasing cell concentration: viability ↓	Natan et al., 2009, [5]
Cumulus or embryonic stem (ES) cells	FD (directly -40°C)	-	EGTA (50 mM) Trehalose (0.5 M)		Membrane integrity (Syto 10/EH) Chromosome analysis	0% membrane integrity Nuclear transfer ES cell lines could be generated	Ono et al., 2008, [147]
Human primary foreskin fibroblast (F12)	AD	Detected by FTIR (not quantified)	No further excipients, removal of culture medium	Trehalose introduction by osmotic or thermal shock Storage conditions Desiccation rate Degree of confluence Fluorescent light	Membrane integrity (trypan blue)	Trehalose: desiccation tolerance for 5 days Vacuum storage: viability ↑ High cellular density: desiccation tolerance ↑ Fluorescent light: viability↓	Puhlev et al., 2001, [94]
Human bone marrow-derived mesenchymal stem cells	FD (precooled -60°)	2.9%	PVP 40 (30%) Trehalose (100 mmol)	Trehalose loading by incubation Rehydration solutions	Membrane integrity (trypan blue)	Adhesion and proliferation impaired Recovery: 69.33%	Zhang et al., 2010, [6]
Fibroblasts	FD (40°C/min)	0.05 g H_2O/g dw	Trehalose (250 mM) BSA 6700 g/mol (9.5%)		DNA damage (Comet assay) Viability (trypan blue)	No viable cells Intact protein secondary structure Trehalose: reduced DNA damage	Zhang et al., 2017, [95]

Table II-5. Approaches of (freeze-) drying eukaryotic cells.

After lyophilization, differentiation and colony formation was proved for mononuclear stem cells and hematopoetic stem and progenitor cells, all derived from umbilical cord blood: Buchanan et al. loaded trehalose via ATP poration into human hematopoietic stem and progenitor cells, and freeze dried them to various levels of residual moisture content. After cytokine addition, the colony forming unit potential and the formation of progenitor cells (e.g. erythrocytic (BFU-E), granulocytic (CFU-GM), or mixed (CFU-GEMM)) of the lyophilized cells was analysed with the MethoCult™ medium by stem cell technologies (Vancouver, Canada). A correlation between residual moisture content and the ability of colony formation was observed, when they removed samples at different stages of the lyophilization process. A decrease in the ability of cells to form colonies with proceeding lyophilization cycle und thus lower residual moisture contents was reported. However, all types of colony formation could be detected even at a residual moisture content of 1.9 ± 0.7%. During storage (25°C, 4 weeks), a decrease of CFU-GM and BFU-E formation was recorded, however, even after storage, cells were still able to form colonies [54]. Also Natan et al. freeze dried mononuclear cells derived from umbilical cord blood and showed a viability of 88% – 91%, after staining cells with the fluorescent dyes Syto-13 and PI. Using also the MethoCult™ medium, they as well identified formation of cell colonies. Due to the very low resulting Tg (11.8°C), they stored the samples for 3 or 7 days at 2-8°C or 25°C and still reported membrane integrity values of above 50%, but did not examine the ability of cells to form colonies after storage [5]. To our knowledge, proliferating and differentiating eukaryotic cells have been only reported for stem cells, derived from umbilical cord blood. Stem cells are small cells, with only little cytoplasm and the ability of intense renewal and proliferation rates. Furthermore, they show little metabolic activity and only very reduced protein synthesis [145], [146]. This total reduction on basic properties is reflected in the simple structure of stem cells, which might facilitate stabilization during freeze-drying. This could be an explanation for the proliferation after lyophilization of eukaryotic cells that has been described for stem cells only. For higher differentiated cells, no viable or proliferating cells were obtained after freeze-drying.

Beside freeze-drying, many alternative drying methods were applied to desiccate eukaryotic cells, mainly air-drying or vacuum-drying. However, as mentioned above, the differences in residual moisture content make it difficult to compare air-dried with lyophilized samples. Especially for air-dried samples, a correlation between residual moisture and viability was shown, with a drop in viability below a certain water content, which may vary depending on the cell type. Chen et al. concluded that a moisture content of minimum 15% is necessary to retain high membrane integrity of NIH 3T3 fibroblasts after air-drying and trehalose loading by H5 pore formation [20]. Acker et al. achieved membrane integrity of above 90% and reported cell growth with 10% residual moisture content, both of which rapidly dropped at lower moisture content levels [17]. Li et al. recovered proliferating human hepatoma cells with a cell membrane integrity of 98.3% after

spin-drying to a water content below 0.12 g H_2O/g dw. Proliferation was determined by incubation of the spin-dried cells in a fully complemented medium and cell counting after 7 days of culture. To withstand desiccation, cells were transfected with genes encoding for two LEA proteins [23].

II.5 Conclusion

Taking all studies into account, there is still no certain advice or approach available on how to stabilize cells during and after drying. It appears to be a scientific field that is still based on empirical testing of various theories and excipients. The success of certain molecules is to a large extent cell and strain specific and no ubiquitous "stabilization cocktail" can be recommended. However, trehalose is undoubtedly the most prominent molecule in stabilizing any type of cells and researchers agree that it is important to load trehalose intracellularly to achieve maximum survival rates. As a result, various loading techniques have established, with resulting intracellular trehalose concentrations between 20 – 500 mM. Apart from trehalose, no other substance could be implemented as a universal protecting agent, though certain excipients such as sucrose or HES showed good results. Crowe et al. and Goodrich et al. hypothesized that a sugar in combination with glass forming excipients, such as macromolecules, would lead to optimal results [10], [42], [46]–[48]. Also polyols such as sorbitol and glycerol were were found to be promising excipients during cryoconservation, but this success could not be transferred to cell drying. With the use of EGCG or arbutin, new excipients obtained high viabilities for certain cell lines but their application did not establish throughout the field of cell (freeze-) drying.

Despite finding the most appropriate excipients, many process parameters can be optimized to find best drying conditions. Drying method, cell density, freezing rate, residual moisture content or rehydration procedure can influence cell survival during and after (freeze-) drying.

Further research is also needed to fully elucidate the damaging processes that occur during desiccation and the stabilizing effects of the described excipients. Enhanced interaction of membrane lipids, accompanied by leakage of intracellular cell components is an accepted explanation for cell damage during drying and it is the prevailing opinion that water replacement by small polar substances is an important mechanism for many molecules to prevent cell injury. Additionally, vitrification of the solution can prevent ice formation and fusion. However, this cannot be the only explanation; otherwise, other hydrophilic molecules such as glycerol, which are also capable of hydrogen bond formation, would be more successful in stabilizing sensitive structures.

Vast differences exist regarding the current success of drying different cell types. With microorganisms, it is possible to obtain cells that retain the ability to form colonies and proliferate

even after lyophilization and reconstitution. Spermatozoa with no intact cell membranes or motility can direct embryogenic development, followed by live offspring of mice and even foals. RBCs were able to maintain their physiological functionality, and *thrombocytes* could response to aggregation agonists after freeze-drying. The most complex and therefore most difficult cells to stabilize are eukaryotic cells. Natan et al. and Buchanan et al. were able to freeze dry stem cells that could differentiate and form colonies after rehydration [5], [54], whereas for more differentiated cells, proliferation was only reported for cells dried to high water contents [17], [23].

II.6 References

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Chapter III

Defining experimental parameters for lyophilization of human keratinocytes

III.1 Introduction

The skin is a crucial protective barrier of the body against the environment and consists of the dermis and epidermis, which is mainly build up from keratinocytes. In the basal layer of the epidermis keratinocytes are in a proliferative state and differentiate with further migration through the epidermis until they end up as corneocytes [1]. Moreover, keratinocytes play a vital role in the wound healing process which can be divided into four steps: inflammation with haemostasis, proliferation and remodelling [2]. During hemostasis, thrombocytes are activated and catalyse the coagulation cascade to form a blood clot and to stop the bleeding. Neutrophils are attracted by cytokines and growth factors and initiate the inflammation phase. Monocytes differentiate to macrophages, migrate into the wound bed and further release cytokines. Macrophages are essential for the cleaning of the wound bed and remove damaged matrix, cell debris and bacteria. Subsequently the formation of extracellular matrix, reepithelialisation, angiogenesis, vessel formation and also nerve repair is part of the remodelling phase. For a in depth description of the different stages during wound healing, we want to refer to this review article by Luis Cañedo-Dorante and Mara Cañedo-Ayala [2].

Keratinocytes are especially involved in the reepithelialisation process, which can be divided into keratinocyte migration and proliferation. As a first step keratinocytes lose their cell-to-matrix adhesions and migrate into the wound bed ("migrating epidermal tongue"), subsequently basal keratinocytes proliferate to further fill the wound gap with new cells. Latter processes are controlled and regulated by various growth factors and cytokines such as EGF, TGF-alpha or HB-TGF, but also Matrix Metalloproteinases (MMP) which are components of the extracellular matrix. During all processes, keratinocytes are in close contact to the surrounding cells such as fibroblasts or immune cells and produce a broad range of signalling molecules for paracrine but also autocrine signal transmission. For example the release of IL-1 or TNF alpha by keratinocytes stimulates proliferation and migration of keratinocytes but also activates fibroblasts [1], [3].

The wound healing process of burn injuries differs from normal wounds and must be handled with special precautions. An increased capillary permeability can lead to protein and fluid leakage to the interstitial place, thus inducing hypovolemia with the demand of fluid replacement. Furthermore, the inflammation phase is prolonged and due to the protein rich plasma at the surface, the burn wounds are prone to infections and require special infection prophylaxis. Conventionally treatment of burn injuries includes skin grafting of full or split thickness grafts, however, this goes along with a bunch of disadvantages such as limited donor site and the risk of scar formation or rejection. Despite these differences in treatment and disease profile, burn wounds also undergo the described stages of wound healing. The central role of keratinocytes in the healing and reepithelialisation process contributed to the application of keratinocytes for burn

trauma as an alternative to conventional treatments [4], [5], [6]. Confluent cultured epithelial autographs (CEA) emerged as a new therapeutic option, where keratinocytes are isolated from a small biopsy of the patient's individual skin, further expanded and transplanted to the burn site. However, drawbacks such as long cultivation time of 3 or 4 weeks, the extreme fragility of the epithelial sheet as well as a high vulnerability to infection, led to doubts about the superiority of confluent keratinocyte sheets compared to conservative treatments [7], [8]. Keratinocytes in a single cell suspension are an alternative that requires less cultivation time and facilitates handling, transport and even storage, as keratinocytes can be frozen until use [9]. The keratinocyte suspension can be simply sprayed on to the wound and convinced in the treatment of burn and normal wounds [10]–[13].

Until now, therapeutic cells such as a keratinocyte suspension are freshly prepared or frozen at -80°C until use. Freeze-drying of cells is a promising preservation technique to simplify storage and transport of therapeutic cells, which can pave the way to a more widespread application and new therapeutic options. However, lyophilization of cells is a very broad field and success or failure is to a large part dependent on cell type and cellular complexity, e.g. the stabilization of eukaryotic cells is more demanding than stabilization of bacteria or spermatozoa (see Chapter II).

In this thesis we will focus on the question if we can successfully stabilize keratinocytes in the dry state to improve storage and transport of keratinocyte suspensions. HaCaT (Human adult low Calcium high Temperature keratinocytes) cells were used as model cell line, as they are immortalised, require less complex cultivation conditions and are a suitable cell model for primary keratinocytes [14]. In this chapter we will focus not on excipient optimization, but on the influence of general freeze-drying parameters and growth conditions of the keratinocytes prior and during lyophilization. It is the aim to define basic lyophilization parameters that will be applied for future experiments.

III.2 Material and Methods

III.2.1 Materials

Hydroxyethyl starch 200/0.5 (HES) was purchased from BOC Sciences (Shirley, NY, USA). Hydroxyectoine (HE), tris buffered saline (TBS: 0.05 M Tris buffered saline (0.138 M NaCl, 0.0027 M KCl), pH 8.0, at 25 °C), dulbeccoo's phosphate buffered saline (PBS: 0.2 g/L KCL, KH_2PO_4, 8 g/L NaCl, 1.15 g/L Na_2HPO_4 (anhydrous)) and $CaCl_2$ were supplied by Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). HEPES and NaCl were ordered from VWR (VWR, Allison Park, PA, USA). HaCaT (Human adult low Calcium high Temperature) keratinocytes were provided from the DKFZ (Heidelberg, Germany)

III.2.2 Cell and sample preparation

Cell preparation: HaCaT keratinocytes were cultivated in EpiLife with 60 µM calcium chloride, supplemented with 5 ml of 100x Supplement S7 (both from Gibco by Thermo Fisher Scientific (Waltham, MA, USA)).

For better cell attachment cell culture flasks were coated with 60 µg/ml PureCol Type I Collagen (Advanced Biomatrix, Carlsbad, CA, USA) for 90 minutes at 37 °C, 5% CO_2.

Doubling time: The cellular doubling time was calculated according following formula:

$$Doubling\ time\ [days] = \frac{Days\ of\ cultivation * \ln(2)}{\ln(\frac{cell\ number\ at\ harvesting}{cell\ number\ at\ seeding})}$$

Sample preparation: For lyophilization of cells in suspension, cells were resuspended in different formulations, all containing 10% (w/v) hydroxyethyl starch (HES) and 5% (w/v) hydroxyectoine (HE), dissolved in EpiLife/S7, PBS, TRIS or HEPES buffer. If not mentioned otherwise, a concentration of 0.5 – $1 \cdot 10^6$ cells/ml was used for freeze-drying. Aliquots of 500 µl were filled in 10 R tubing vials (MGlas AG, Muennerstadt, Germany) and semi-stoppered with lyophilization stoppers (FluroTec® rubber stopper; West Pharmaceuticals, Eschweiler, Germany). The low filling volume of 500 µl is due to the desired application. The lyophilized product should get applied and reconstituted directly on the wound bed, which requires a thin, disk like lyophilized product. However, a volume of 500 µl provides also enough solid material for subsequent physicochemical analysis and allows direct analysis of lyophilized cells without prior upconcentration.

For lyophilization of adherent cells, cells were seeded in a concentration of 4000, 7000 or 10000 cells/cm^2 in a 6-well plate (TPP products, Trasadingen, Switzerland) and were cultivated for 24 hours at 37°C, 5% CO_2. Prior to freeze-drying, growth medium was aspirated and cells were washed once with PBS. For lyophilization, 1 ml of respective formulation was added per well and cells were freeze-dried directly in the well plate.

Lyophilization: A Christ 2-6D laboratory scale freeze-dryer was used (Martin Christ, Osterode am Harz, Germany). With an equilibrium step of 30 minutes at -5 °C, samples were cooled in a constant freezing rate of 1 K/min down to -50 °C. Temperature was hold for 90 minutes at -50 °C. Primary drying was carried out at -20°C with a vacuum set at 0.25 mbar for 7 hours. Secondary drying was performed at 20°C and 0.25 mbar for 4 hours with a heating ramp of 0.1 K/min until 0°C and 0.4 K/min until 20°C. Samples were stoppered at 600 mbar. Sample temperature was monitored using *Wireless Product Temperature Measurement Sensors* from Martin Christ (Osterode am Harz, Germany). Chamber pressure was controlled with a pirani and a capacitance manometer.

Freeze-thaw cycle: Cells were frozen with the same temperature program as for freeze-drying cells. After 2 hours at -50°C cells were thawed with a heating ramp of 0.1 K/min until 5°C.

III.2.3 Osmolality and pH

Osmolality: The osmolality was measured with an Osmomat 030-D (Gonotec, Berlin, Germany) using 20 µl sample volume.

pH measurements: The pH measurements were performed with an InLab Expert Pro-ISM pH electrode (Mettler Toledo, Germany). Before measurement of the samples, the pH electrode was calibrated at each temperature with two calibration buffers pH 2 at 25 °C and pH 7 at 25 °C (Bernd Kraft, Germany) using the pH values provided from the manufacturer for the respective temperature.

III.2.4 Analysis of cells

Reconstitution: If not described differently, freeze-dried samples were reconstituted in the 1.5 x volume of the originally lyophilized volume with EpiLife/S7 (37°C). For example 500 µl freeze-dried cell suspension was reconstituted with 750 µl EpiLife/S7.

Cell membrane integrity and total cell recovery: Cell membrane integrity and total cell recovery of HaCat keratinocytes after lyophilization was quantified with a Vi-Cell™ XR Cell Viability Analyzer (Beckmann Coulter, Krefeld, Germany), using trypan blue, a membrane impermeable dye, staining cells with impaired cell membrane blue. After reconstitution, aliquots of 550 µl of cell suspension were measured directly without dilution. Cells were automatically counted and categorised into cells with intact and impaired cell membrane by their trypan blue exclusion with the software integrated in the Vi-Cell™ XR. Recovery of cells was calculated as the total number of cells after freeze-drying divided by the number of total cells before freeze-drying, multiplied by the factor 100, regardless if their membrane was impaired or not.

Calcein-AM and EH staining: After lyophilization of adherent cells, cells were reconstituted with 1.5 ml EpiLife/S7 (37°C). The supernatant was aspirated, cells were washed once with PBS and were incubated for 24 hours with 750 µl EpiLife/S7 at 37 °C, 5% CO_2. Cells were washed two times with PBS and stained with 0.25 µM Calcein-Acetoxymethylester (Calcein-AM) and 4 µM Ethidium-Homodimer-1 (LIVE/DEAD™ Viability kit by molecular probes by Thermo Fisher Scientific, Waltham, MA, USA) for 20 minutes at RT. Subsequently cells were washed two times with PBS and stained with 5 µg/ml Hoechst 33342 (molecular probes by Thermo Fisher Scientific, Waltham, MA, USA) for 15 minutes at RT. After two washing steps with PBS cells were analyzed directly in the well plate with a Keyence BZ8100 Fluorescence microscope (Keyence, Osaka, Japan) equipped with a Nikon SPlan Fluor 10x/0.45 objective (Nikon, Japan).

Three pictures were taken per formulation and time point and subsequent evaluation was performed with the software Fiji (*Fiji Is Just ImageJ*), as depicted in Figure III-1 [15].

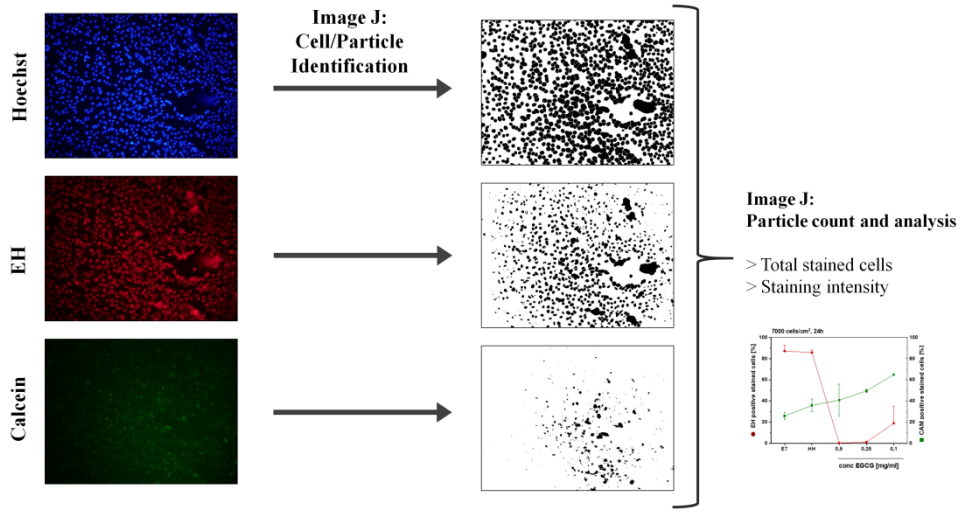


Figure III-1. Exemplary evaluation of cell staining with the software Fiji.

To automatically count the total number of stained cells, all cells with a colour intensity above a certain threshold were depictured as black particles. The threshold for counting cells/particles was kept consistent to guarantee comparability. Subsequently particle number and staining intensity of the particles were analyzed and the percentage of positive stained cells determined by the following equation:

$$EH/Calcein\ \ positive\ stained\ cells\ [\%] = \frac{EH/Calcein\ \ stained\ cells}{Hoechst\ stained\ cells} * 100$$

Figure III-2 shows an exemplary macro to automatically count positively cells stained with Calcein-AM.

```
name=getTitle;
dir = getDirectory("image");
run("Split Channels");
selectWindow(name+" (green)");
name2=getTitle;
run("Duplicate...", "title="+name+"_DUPLICATE");
name3=getTitle;
setThreshold(46, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Watershed");
run("Set Measurements...", "area mean min display redirect=["+ name2 +"] decimal=3");
run("Analyze Particles...", "size=15-Infinity circularity=0.2-1.00 display clear
summarize");
selectWindow(name+" (red)");
run("Close");
selectWindow(name+" (green)");
run("Close");
selectWindow(name+" (blue)");
run("Close");
selectWindow(name3);
saveAs("jpeg");
run("Close");
selectWindow("Results");
saveAs("Measurements", dir + name +"Results.csv");
run("Close");
```

Figure III-2. Exemplary macro applied to analyze the number of Calcein positive stained cells and staining intensity. Data processed with Fiji.

III.3 Results

III.3.1 Freezing

As described in Chapter II, the freezing rate has big influence on cell survival and membrane integrity. Subsequently we compared an intermediate freezing rate (1 K/min) with rapid freezing by immersion of the sample in liquid nitrogen (Figure III-3). No difference in total cell recovery was observed, but membrane integrity dropped from 13.5 ± 3.9% for intermediate freezing to 4.6 ± 0.7% after rapid freezing. The immersion of cells in LN_2 caused a clear damage of the cell membrane and an intermediate cooling rate of 1 K/min was therefore applied for further experiments.

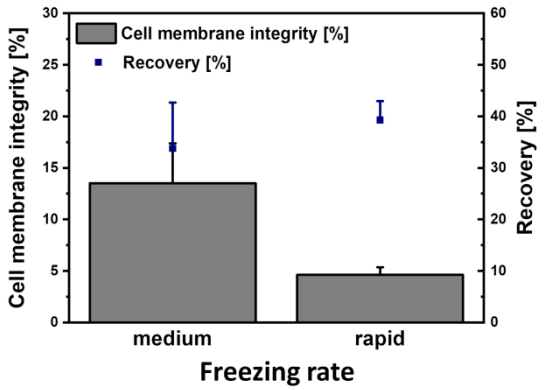


Figure III-3: Cell membrane integrity (bars) recovery (squares) of cells freeze-dried in formulations containing 10% HES and 5% HE in EpiLife/S7. Cells were frozen with a medium freezing rate (1 K/min) or rapidly by immersion in LN_2.

III.3.2 Reconstitution

The reconstitution step is not particular part of the lyophilization process itself; however, it has big impact on cell membrane integrity and cell survival after freeze-drying. To define the optimum rehydration parameters, we tested the influence of rehydration medium (Figure III-4 A), rehydration volume (Figure III-4 B) and also an upstream preincubation step (Figure III-4 C). If not mentioned otherwise, samples were reconstituted with the 1.5 x volume, with EpiLife/S7 as rehydration medium (for example 500 µl of lyophilizate were reconstituted in 750 µl of reconstitution volume). A temperature of 37°C was chosen to avoid a phase transition of the cell membrane during reconstitution.

The comparison of EpiLife/S7, PBS and HPW as rehydration buffers clearly showed detrimental effects for PBS. EpiLife/S7 and HPW as rehydration medium resulted in over 30% cell membrane integrity rates, whereas PBS could only reach 20%. We also compared a 1.5 x with a 3 x reconstitution volume. The higher sample dilution led to decreased membrane integrity values, even if the recovery rate remains stable. A 1.5 x reconstitution volume was chosen as the

minimum tested volume, to avoid a too concentrated solution after reconstitution to enable a direct measurement with the ViCell XR™. Furthermore a formulation with 10% HES and 5% HE leads to high osmolality which we preferred to further dilute with a higher reconstitution volume.

Wolkers et al. described remarkable effects of a prehydration step prior to reconstitution of lyophilized platelets [16], however, these results could not be reported for lyophilized HaCat keratinocytes. Samples were incubated at 37°C, 5% CO_2 with saturated moisture prior to reconstitution. With longer incubation time cell membrane integrity clearly decreased. Already after 30 minutes of incubation only 12.5 ± 3.6% of all cells exhibited intact cell membrane integrity which further dropped to 5.4 ± 2.1 % after 60 minutes of preincubation. Sample mass increased with incubation time, confirming water absorption by the lyophilizates. For further experiments, reconstitution was performed at 37°C, with 1.5 x volume of EpiLife/S7 without preincubation.

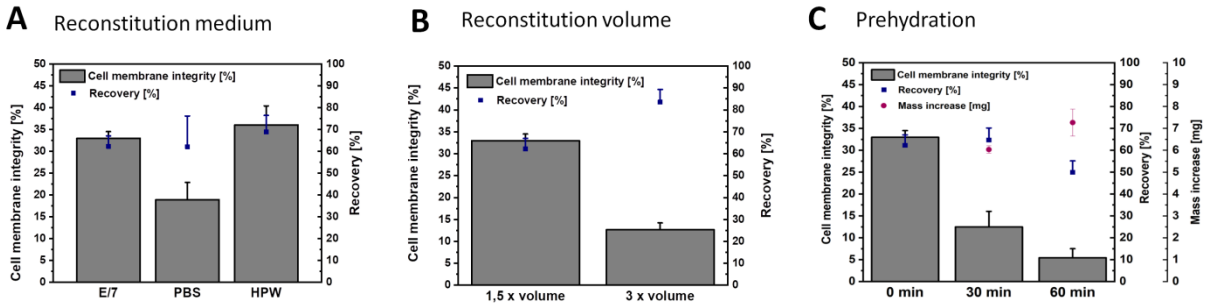


Figure III-4: Cell membrane integrity (bars), recovery (blue squares) of cells after freeze-drying in a formulation containing 10% HES and 5% HE in EpiLife/S7. Cells were reconstituted **(A)** in EpiLife/S7, HPW or PBS, **(B)** with the 1.5 x or 3 x volume in EpiLife/S7 or **(C)** directly after lyophilization or after preincubation for 30 or 60 minutes at 37°C in saturated moisture and 5% CO_2. Mass increase depicted as red circles.

III.3.3 Cellular condition

Despite freezing or rehydration parameters, also the influence of the cellular condition before freeze-drying was investigated.

III.3.3.1 Calcium level

HaCat keratinocytes were cultivated in EpiLife/S7 medium with low Calcium (60 µM) and are known to undergo differentiation at higher calcium concentrations [17], [14]. After differentiation, keratinocytes usually rapidly die, which does not allow long-term cultivation. But unlike primary keratinocytes, HaCat keratinocytes are still able to proliferate and also maintain their clonogenic potential if higher amounts of calcium are added [18]. To address the question whether calcium induced differentiation enhances the potential to maintain membrane integrity we added 0, 1.8 or 2.5 mM calcium to the cell culture medium EpiLife/S7.

The microscopic images in Figure III-5 A show the morphological changes of HaCaT keratinocytes cultivated in EpiLife/S7 with the addition of different calcium amounts. After two days of cultivation only minor changes are visible, but cells with the addition of calcium seem to grow in closer contact to other cells. After four days, the differences become clearly visible and cells with calcium form island like clusters with strong association to adjacent cells. Subsequent lyophilization of cells cultivated for 7 days with calcium addition showed an increase of almost 10 percentage points in membrane integrity (Figure III-5 B). No difference between 1.8 and 2.5 mM calcium was observed, despite lower cell recovery for 2.5 mM Ca^{2+}.

However, the addition of calcium does not only change the morphology and drying behaviour of HaCat keratinocytes, also protein expression is altered. Differentiated cells are described to maintain a higher Keratin 1 and 10 expression, whereas keratin 14 expression decreases [14], [19]. To maintain HaCat keratinocytes in a stable, basal like pheno- and genotype, we decided against a supplementation of EpiLife/S7 with calcium for further experiments.

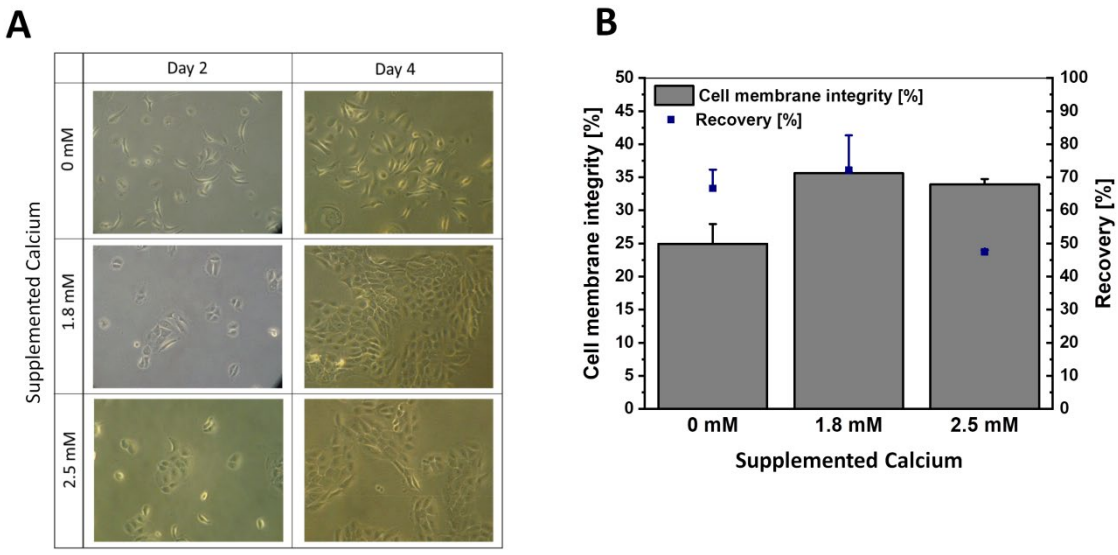


Figure III-5. (A) Representative microscopic images of cells grown in EpiLife/S7 with the addition of 0, 1.8 or 2.5 mM calcium after 2 and 4 days of cultivation**. (B)** Cell membrane integrity (bars), recovery (squares) of cells after freeze-drying in formulations containing 10% HES and 5% HE in EpiLife/S7 after cultivation of 7 days in EpiLife/S7 with the addition of 0, 1.8 or 2.5 mM calcium.

III.3.3.2 Cell density

Despite calcium addition, other cellular properties such as the cell density during drying were analyzed. In particular we focused on the growth density of adherent cells and the cell concentration of cells in suspension. After harvesting, cells were diluted to $5 \cdot 10^5$, $1 \cdot 10^6$ and $2 \cdot 10^6$ cells/ml in a formulation containing 10% HES and 5% HE in EpiLife/S7 prior to freeze-drying cells in suspension. For all cell concentrations, no difference was observed for cell membrane integrity, despite a lower cell recovery for $5 \cdot 10^5$ cells/ml (see Figure III-6 A).

For freeze-drying adherent cells, cells were seeded in concentrations of 4000, 7000 and 10000 cells/cm^2, leading to different growth densities. After 24 hours of cultivation, cells were freeze-dried with 10% HES and 5% HE in EpiLife/S7. Intracellular esterase activity and membrane integrity were determined with Calcein-Acetoxymethyl ester (Calcein-AM) and Ethidiumhomodimer-1 (EH). Calcein-Acetoxymethylester is a fluorescent precursor, which fluorescence is switched on after intracellular cleavage of the ester. Resulting Calcein is trapped and accumulated intracellularly and is therefore labelling metabolic active cells with intact intracellular esterases. EH is a membrane impermeable high affinity nucleic acid dye, which emits red fluorescence if bound to nucleic acids and is therefore labelling cells without intact cell membranes. EH together with of Calcein-AM is a widely established pair of fluorescent dyes to distinguish between living and dead cells.

For adherent cells, a higher growth density seems to have a positive influence on cell survival (Figure III-6 B). A density of 4000 cells/cm^2 resulted in only 50% of cells positively stained with Calcein, whereas for higher growth densities more than 75% of the cells were positive labelled. A similar trend is visible for EH staining. At higher growth densities (10000 cells/cm^2), less cells are stained with EH, indicating higher membrane integrity.

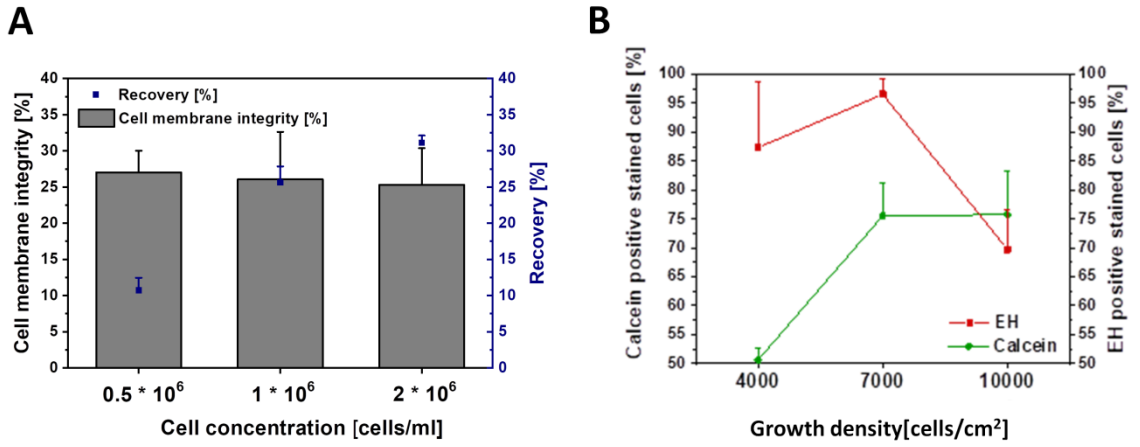


Figure III-6. (A) Cell membrane integrity (bars), recovery (squares) of cells after freeze-drying cells in suspension in formulations containing 10% HES and 5% HE in EpiLife/S7. Cells were freeze-dried in different cell concentrations. **(B)** Percentage of EH (red squares) and Calcein (green circles) positive stained cells after freeze-drying, reconstitution and staining after 24 hours of incubation. Cells were seeded in densities of 4000, 7000 and 10000 cells/cm^2 and freeze-dried adherent, with 10% HES, 5% HE in EpiLife/S7. Pictures were analyzed with the software Fiji.

III.3.3.3 Growth condition

For a more comprehensive overview of the effect of cellular conditions before and during freeze-drying, we analyzed between 20 and 25 different freeze-drying cycles. Prior to drying, cells were resuspended in 10% HES and 5% HE in EpiLife/S7. Besides cell concentration, also the growth density (cells/cm^2) at the time point of harvest, cellular doubling time and cellular passage number

were observed and correlations between latter parameters and cell membrane integrity were investigated.

Previous findings were confirmed and no difference between cell concentration for freeze-drying cells in suspension, ranging from $2.0 \cdot 10^5$ to $1.4 \cdot 10^6$ cells/ml was detected (Figure III-7 A) and also no changes regarding the growth density at the time point of harvest were observed (Figure III-7 B). No clear relationship between doubling time and membrane integrity is present (Figure III-7 C) and also for higher passage numbers no trend towards lower or even higher membrane integrity values is present (Figure III-7 D). To conclude, the analyzed parameters do not influence the outcome after lyophilization of cells in suspension and can be therefore neglected for future cultivation conditions. This gives room to cultivate cells in a broader range of conditions without the need to exclude a batch because one parameter is out of scope.

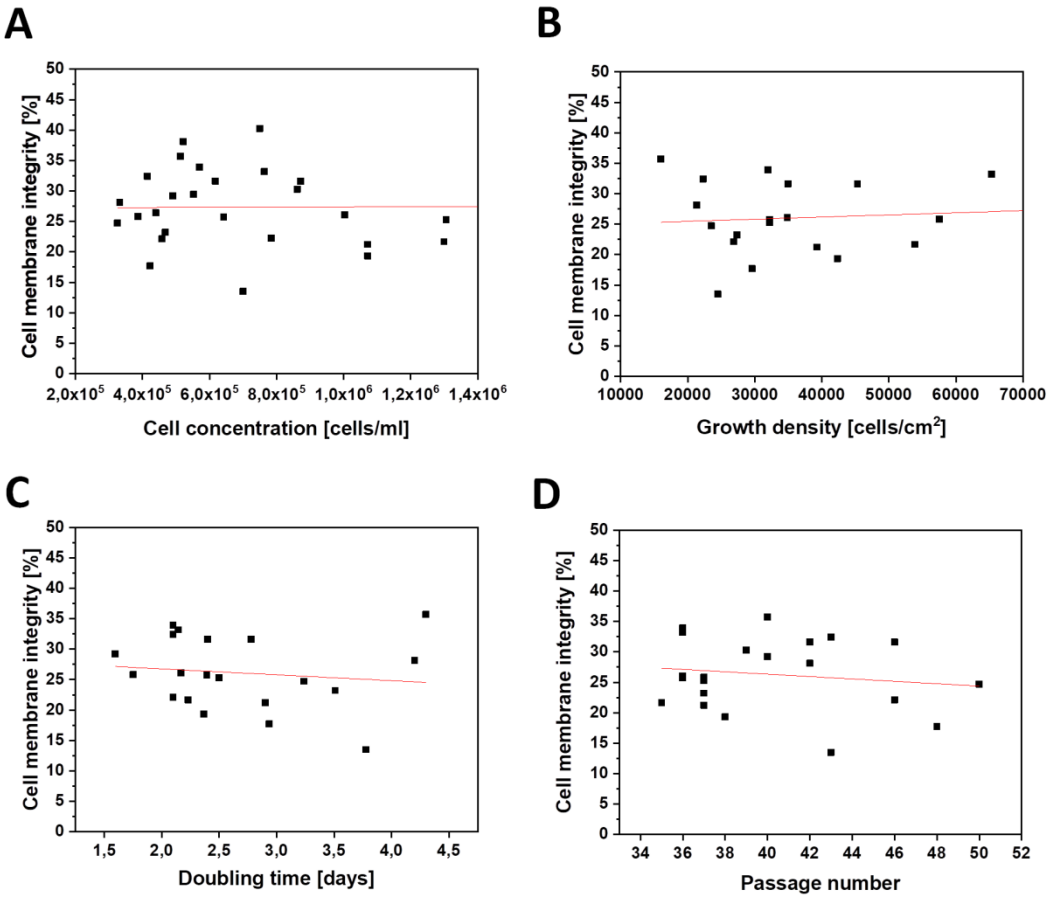


Figure III-7. Cell membrane integrity values after freeze-drying of HaCat keratinocytes resuspended in 10% HES and 5% HE in EpiLife/S7. Cells were analyzed regarding **(A)** Cell concentration, **(B)** Growth density, **(C)** Doubling time or **(D)** Passage number. Red line represents trend line.

III.3.4 From cell culture medium EpiLife/S7 to HEPES buffer

The cell culture medium EpiLife contains various excipients in unknown concentrations (see Table III-1). Besides a variety of amino acids and vitamins, also salts, nitrogen compounds or

nucleosides are included. This diversity of chemically very different substances makes it difficult to determine unwanted effects or ascribe positive effects to certain compounds. Also in case of a risk assessment or an approval of a drug product it is advantageous to know exact concentration and composition of the formulation, which cannot be provided for EpiLife. Furthermore the supplement S7 contains unknown substances as the composition is not disclosed from the manufacturer.

Ingredients in EpiLife

Amino acids	Vitamins	Salts	Others
L-Arginine HCl	Riboflavin	Ammonium- molybdate	Adenine-HCl
L-Cystine	Vitamin B12	Ammonium-metavanadate	D-Glucose
L-Histitinde-HCl	D-Biotin	$CaCl_2$	DL-alpha-Lipoic Acid
L-Isoleucin	Thiamine – HCl	$CuSO_4 \cdot 5\ H_20$	Ethanolamine
L-Leucine	D-Pantothenic Acid	$FeSO_4 \cdot 7\ H_20$	O-Phosphorylethanolamine
L-Lysine	D-Calcium Pantothenate	$MgCl_2 \cdot 6\ H_20$	HEPES
L-Methionine	Folic Acid	$MnSO_4 \cdot H_20$	Putrescine
L-Phenylalanine	Pyridoxal HCl	$NiCl_2 \cdot 6\ H_20$	Sodiumpyruvate
L-Threonine		KCl	Thymidine
L-Tryptophan		$NaHCO_3$	Phenolred
L-Tyrosine		NaCl	Niacinamide
L-Glutamine		Sodiummetasilicate	i-Inositol
		Sodium Phosphate Dibasic	Choline Chloride
		Na_2SeO_3	
		$SnCl_2 \cdot 2\ H_20$	
		$ZnSO_4 \cdot 7\ H_20$	

Table III-1. Ingredients in EpiLife cell culture medium. All ingredients in unknown concentrations.

We therefore searched for an alternative basis with known ingredients and a reduced number of potential active components. We screened PBS, TRIS and HEPES buffer, since they are commonly used in cell culture. pH values and osmolality of compared buffers and EpiLife/S7 are shown in Table III-2. It can be noticed that EpiLife/S7 shows higher pH values, which is due to its open carbonate buffer system. This buffer system maintains a balanced pH based on an exchange between the CO_2 contained in the incubator atmosphere (5% CO_2) and the CO_2 in the culture medium. Therefore at lower CO_2 content – such as in the atmosphere – higher pH values adjust than under cell culture conditions. PBS was used at a pH of 7.4, because this is the optimised pH

for cell culture conditions. The pH of HEPES and TRIS buffer was adjusted also to pH 7.4 for an optimal comparison with PBS, but of course for direct comparison with EpiLife/S7 a pH of 8 would be preferred.

Phosphate buffered saline (PBS) (Sigma Aldrich, St. Louis, MO, USA), without calcium or magnesium was the first buffer that was tested, as it is a widely used buffer in cell culture at a pH of 7.4. PBS is applied for cell washing, to maintain a physiological pH, balanced osmotic pressure and normal cell metabolism [20]. Cells were resuspended in 10 % HES and 5% HE formulated either PBS or EpiLife/S7. Cell membrane integrity and recovery were measured after one freeze-thaw cycle and after freeze-drying (Figure III-8 A). No difference after freezing and thawing was observed, whereas a decrease in cell membrane integrity from 25.8 ± 6.7% in EpiLife/S7 to 6.0 ± 3.7% in PBS after lyophilization occurred. Also recovery rate decreased and proved PBS as an unfavourable basis for the lyophilization formulation. Subsequently other buffers were examined and HEPES and also TRIS buffer were chosen. Both are also commonly used in cell culture and belong to the so called "Good's buffers". Good et al. described several characteristics of "Good's buffers", such as low toxicity, cell membrane impermeability, no interference with chemical reactions and low absorbance between 240 and 700 nm, which make them especially suitable for cell culture and biological research analysis [21].

	Before HES/HE addition		**After HES/HE addition**	
	pH	**Osmolality [mosm/kg]**	**pH**	**Osmolality [mosm/kg]**
TRIS (25 mM, 150 mM NaCl)	7.39	294	7.31	662
HEPES (25 mM, 150 mM NaCl)	7.39	310	7.29	645
PBS	7.39	284	7.06	642
EpiLife/S7	7.70	333	8.1	765

Table III-2. pH and osmolality of used buffers.

HEPES buffer maintained highest levels of cell membrane integrity compared to PBS or TRIS buffer, however, EpiLife/S7 still results in higher absolute values (Figure III-8 B), which could be due to its higher pH. Since HEPES buffer shows little superiority compared to TRIS buffer, we further tried to optimise the concentration and osmolality of HEPES buffer.

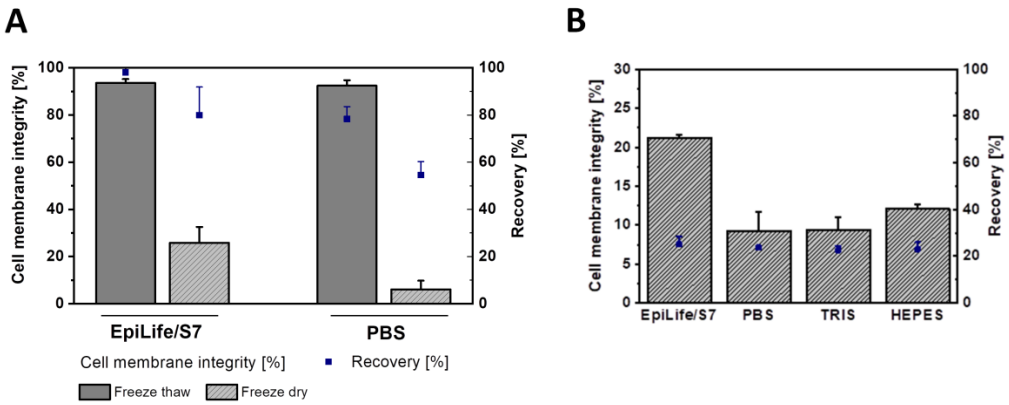
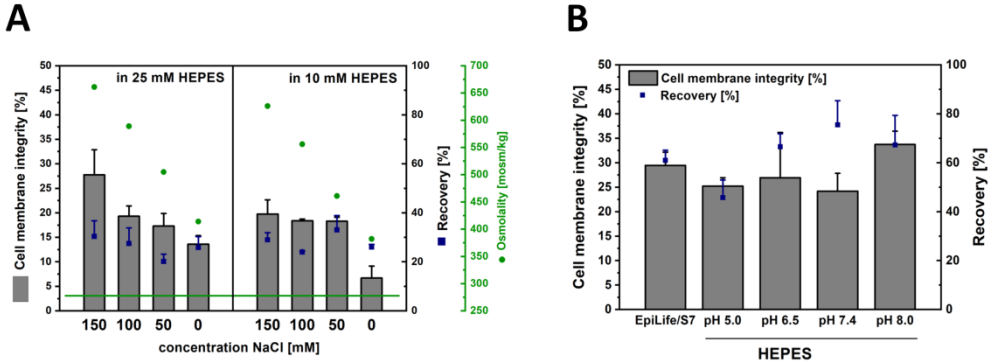


Figure III-8. Cell membrane integrity (bars) and recovery (squares) of cells after freeze-thawing (grey bars) or freeze-drying (striped bars) in formulations containing 10% HES and 5% HE **(A)** in EpiLife/S7 or PBS or **(B)** in EpiLife/S7, PBS, TRIS or HEPES buffer.

Optimising the formulation and pH of HEPES buffer should reveal if it is possible to achieve comparable results with HEPES buffer as with EpiLife/S7. Different HEPES concentrations (25 mM and 10 mM) in combination with varying amounts of NaCl (0 - 150 mM) were tested (Figure III-9 A). 10% HES and 5% HE were added to all formulations and the pH was adjusted to 7.4. Highest cell membrane integrity was achieved for 25 mM HEPES with 150 mM additional NaCl. A lower NaCl concentration resulted in membrane integrity rates below 20% and even below 10% for 10 mM HEPES without NaCl. For 25 mM HEPES buffer a correlation of membrane integrity with osmolality was observed, indicating that a hypertonic solution is more suitable for cell survival. This is also emphasised by the low membrane integrity observed for 10 mM HEPES without NaCl.

Figure III-9 B illustrates membrane integrity and recovery for cells freeze-dried in 10% HES and 5% HE in HEPES buffer (25 mM, 150 mM NaCl) with a pH ranging from 5.0 to 8.0. Even if no big differences were observed, it appears that at a higher pH higher membrane integrity rates were observed, with the highest values of 33.7 ± 2.7% for pH 8.0. For further experiments, EpiLife/S7 was replaced by HEPES buffer with 25 mM HEPES, 150 mM NaCl, set to a pH of 8.0.



FigureIII-9: Cell membrane integrity (bars) recovery (squares) and osmolality (circles) of cells freeze-dried in formulations containing 10% HES and 5% HE **(A)** in 10 or 25 mM HEPES buffer with 0 – 150 mM NaCl or **(B)** in EpiLife/S7 or HEPES buffer (25 mM, 150 mM NaCl) with a pH from 5.0 to 8.0

III.4 Discussion

Our aim was to optimise a freeze-drying process to stabilize human keratinocytes in a dry state and to contribute therefore to a more widespread application of cell based therapies, in particular keratinocyte suspensions for burn wounds and other skin lesions. HaCat keratinocytes were used as a model cell line to simplify culture and handling conditions. In the present chapter we defined the experimental procedure of the freezing and reconstitution process as well as the influence of different growth conditions and cell densities before and during drying. The application of certain excipients and the influence of drying time and residual moisture content will be discussed in the following Chapters IV-VI. Furthermore the formulation was simplified and HEPES buffer was implemented as basis component instead of cell culture medium EpiLife/S7 which contains a broad number of ingredients with unknown concentration.

Freezing: We could prove that fast freezing by immersion in LN_2 drastically reduces membrane integrity of lyophilized cells and that an intermediate freezing rate (1 K/min) is favoured. This is in accordance with literature findings, where rapid freezing is described to induce intracellular freezing events, which damage the plasma membrane and cause severe cell damage [22]. Since the formulation containing 10% HES and 5% HE in EpiLife/S7 or HEPES buffer already has a high osmolality, we excluded slow freezing rates to avoid additional osmotic stress and cell dehydration [23].

Reconstitution: To optimise the reconstitution step, reconstitution volume, reconstitution solution and prehydration prior to complete reconstitution were analyzed. PBS as reconstitution medium showed adverse effects compared to HPW or EpiLife/S7, which is especially interesting, as many researchers found that water as rehydration medium is less suitable [24], [25]. Prior research suggests that higher reconstitution volumes can promote faster reconstitution times and therefore avoid the occurrence of detrimental concentration effects, caused by partial dissolved cake components [24]. However, this could not be confirmed by our experiments and higher reconstitution volume (3 x the original volume) was less effective than a 1.5 x volume. Some authors have also reported that a prehydration step prior to complete rehydration can introduce small amounts of water to the cell membrane. Therefore the membrane can get rehydrated without intracellular components leaking from the cytoplasm, which could reduce damage and protein loss during reconstitution [16], [26]. Contrary to literature findings, our experiments showed that a prehydration step of 30 or 60 minutes in moisture saturated air drastically decreased membrane integrity. However, the reconstitution studies in the literature were performed with platelets, which lack a nucleus and in general differ very much from eukaryotic cells. Furthermore the reconstitution process is also dependent from cake composition and structure and needs to be determined individually for every cell type and formulation. To conclude, our findings suggest

that the optimum rehydration procedure for HaCaT keratinocytes freeze-dried in 10% HES and 5% HE in EpiLife/S7 is either HPW or EpiLife/S7 in a 1.5 x volume without prehydration.

Cellular condition: Cultivation of HaCat keratinocytes in presence of higher calcium concentrations led to a change in morphology and to an increased membrane integrity after lyophilization. Also freeze-drying adherent cells at higher cell densities led to more Calcein and less EH positive stained cells. The literature review shows that calcium addition as well as cultivating HaCat keratinocytes above 80% confluency can induce keratinocyte differentiation, with a change in cytokine and differentiation marker production [19]. Moreover, we could show that unlike primary human keratinocytes, HaCat keratinocytes increase their proliferation rate upon calcium addition (data not shown), which is in accordance with the findings of Miceallef et al. [27]. Therefore, differentiation of HaCat keratinocytes might be the reason for the better cell preservation after freeze-drying for both Ca^{2+} addition and cultivation at higher cell densities. Differentiation, coming along with a change in growth behaviour, cytokine pattern and also the formation of desmosomes and tight junctions could lead to cellular structures that are more resistant against external stresses. Despite latter findings we decided to cultivate keratinocytes without calcium addition as we favour the characteristics of undifferentiated cells, which have a higher ability to induce cell growth and migrative behaviour of adjacent cells. Additionally, HaCat keratinocytes are only used as model cell line and primary keratinocytes will be used for a therapeutic application, which do not proliferate in the differentiated state.

To determine the optimum cell density for freeze-drying keratinocytes in suspension, we compared cell densities ranging from $2.0 \cdot 10^5$ to $2 \cdot 10^6$ cells/ml but did not notice any differences in drying behaviour. Freeze-drying cells in higher concentrations is time and material saving, as more cell material can be obtained in fewer lyophilization processes. As a compromise between cultivation time, effective yield and to obtain sufficient cells for subsequent analysis, we freeze-dried cells in a concentration range of $0.5 – 1 \cdot 10^6$ cells/ml. No effect on membrane integrity or cell recovery was observed for other cultivation conditions, such as cellular doubling time, passage number or growth density when cells were freeze-dried in suspension. We therefore set no specific target corridor for these parameters.

Replacement of EpiLife/S7: EpiLife/S7 contains many ingredients with unknown concentrations and moreover the formulation of the supplement S7 is not disclosed. To replace this multi-component cell culture medium as basis for the lyophilization formulation, different alternative buffer systems were tested. PBS led to equal membrane integrity values after freeze-thawing, however, after freeze-drying a drastic decrease in membrane integrity and recovery rate was observed with PBS. Previous studies have shown that PBS undergoes a pH shift during freezing due to crystallization of $Na_2HPO_4 \cdot 12H_2O$, depending on concentration and initial pH, which

could lead to further cell damage during drying [28]. However, HEPES buffer emerged to be an acceptable alternative for EpiLife/S7 and resulted in similar membrane integrity values. HEPES buffer at a pH of 8.0, a concentration of 25 mM HEPES and 150 mM NaCl, was superior compared to lower pH values and lower NaCl or HEPES concentrations. With the addition of 10% HES and 5% HE a hypertonic solution featured an osmolality of 660 mosm/kg. Interestingly, this hyperosmolality seems to have no detrimental effect, even if freezing of extracellular water will contribute to a further upconcentration of the resulting solutes. The reverse was true and lower NaCl or HEPES concentrations led to lower membrane integrity values.

Conclusion: Freeze-drying cells is an interplay between the optimization of the technical aspects of a lyophilization cycle, finding the best growth conditions prior to lyophilization and the implementation of a highly effective excipient composition. It is beyond the scope of this thesis to perform an in depth analysis and optimization of all possible setscrews. We therefore tried to set a framework of general parameters that are applied for following experiments, which are summarized in Table III-3.

Parameter	**Applied condition**
Calcium	No additional Calcium
Cell density	0.5 – $1 \cdot 10^6$ cells/ml
Freezing rate	1 K/min
Rehydration	1.5 x volume, 37°C, EpiLife/S7 or HPW
	no prehydration
Basis buffer	25 mM HEPES with 150 mM NaCl (pH 8)
	EpiLife/S7

Table III-3. Applied cultivation, lyophilization and reconstitution conditions for freeze-drying HaCat keratinocytes.

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Chapter IV

DMSO as new, counterintuitive excipient for freeze-drying human keratinocytes

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Abstract

DMSO is widely used as powerful cryoprotectant for the storage and transport of frozen cells. Beyond this established application of DMSO, we could now show that it has also promising effects in the field of lyophilization of therapeutic cells Freeze-drying of HaCaT keratinocytes in 10% HES, 5% HE and in presence of DMSO led to an increase in cell membrane integrity from 25.3 ± 2.7 % without DMSO to 41.4 ± 4.3 % with 2% DMSO, as determined by trypan blue exclusion. Interruption of the lyophilization cycle at different sampling points showed a rapid decrease of cell membrane integrity below a critical residual moisture content. DMSO was able to stabilize cell membranes below this moisture level up to a final residual moisture content of less than 1%. Furthermore, DMSO increased the total protein content of cells after freeze-drying and subsequent SDS PAGE analysis indicated that certain abundant proteins were better preserved with the use of DMSO. Owed to its low vapour pressure, a significant part of DMSO is not removed during freeze-drying and remains as plasticiser in the lyophilized cake. However, a Tg above 60°C for 2% DMSO indicates that samples can still be stored at temperatures of 2-8 °C. Also, no macroscopic or microscopic collapse could be observed by SEM or BET measurements and DMSO addition led even to more elegant cakes with reduced cake cracking. Thus, with a better preservation of cell membranes and cellular structures, DMSO can contribute to the still unsolved problem of freeze-drying cells of higher complexity.

IV.1 Introduction

Application of cell therapies, such as stem cell transfer, tissue engineering, keratinocytes for wound healing, blood transfer or reproduction medicine, is a rapidly growing therapeutic field [1]. These manifold applications and the increasing demand of regenerative medicine and blood transfusions require a constant supply of active cells. Therapeutic cells must be widely distributed and rapidly available, but due to their very short shelf life they must be used within days by federal regulations [2], [3], [4]. To satisfy these requirements, a successful preparation method of cells for long term storage is needed, thus allowing them to be available on demand. Nowadays, the most common way for transport and storage of active cells is cryopreservation at sub-zero temperatures, going along with high costs and energy input. Also transient warming events or cross-contamination are critical factors, which require additional precautions when handling frozen products [3], [5], [6].

A smart way to overcome latter problems could be lyophilization of therapeutic cells. Freeze-drying as preservation method offers various advantages including increased sample stability at higher temperatures, easy transportation and controlled and fast reconstitution processes [7]. However, cellular damage can be induced by all different steps of a lyophilization cycle and many efforts have been made to successfully replace the removed water and to stabilize cells in the dry state. A wide range of excipients was screened and sugars, polyols, amino acids, antioxidants, macromolecules and other compounds were implemented as stabilizers [7], [8]. Despite the fact that many milestones have been reached, lyophilization of eukaryotic cells still remains an extremely challenging field with limited success so far. To our knowledge, besides stem cells, eukaryotic cells that proliferate after lyophilization could not be obtained yet. It is therefore necessary to apply new approaches for a better stabilization of cells in the dry state and we want to introduce DMSO as an additional lyoprotectant for the lyophilization of cells.

For freezing and the storage of frozen cells DMSO is widely described in literature [9]. DMSO is a small aprotic molecule with relatively low toxicity (LD_{50}: 2.5 to 8.9 g/kg for different species), which can easily permeate biological membranes. Its high boiling point (189°C) indicates high association of the single molecules. Besides, DMSO is a hydrogen bond acceptor and it was shown that DMSO and water molecules highly interact. Nevertheless DMSO is also described to show high self-association behaviour, most likely due to dipolar forces and molecular associations [9], [10]. Also a chain like structure with association of oxygen to sulphur atoms has been reported to explain its high boiling point and high latent heat of vaporisation [9]. With its resulting very low vapour pressure (0.61 mmHg at 25°C) it is doubtful if DMSO can be removed during freeze-drying and it is likely that a significant amount of the originally applied DMSO will

remain in the lyophilized cake [11]. Subsequently the question arises, whether an acceptable lyophilized product can be achieved in the presence of DMSO.

During cryopreservation DMSO does not only depress the freezing point of the aqueous solution, but also – according to the so called vitrification hypothesis – promotes vitrification and therefore inhibits ice crystal formation [12], [13]. Other protection mechanisms for DMSO are proposed and many researchers focused on the question, how DMSO interacts with lipid bilayers. For example Notman et al. showed with molecular dynamics simulations the interaction of DMSO with cell membranes and proved a concentration dependant influence of DMSO on phospholipid membranes. In low concentrations DMSO leads to membrane thinning, whereas higher concentrations promote pore formation and even membrane disintegration [14], [15]. Furthermore it was shown that DMSO maintains membrane fluidity during freezing and increases cell membrane permeability by interaction with the hydrophilic region of the membrane lipids [9].

However, all research about DMSO and its interaction with water and membranes only faced the question of its behaviour in the frozen or liquid state, but to our knowledge freeze-drying of cells in the presence of DMSO has not been studied systematically. Our research therefore addresses the question, whether freeze-drying in the presence of DMSO is feasible and to what extent the physicochemical properties of the lyophilized cakes are affected. Moreover, it is in our interest to investigate, if DMSO is not only a powerful cryoprotectant but can also further stabilize cells during freeze-drying. In particular the effect of DMSO on the cell membrane is of interest, as especially the sensitive cell membrane gets highly damaged during freeze-drying. Herein we present our recent work in freeze-drying HaCaT keratinocytes in the presence 10% HES, 5% HE and low concentrations of DMSO and its effect on cellular membrane integrity and protein condition, as well as on the physicochemical properties of the lyophilized matrix.

IV.2 Material and Methods

IV.2.1 Materials

Hydroxyethyl starch 200/0.5 (HES) was purchased from BOC Sciences (Shirley, NY, USA). Hydroxyectoine (HE), tris buffered saline (TBS: 0.05 M Tris buffered saline (0.138 M NaCl, 0.0027 M KCl), pH 8.0, at 25 °C), formaldehyde (36% in water), sodium dodecyl sulphate (SDS), dulbeccoo's phosphate buffered saline (PBS: 0.2 g/L KCL, KH_2PO_4, 8 g/l NaCl, 1.15 g/l Na_2HPO_4 (anhydrous)) and polysorbate 20 was ordered from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). HEPES and NaCl were ordered from VWR (VWR, Allison Park, PA, USA). HaCaT (Human adult low Calcium high Temperature) keratinocytes were purchased from the DKFZ (Heidelberg, Germany).

IV.2.2 Cell and sample preparation

Cell preparation: HaCaT keratinocytes were cultivated in EpiLife with 60 µM calcium chloride, supplemented with 5 ml of 100x Supplement S7 (both from Gibco by Thermo Fisher Scientific (Waltham, MA, USA)). For better cell attachment cell culture flasks were coated with 60 µg/ml PureCol Type I Collagen (Advanced Biomatrix, Carlsbad, CA, USA) for 90 minutes at 37 °C, 5% CO_2.

Sample preparation: For lyophilization of cells in suspension, cells were resuspended in different formulations, containing 10% (w/v) hydroxyethyl starch (HES) and 5% (w/v) hydroxyectoine (HE), dissolved in HEPES buffer (25 mM HEPES, 150 mM NaCl, pH 8.0). Between 0% and 4% (v/v) DMSO were added and cells were freeze-dried in a concentration of 0.5 – $1 \cdot 10^6$ cells/ml. Aliquots of 500 µl were filled in 10 R tubing vials (MGlas AG, Muennerstadt, Germany) and semi-stoppered with lyophilization stoppers (FluroTec® rubber stopper; West Pharmaceuticals, Eschweiler, Germany). In earlier experiments, this cell concentration showed to be adequate to obtain sufficient cell and protein content for further cellular analysis. A volume of 500 µl provides also enough solid material for subsequent physicochemical analysis and allows direct analysis of lyophilized cells without prior upconcentration.

For lyophilization of adherent cells, cells were grown in a 6-well plate (TPP products, Trasadingen, Switzerland) or on round glass coverslips (d =13 mm, VWR, Allison Park, PA, USA) in a 24-well plate (VWR, Allison Park, PA, USA) for 24 hours. Prior to freeze-drying, growth medium was aspirated and cells were washed once with PBS. For lyophilization, 1.5 ml of respective formulation was added per well and cells were freeze-dried directly in the well plate.

Lyophilization: A Christ 2-6D laboratory scale freeze-dryer was used (Martin Christ, Osterode am Harz, Germany). With an equilibrium step of 30 minutes at -5 °C, samples were cooled in a constant freezing rate of 1 K/min down to -50 °C. Temperature was hold for 90 minutes at -50 °C. Primary drying was carried out at -35°C with a vacuum set to 0.01 mbar for 20 hours. Secondary drying was performed at 20°C and 0.01 mbar for 5 hours with a heating ramp of 0.1 K/min until 0°C and 0.2 K/min until 20°C. Samples were stoppered at 600 mbar. Sample temperature was monitored using *Wireless Product Temperature Measurement Sensors* from Martin Christ (Osterode am Harz, Germany) and chamber pressure was controlled with a pirani and a capacitance manometer.

To remove samples within the freeze-drying cycle, shelf temperature was set to -50°C during primary drying or to -10°C during secondary drying to interrupt the drying process. All samples were stoppered at 600 mbar and stored at -80°C until analysis. To resume the drying process,

remaining vials were reopened manually to a semi stoppered position and the freeze-drying cycle was continued at previously set drying conditions.

IV.2.3 Physicochemical analysis

For non-cellular experiments, placebo samples were prepared and analyzed.

Residual moisture content: For the determination of residual moisture content of dry samples, a headspace Karl Fischer titrator was used (Aqua 40.00, Elektrochemie Halle, Halle, Germany). Between 10 and 30 mg of sample was transferred under dry atmosphere (relative humidity below 10%) into 2R vials and stoppered. The samples were heated to 100°C and analyzed via headspace Karl Fischer titration. Residual moisture content of collapsed or partial dried samples was determined gravimetrically.

Specific surface area (SSA): Specific surface area was determined using Brunauer–Emmet–Teller krypton gas adsorption in a liquid nitrogen bath at 77.3 K (Autosorb 1, Quantachrome, Odelzhausen, Germany). Approximately 75 mg of a sample was gently crushed with a spatula and weighed into a glass tube. Before measurement, an outgassing step was performed for at least 2 hours at room temperature. An 11-point gas adsorption curve was measured, covering a p/p0 ratio of 0.05-0.30. Data evaluation was performed according to the multipoint Brunauer–Emmet–Teller method fit of the Autosorb 1 software.

Dynamic scanning calorimetry (DSC): A Netzsch DSC 204 Phoenix (Selb, Germany) was used to measure the glass transition temperature of the maximal freeze-concentrated solution (Tg'). Aliquots of 20 µl of each solution was transferred into an aluminium sample pan and crimped. Samples were cooled with 10 K/min from 20 to -100°C and after analysis, Tg' was determined with the Netzsch Proteus software.

Glass transition temperatures of the dried cake (Tg) were measured using a Mettler Toledo DSC 821e (Gießen, Germany). Under dry atmosphere, approximately 5 mg of dry cake was transferred into an aluminium sample pan and crimped. Samples were heated from -40 to 140 °C with a heating rate of 10 K/min and after analysis, Tg' was determined with the Mettler STARe software.

Residual DMSO analysis: As described previously, residual DMSO content was analyzed by static headspace gas chromatography-mass spectrometry [16]. An Agilent Technologies 7890B gas chromatograph (Waldbronn, Germany), equipped with an Agilent J&W DB-624 UI ultra inert capillary column (6% cyanopropyl phenyl and 94% polydimethylsiloxane, 30 m × 0.25 mm × 1.4 µm) and an Agilent Technologies 7010B triple quadrupole detector with high efficiency source was used for analysis.

Microscopic characterisation: Scanning electron microscopy (SEM) was used to study the morphology of the samples and to detect microscopic collapse. A JSM-6500F Field Emission Electron Microscope (Jeol, Eching, Germany) was used. The freeze-dried cake was broken with a spatula under dry atmosphere and the generated pieces were fixed with carbon conductive cement on a sample holder such that the breakage border could be analyzed.

Macroscopic characterisation: To analyze the macroscopic cake structure and the extent of cracking during lyophilization, samples were freeze-dried in glass dishes of 30 mm diameter with a filling volume of 1 ml. For better visualisation of cracks, samples were placed on a white LED tray with a black cover with a 30 mm opening in the middle. A carton box was placed around the LED tray to avoid excess light. Pictures were taken with a Nikon D5300 camera (Nikon Corporation, Tokyo, Japan) from the top of the box, perpendicular to the sample.

IV.2.4 Analysis of cells

Reconstitution: Freeze-dried samples were reconstituted in the 1.5 x volume of the originally lyophilized volume with HEPES buffer (37°C, 25 mM HEPES, 150 mM NaCl at pH 8.0). For example 500 µl freeze-dried volume was reconstituted with 750 µl HEPES buffer. Partially dried samples or frozen samples were rapidly thawed and diluted with HEPES buffer to a total volume of 750 µl.

Cell membrane integrity and total cell recovery: Cell membrane integrity and total cell recovery of HaCaT keratinocytes after lyophilization was quantified with a Vi-Cell™ XR Cell Viability Analyzer (Beckmann Coulter, Krefeld, Germany), using trypan blue, a membrane impermeable dye, staining cells with impaired cell membrane blue. After reconstitution with 750 µl HEPES buffer, aliquots of 550 µl of cell suspension were measured directly without dilution. Cells were automatically counted and categorized into viable and dead by their trypan blue exclusion with the software integrated in the Vi-Cell™ XR, thus giving the total percentage of membrane intact cells. Recovery of cells was calculated as the total number of cells counted after freeze-drying divided by the number of total cells before freeze-drying and multiplied by the factor 100, regardless if their membrane was impaired or not.

Cell membrane visualization: 10,000 HaCaT keratinocytes per sample were seeded on sterilized 13 mm glass coverslips and freeze-dried after 48 hours of incubation at 37°C, 5% CO_2 as described above. After reconstitution, cells were fixed with 4% formaldehyde in PBS for 15 minutes at RT. Subsequently cells were permeabilized with 0.5% Triton X in PBS and 0.25 µm Rhodamine Phalloidin (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) was added for 40 minutes at RT. To stain the nucleus, cells were incubated with DAPI at a final concentration of 0.5 µg/ml in PBS for 20 minutes at RT. Between the single steps, cells were

washed three times with PBS. Cells were mounted using FluorSave reagent (Merck Millipore, Billerica, MA, USA) and pictures were taken with a SP8 inverted scanning confocal microscope (Leica Camera, Wetzlar, Germany).

Calcein-AM confocal imaging: 50,000 HaCaT keratinocytes per sample were seeded on sterilized 13 mm glass coverslips and freeze-dried as describes above. After reconstitution, cells were washed three times with PBS and stained with 0.5 µM Calcein-AM Viability Dye (eBioscience™ by Thermo Fisher Scientific, Waltham, MA, USA) for 10 minutes at RT. Subsequently cells were fixed with 4% formaldehyde in PBS for 15 minutes at RT and stained with 5 µg/ml Hoechst 33342 (molecular probes by Thermo Fisher Scientific, Waltham, MA, USA) for 15 minutes at RT. Between the single steps, cells were washed three times with PBS and finally cells were mounted using FluorSave reagent (Merck Millipore, Billerica, MA, USA). Analysis was performed with a SP8 inverted scanning confocal microscope (Leica Camera, Wetzlar, Germany).

Calcein-AM flow cytometry: 250,000 cells per vial were freeze-dried in suspension as described above. After reconstitution, cells were washed two times with PBS and resuspended in 100 µl PBS containing 0.1 µM Calcein-AM Viability Dye. After incubation for 20 minutes at RT, cells were washed two times and resuspended in PBS containing 2 mM EDTA. As a control 75,000 non freeze-dried cells were treated the same way as described above. Cells were analyzed using an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA) set to 488 nm excitation and 530/30 nm bandpass emission wavelength. All samples were gated based on morphology according to forward/sideward scattering.

EGFR confocal imaging: 50,000 HaCaT keratinocytes were seeded and prepared as cells for Calcein-AM staining. After reconstitution, cells were washed three times with PBS and fixed with 4% formaldehyde in PBS for 15 minutes at RT. For permeabilization 0.3% polysorbate 20 in PBS was added for 30 minutes. Blocking was performed for 1 hour at RT with 5% BSA in TBST. Cells were stained with 4 µg/ml EGFR antibody conjugated with Alexa Fluor® 488 (Santa Cruz Biotechnology, Dallas, TX, USA) for 24 hours at 4 °C and finally DAPI was added at a final concentration of 0.5 µg/ml in PBS and incubated for 20 minutes. Between the single steps, cells were washed three times with PBS. Cells were mounted using FluorSave reagent (Merck Millipore, Billerica, MA, USA) and were analyzed with a SP8 inverted scanning confocal microscope (Leica Camera, Wetzlar, Germany).

EGFR flow cytometry: 250,000 cells per vial were freeze-dried in suspension as described above. After reconstitution, cells were washed two times with PBS and blocking was carried out by incubating cells with TBST containing 5% BSA for 1 hour. Cells were washed two times, centrifuged and resuspended in 50 µl TBST with 5% BSA. Aliquots of 2.5 µl of EGFR antibody conjugated with Alexa Fluor® 488 (Santa Cruz Biotechnology, Dallas, TX, USA) was added per

sample and incubated for 2 hours at 4°C. As a control 75,000 non freeze-dried cells were treated the same way as described above. An isotype control (normal mouse IgG2a Alexa Fluor® 488, Santa Cruz Biotechnology) was applied to proof specific binding of the antibody. Flow cytometry analysis was carried out as described above.

Lysis of cells: 300,000 cells were plated per well of a 6-well plate (TPP products, Trasadingen, Switzerland) 24 hours prior to experiment and were incubated at 37 °C, 5% CO_2. Cells were washed once with PBS, 1.5 ml of formulation was added to each well and cells were freeze-dried directly in the well plate. After reconstitution, the supernatant was aspirated and cells were washed three times with ice cold PBS. 50 µl of lysis buffer (RIPA buffer (Sigma Aldrich, St. Louis, MO, USA) with 1× phosphatase inhibitor PhosSTOP and 1× protease inhibitor cOmplete™ ULTRA Tablets, both Roche Diagnostics (Rotkreuz, Switzerland) was added per well and incubated for 30 minutes on an orbital shaker on ice. Subsequently cells were scraped, transferred into Eppendorf reaction tubes (Greiner AG, Kremsmünster, Austria) and centrifuged for 10 minutes at 16,000 g and 4 °C. The protein lysate was used for the analysis of the total protein content, as well as for SDS-PAGE with subsequent protein staining.

BCA assay: To determine the total protein content after freeze-drying, a BCA assay of the protein lysate was performed according the manufacturer's instructions, using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Gel electrophoresis: Aliquots of 13 µg of protein lysate were mixed with loading buffer (4x Laemmli Loading buffer, VWR, Allison Park, PA, USA) and water to a volume of 30 µl and heated for 5 minutes at 95 °C. Aliquots of 25 µl of latter mixture were loaded in each well of a 10% tris-glycine gel (Novex™ WedgeWell, Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) and 5 µl of PageRuler™ Plus Prestained Protein Ladder (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) were loaded in the first and the last well. The gel was run for 90 minutes at 100 V in 1× Rotiphorese® running buffer (Carl Roth, Karlsruhe, Germany). After the run, the gel was rinsed twice in deionised water and stained for 1 hour in Brilliant Blue G solution (Sigma Aldrich, St. Louis, MO, USA). Subsequently the gel was destained (destaining solution: 50% HPW, 40% methanol, 10% acetic acid) for 24 hours. Pictures were taken with a Biorad Chemidoc (Bio-Rad Laboratories, Hercules, CA, USA). Intensities of certain protein bands were analyzed and integrated with the software Image Lab 6.0.1.

Cytotoxicity of DMSO: The cytotoxicity of DMSO was tested via MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. 8,000 HaCaT keratinocytes were plated to each well of a transparent 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA) and were grown for 24 hours at 37 °C, 5% CO_2. Aliquots of 100 µl of all tested formulations were prewarmed to 37 °C and added to the wells, respectively. As a positive control EpiLife/S7

medium and as negative control 5% SDS in PBS was used. After respective incubation times, medium was aspirated and 100 µl of MTT-containing medium (0.5 mg/ml MTT in EpiLife/S7 medium) was added to each well. Cells were incubated for another 3 hours at 37 °C, 5% CO_2. Subsequently, the cell culture medium was carefully removed and 200 µl of *iso*-propanol was added to each well to dissolve all formed formazan crystals. After fully dissolution of all crystals, the well plate was centrifuged at 2,000 g for 5 minutes and 150 µl of the solution was transferred to another transparent 96-well plate. The optical density at 570 nm was measured with a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany).

IV.3 Results

IV.3.1 Freeze-drying formulation

In previous experiments, various excipients, including sugars, polyols and antioxidants were screened and best results were achieved with a combination of 10% hydroxyethyl starch (HES) with 5% hydroxyectoin (HE), formulated in HEPES buffer (25 mM, 150 mM NaCl, pH 8).

HES has been widely used as excipient to stabilize cells during freeze-drying, though it is not supposed to penetrate cell membranes. Due to its vitrification properties, HES can inhibit fusion of the cell membrane during drying. Additionally, HES has strong water binding properties and increases the viscosity of a solution which leads to reduced movement and osmotic stress during drying [17], [18]. Moreover, as a large polysaccharide HES is supposed to have a relatively high Tg, which is beneficial for freeze-drying and subsequent storage [19].

The tetrahydropyrimidine derivative hydroxyectoine was found in gram-positive halophilic/halotolerant bacteria and is supposed to have stabilizing effect on protein and DNA structure [20]. Hydroxyethyl starch cannot be used as the only excipient, but in combination hydroxyectoine, good results in freeze-drying HaCaT keratinocytes were achieved.

IV.3.2 Effect of DMSO on the physicochemical properties of the freeze-dried matrix

IV.3.2.1 Glass transition of the maximally freeze-concentrated solution

In the following we show the influence of DMSO added to a placebo formulation, containing 5% HE and 10% HES in HEPES buffer, on the physicochemical properties of the resulting freeze-dried matrix. Between 0 and 4% DMSO (v/v) were added, respectively.

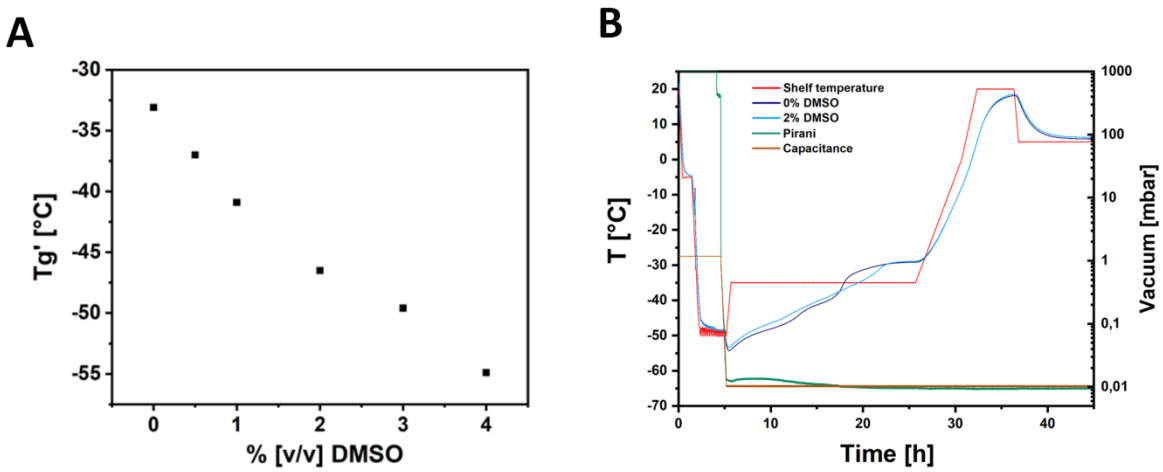


Figure IV-1. (A) Glass transition temperatures of the maximally freeze-concentrated solution (Tg') of 10% HES and 5% HE in HEPES buffer containing between 0% and 4% (v/v) DMSO, respectively. **(B)** Thermocouple and pressure readouts of the freeze-drying cycle. The green and brown lines represent the pirani (green) and capacitance (brown) manometers. Shelf temperature is depicted in red. Dark and light blue lines are the thermocouple readouts for formulations containing 10% HES, 5% HE, with (dark blue) and without (light blue) 2% DMSO.

With increasing amount of DMSO, the glass transition of the maximal freeze-concentrated solution (Tg') decreases in a linear way (see Figure IV-1 A). Thermograms can be found in the supplementary data (see Figure IV-S.1). As freeze-drying should be carried out at temperatures below collapse temperature, which is typically 2-3 degrees above Tg', freeze-drying formulations with DMSO requires especially low product temperatures during primary drying [21]. DSC measurements showed a glass transition of -46.5°C for 10% HES and 5% HE in HEPES, containing 2% DMSO. Thereby, to avoid collapse a product temperature below -48.5°C should be aimed during primary drying. This was achieved with a very conservative freeze-drying cycle with a shelf temperature set to -35 °C and vacuum to 0.01 mbar (Figure IV-1 B). It appears that drying with and without DMSO has no impact on the drying behaviour itself, as both product temperatures move within the same temperature range during drying. Indicated by the thermocouple readouts, DMSO addition leads to a reduction of drying time of approximately 3 hours. The short overall drying time is a result of the low filling volume of 0.5 ml, with a filling height of only 2.2 mm.

IV.3.2.2 Residual DMSO after freeze-drying

The very low vapour pressure of 0.61 mmHg at 25°C of DMSO already suggested residual DMSO in the freeze-dried product [11], [9]. The exact amount of DMSO after lyophilization was determined by GC-MS analysis. Figure IV-2 A shows that irrespective of the amount of originally applied DMSO almost the entire DMSO is retained in the cake after lyophilization. This is in accordance with the sample mass after lyophilization, which increases with higher residual DMSO (Figure IV-2 B).

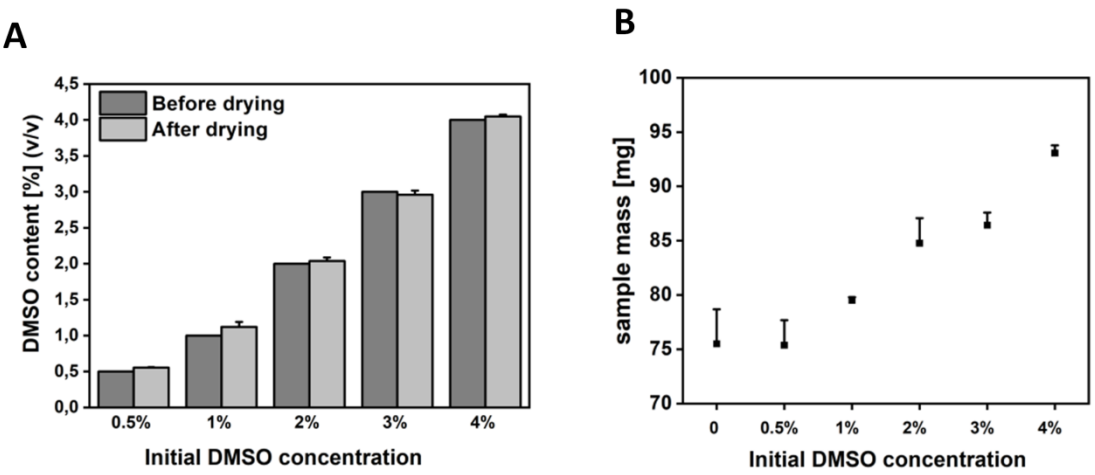


Figure IV-2. (A) DMSO content before and after lyophilization for formulations of 10% HES and 5% HE in HEPES, containing between 0.5% and 4% (v/v) DMSO, respectively. DMSO content before freeze-drying was calculated by the amount of added DMSO. DMSO content after freeze-drying was determined by GC-MS analysis. **(B)** Sample mass after lyophilization for formulations of 10% HES and 5% HE in HEPES, containing between 0% and 4% (v/v) DMSO, respectively.

IV.3.2.3 Residual moisture and glass transition temperature

For formulations containing 10% HES and 5% HE with 0 and 4% DMSO, respectively, the residual moisture (RM) content and glass transition temperatures (Tg) of the dried cakes were analyzed. As expected, higher amounts of DMSO led to significant lower glass transition temperatures (Figure IV-3). Tg decreases from 101.6 ± 1.6°C for HES/HE without DMSO to 31.8 ± 1.0°C for formulations containing 4% DMSO. For 2% DMSO Tg is still above 60°C, which is therefore no obstacle for an intended storage temperature of 2-8°C. Thermograms of the lyophilizates are provided in the supplementary data (Figure IV-S.2). These results match with the previous findings that DMSO is not removed during drying and acts as plasticiser in the lyophilized cake.

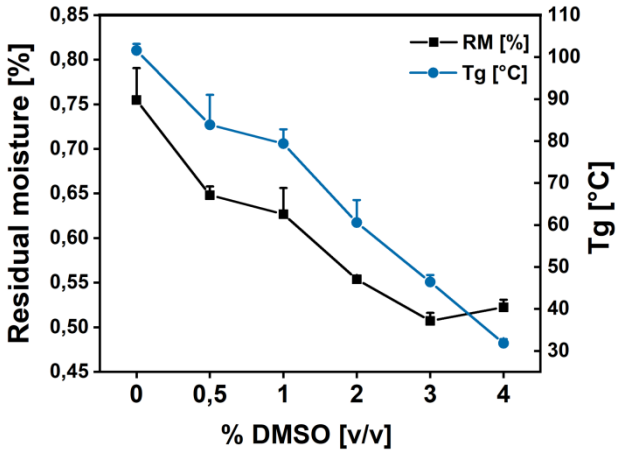


Figure IV-3. Residual moisture content (black squares) and Tg (blue circles) for formulations containing 10% HES and 5% HE in HEPES buffer and between 0 and 4% (v/v) DMSO, respectively.

Interestingly, Tg is not correlating with the residual moisture content and RM is decreasing parallel with Tg. Overall, all products resulted in low residual moisture contents which is again owed to the low filling volume and height. RM content is a critical parameter for storage, but with values below 1% a well preserved product can be achieved and in a later section the influence of RM content on a cellular level will be discussed.

IV.3.2.4 Macroscopic appearance

No macroscopic collapse was observed for formulations containing 0, 2 or 4% DMSO (see Figure IV-4 A). Furthermore, the addition of DMSO contributed to a reduction of cake cracking and more elegant cakes were obtained. This is even better visible if the cakes were freeze-dried in glass dishes and visualized on a white tray (see Figure IV-4 B). An elegant cake appearance is an important goal and quality attribute in the development of a lyophilized drug product. However, there is also growing evidence, that cake appearance is a less critical aspect regarding protein and storage stability [22].

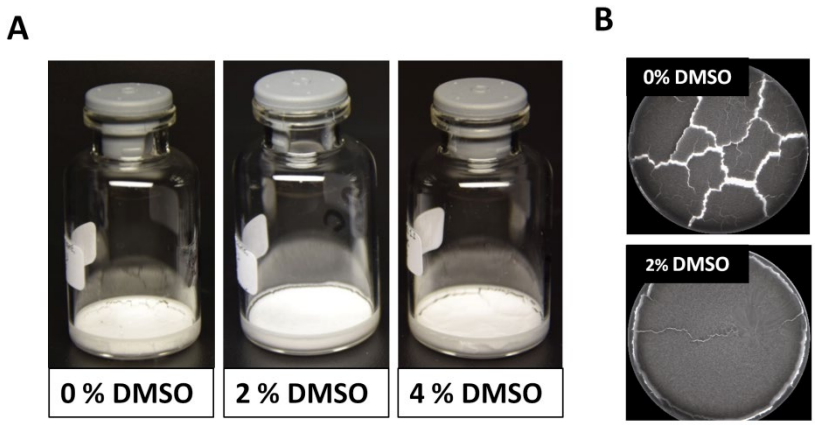


Figure IV-4. (A) Representative photographs of formulations after freeze-drying containing 10% HES and 5% HE in HEPES buffer and 0, 2 and 4% DMSO, respectively. **(B)** Visualization of cake structure of formulations with 0 and 2% DMSO, freeze-dried in glass petri dishes and visualized under a white tray.

IV.3.2.5 Identification of collapse

Even if no visible macroscopic collapse was detected, also microscopic collapse needs to be excluded. During collapse, a critical temperature in primary drying is exceeded, enabling viscous flow of the amorphous freeze-concentrated phase and the resulting loss in pore structure can be detected by SEM pictures of the inner cake structure [23], [24]. Additionally, microcollapse can influence the specific surface area (SSA) of the lyophilizate and a loss in its porous structure can be revealed with a decrease of the surface area. However, also changes in crystallization behaviour during freezing can alter the cake structure and subsequent change the specific surface area.

SSA of formulations containing 10% HES and 5% HE and between 0 and 4% DMSO decreased with increasing content of applied DMSO (Figure IV-5 A). Between 0 and 1% DMSO only a slight reduction in the surface area was measured, whereas for higher DMSO content the SSA decreased more clearly. However, SEM pictures do not show microscopic differences between 0 and 2% DMSO, whereas for 4% DMSO a melted pore structure was observed (Figure IV-5 B). This proves that collapse is only a critical for higher DMSO concentrations, whereas 2% DMSO result in elegant, non-collapsed cakes.

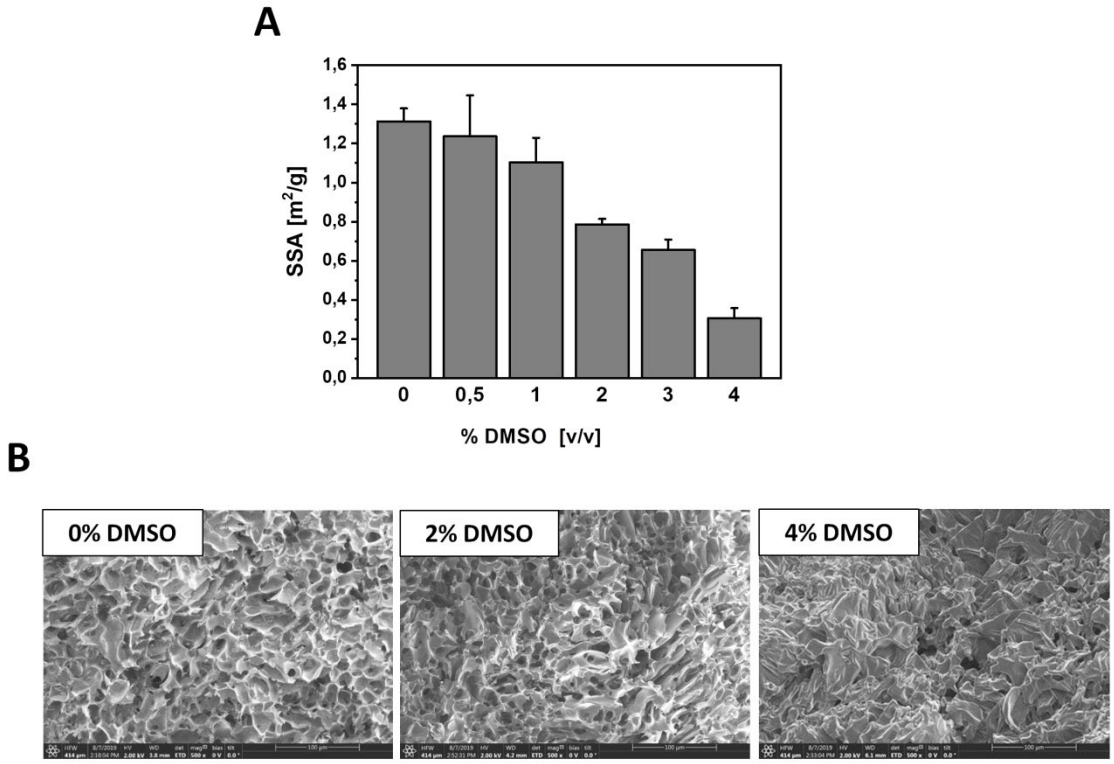


Figure IV-5. (A) SSA for formulations containing 10% HES and 5% HE in HEPES buffer and between 0 and 4% DMSO, respectively. **(B)** SEM images of representative cake structures, containing 10% HES and 5% HE in HEPES buffer, with 0, 2 and 4% DMSO, respectively.

ÍV.3.3 Effect of DMSO on freeze-drying of cells

IV.3.3.1 Toxicity of DMSO

The relatively low toxicity of DMSO paved the way to its widespread application in cell biology [25], [26], [27]. Nevertheless, we wanted to study the influence of DMSO on cell viability and metabolism during short time exposure, as applied during the freeze-drying procedure. Prior to freeze-drying, cells were suspended in different formulations containing 10% HES and 5% HE and 0 – 4% DMSO for typically 30 to 60 minutes, before they are frozen in the freeze-dryer. To address the question whether this treatment influences cell viability, a MTT assay was conducted. Cells were incubated for 30 minutes, 1 hour 45 minutes, 2 hours 45 minutes and 3 hours 45 minutes with formulations of 10% HES and 5% HE in HEPES buffer with 0 - 4% DMSO,

respectively. As a positive control, cell culture medium EpiLife/S7 and as a negative control 5% SDS in PBS was used.

All formulations, including 0% DMSO, showed a decreased metabolic activity of the cells already after 30 minutes of incubation (see Figure IV-6). This can be explained by the high osmolality of 645 mosm/kg, as all formulations contained 10% HES and 5% HE in HEPES buffer with 150 mM NaCl. In a prior formulation development different osmolalities were analyzed and a hypertonic solution (645 mosm/kg) achieved best results.

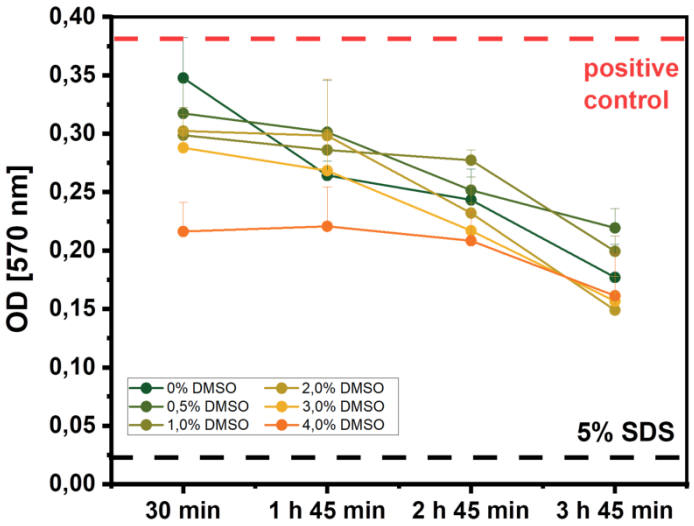


Figure IV-6. MTT assay for formulations containing 10% HES and 5% HE in HEPES buffer and 0 – 4% DMSO, respectively, after incubation times of 30 minutes, 1 hour 45 minutes, 2 hours 45 minutes and 3 hours 45 minutes. As a positive control, cell culture medium EpiLife/S7 and as a negative control 5% SDS in PBS was used. The absorption at 570 nm was set as detection wavelength of the formed and solubilised formazan salt.

Formulations with 4% DMSO showed the most prominent decrease in metabolic activity already after 30 minutes, pointing out a toxic effect of 4% DMSO. The metabolic activity remained stable until 2 hours 45 minutes incubation time, whereupon a further decline in viability was observed. For formulations containing 0, 1, 2 and 3% DMSO, metabolic activity also decreased with longer incubation times, but no difference between the individual formulations was detectable. This leads to the conclusion, that 1, 2 or 3% DMSO has no further negative impact on cellular survival or metabolic activity, because these formulations reduce metabolic activity to the same extent as 0% DMSO. Nevertheless, with longer incubation time, metabolic activity also decreases for formulations containing 0 – 3% DMSO, especially after about 2 hours 45 minutes. To avoid unwanted cellular damage, it is therefore necessary to reduce the exposure time of cells to the hypertonic lyophilization formulations and fast handling is required. However, with an estimated exposure time of 30 – 60 minutes to the respective formulations before cells are frozen, these concerns can be widely neglected.

IV.3.3.2 Cell membrane integrity

Cell membranes play a crucial role in cellular survival, as only intact cell membranes prevent intracellular components from leakage and maintain essential functions of the cells by compartmentation. Even if freeze-dried cells are not able to proliferate after reconstitution, the cell membrane integrity can be used to get a first insight of the cellular status after freeze-drying. It is therefore of major importance to analyze the condition and integrity of the cell membrane. We used the dye exclusion of trypan blue to quantify the percentage of membrane intact cells.

Figure IV-7 A illustrates cell membrane integrity measured via trypan blue exclusion with the automatic cell counter ViCell XR™. Addition of 2% DMSO led to an increase in cell membrane integrity from 25.3 ± 2.7% without DMSO to 41.4 ± 4.3% with 2% DMSO. Higher amounts of DMSO showed no further increase. Recovery of all cells could be enhanced from 41.7 ± 3.2% without DMSO to 58.1 ± 8.8% with 2% DMSO. To keep the load of organic solvents as low as possible and taking the results of the MTT assay and the physicochemical measurements into account, the lowest effective concentration, which was 2% DMSO, was used for further experiments.

For a more detailed insight into the drying process itself, the lyophilization cycle was interrupted at different time points and samples were analyzed regarding cell membrane integrity and residual moisture content (see Figure IV-7 B). Figure IV-7 C and D depict the course of membrane integrity and residual moisture content with proceeding lyophilization cycle for samples freeze-dried in 10% HES, 5% HE in HEPES buffer without (Figure IV-7 C) and with 2% DMSO (Figure IV-7 D). With longer drying time, RM content decreased and as expected, a distinct correlation between residual moisture content and membrane integrity was observed which was true for both formulations. At a high water content of above 50%, cell membrane integrity was only little affected and still ranges between 70 and 90%. However, it must be stressed that samples containing such large amounts of water do not form a dry cake and must be considered as upconcentrated, viscous solutions instead of dry lyophilizates and cannot be stored in this physical state. With proceeding primary drying time, samples with lower residual moisture content and lower cell membrane integrity were obtained. Cells dried without DMSO show a faster decrease in membrane integrity with lower definite values than cell that are dried with 2% DMSO. Already after 10 hours of primary drying, cells freeze-dried without DMSO showed membrane integrity values below 30%, whereas cells dried with 2% DMSO exhibited membrane integrity values of 45.5 ± 4.6%.

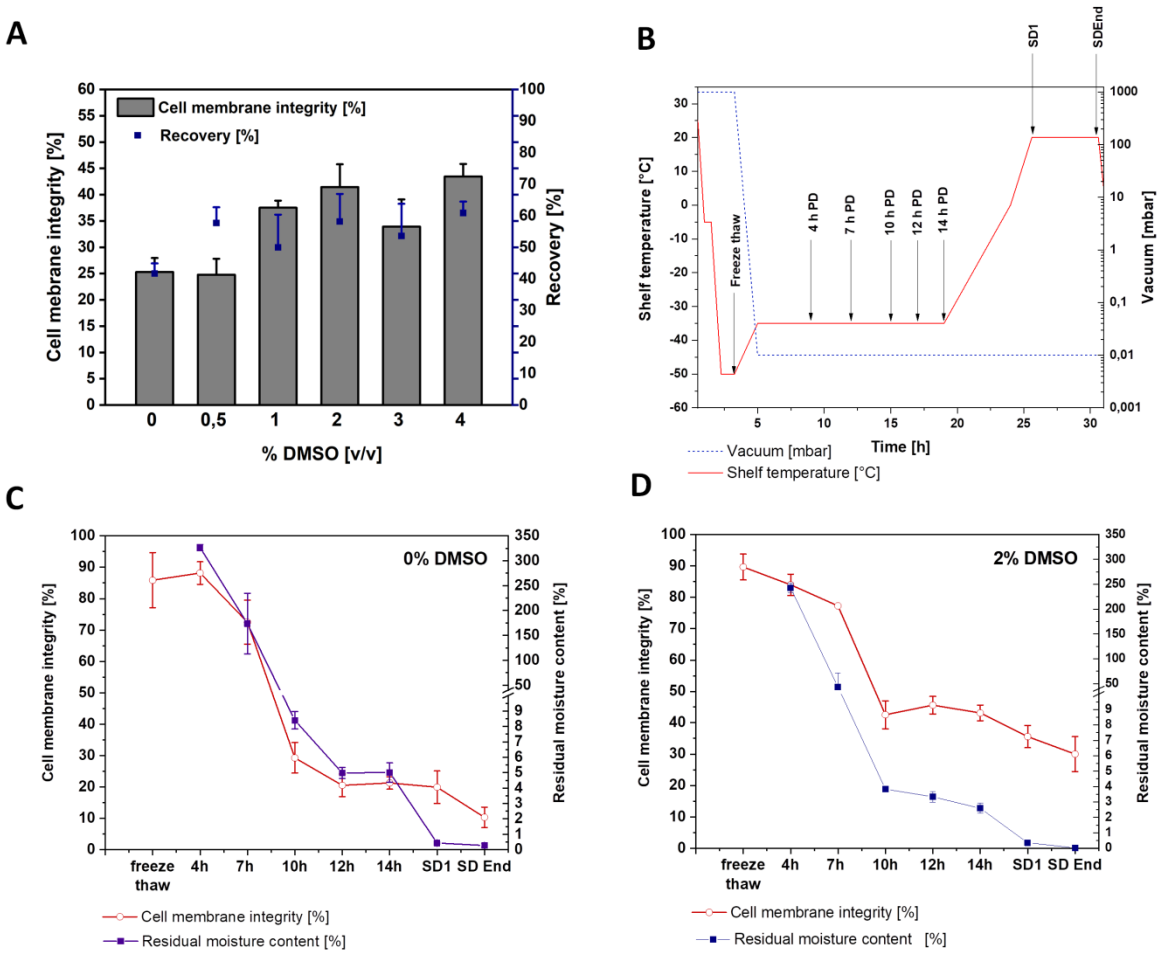


Figure IV-7. (A) Cell membrane integrity and recovery of cells freeze-dried in formulations containing 10% HES and 5% HE in HEPES buffer with 0 – 4% DMSO, respectively. Measured with ViCell XR™. **(B)** Applied lyophilization cycle. The red line represents shelf temperature and the dashed blue line the applied chamber pressure. Arrows illustrate sampling points that were carried out during the freeze-drying cycle. **(C,D)** Cell membrane integrity and residual moisture content of cells freeze-dried in formulations containing 10% HES and 5% HE in HEPES buffer **(D)** with and **(C)** without 2% DMSO at different sampling points during the freeze-drying cycle.

With proceeding primary drying, membrane integrity for samples without DMSO further decreased to 21.1 ± 2.0% membrane integrity, whereas samples with 2% DMSO remained at stable values of around 45% membrane integrity. Little further reduction of membrane integrity during secondary drying was observed for both formulations; still cells dried with 2% DMSO resulted in higher absolute membrane integrity values. These findings demonstrate that the addition of 2% DMSO contributes to enhanced stabilization of cells, if cells are dried to meaningfully low residual moisture contents, as required for long term storage.

IV.3.3.3 Cell membrane visualization

For a closer look at the membrane structure, phalloidin labelled with rhodamine was used to stain the actin skeleton of cells before and after freeze-drying. The actin cytoskeleton is mainly responsible for diverse motility processes and cellular migration. However, it is also associated with the cell membrane and can define cell shape and mechanical properties [28]. We expected to

get a more detailed view of the damages of the cell membrane after lyophilization, if the cytoskeleton is visualized. Figure IV-8 represents morphology of cells before and after freeze-drying and clear differences are noticeable.

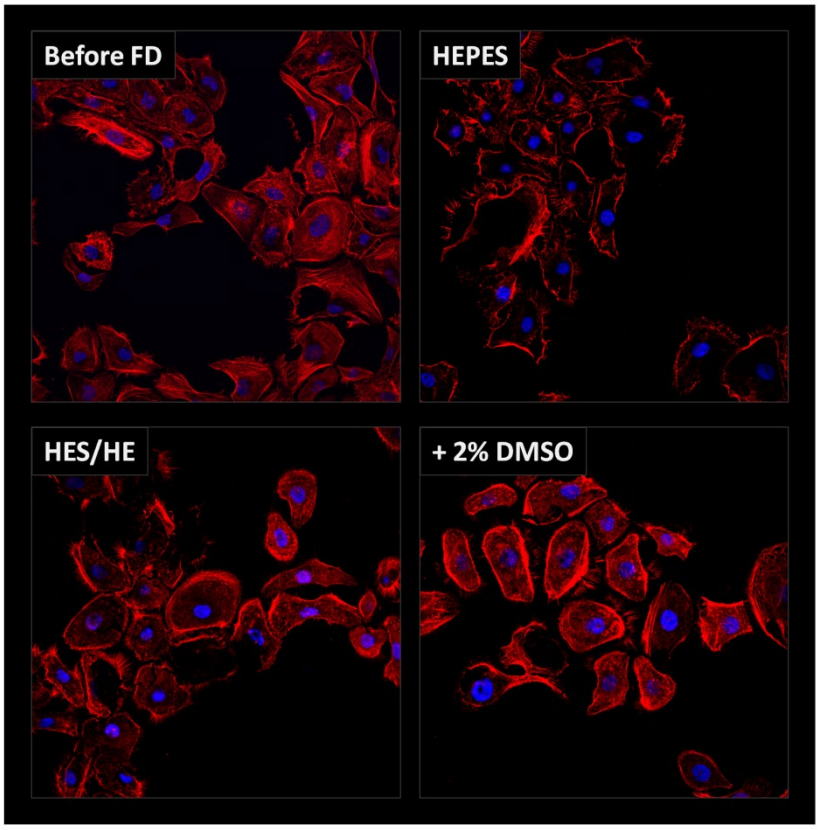


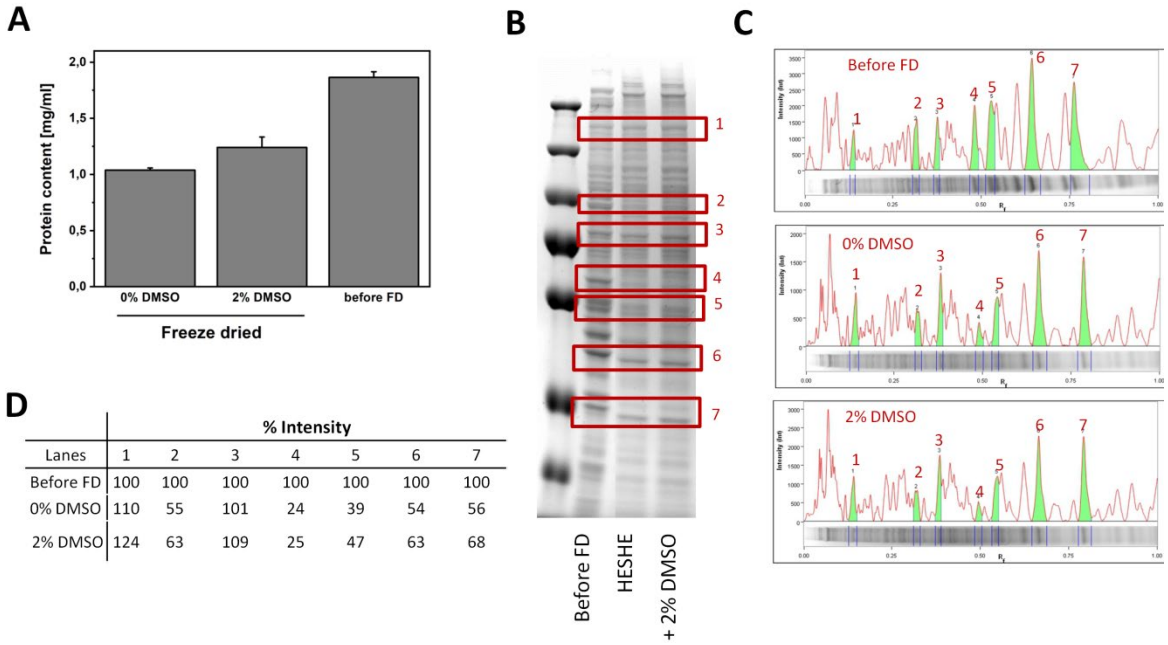
Figure IV-8. Representative confocal microscopy pictures of adherent cells before and freeze-drying in 10% HES and 5% HE in HEPES buffer, with and without 2% DMSO. Cells were stained with Rhodamie Phalloidin and DAPI. Red: Rhodamine Phalloidin, blue. DAPI.

Before drying, cells show a defined cytoskeleton with no damages or ruptures within the cell membrane. Cells are in contact with each other and the lamellopodia are slender and visible as cellular protrusion at the outside of the cell membrane. Freeze-drying only in HEPES buffer resulted in cells with a highly damaged cell membrane measured with trypan blue exclusion but it was also visible after staining of the cytoskeleton. The cell membrane is broken and at some parts only cell fragments without any nuclei can be detected. Only few lamellopodia are visible which are fragmented and also the overall staining of the inner cytoskeleton is reduced. It appears that cells are such extremely damaged, that the nucleus and parts of the cytoskeleton of the inside of the cell were washed out. Freeze-drying in presence of 10% HES and 5% HE reduced the overall cell damage and more cells with only little damage and staining of the inner cytoskeleton are present. Yet, also cells without nucleus or ruptured membranes are visible. However, best preservation of the cellular structure was achieved with the addition of 2% DMSO. Fewer cells are broken, only some cells without nucleus and also the lamellopodia are to a higher number

intact. Nevertheless, all freeze-dried cells have damaged membrane parts and seem to be more retracted from each other with less contact to adjacent cells. Even if cells are still damaged, additional DMSO seems to protect cell membranes and cytoskeleton in an efficient way from intense damage during drying.

IV.3.3.4 Total protein content and protein pattern

Understanding the cellular condition after freeze-drying requires the analysis of additional aspects besides cell membrane integrity. Focusing on the total protein content and protein pattern before and after freeze-drying may give further insight into the extent of cell damage during freeze-drying. Protein degradation into small peptides and leakage into the extracellular space can be determined by a BCA assay, quantifying larger proteins remaining intracellular. Smaller protein fragments can exit the cells if the membrane gets perforated during drying.



D

	% Intensity						
Lanes	1	2	3	4	5	6	7
Before FD	100	100	100	100	100	100	100
0% DMSO	110	55	101	24	39	54	56
2% DMSO	124	63	109	25	47	63	68

Figure IV-9. (A) Total protein content of lyophilized and non-lyophilized cells after cell lysis, determined with a BCA assay. Cells were freeze-dried in 10% HES and 5% HE in HEPES buffer with and without 2% DMSO. **(B)** Protein pattern of lyophilized and non-lyophilized cells after separation in a 10% Tris-Glycine gel under reducing conditions. Proteins were stained with Brilliant Blue G. Red boxes highlight changes in protein pattern. **(C)** Lane profiles of the protein pattern. Green parts highlight integrated peaks. **(D)** Band intensities after freeze-drying normalized to band intensities before freeze-drying. Integrated and analyzed with the software Image Lab 6.0.1.

Figure IV-9 A illustrates the total protein content of living cells before freeze-drying and cells freeze-dried in 10% HES and 5% HE, with and without 2% DMSO. Protein concentration decreases from 1.86 ± 0.05 mg/ml before freeze-drying to 1.24 ± 0.09 mg/ml with 2% DMSO and 1.04 ± 0.02 mg/ml without DMSO. These results show that a considerable part of the proteome cannot be retrieved after freeze-drying. With addition of 2% DMSO it was possible to increase the amount of total intracellular protein after freeze-drying by 16.3%. This is in accordance with

the findings of enhanced membrane integrity, as a higher membrane integrity can restrain intact protein and protein fragments intracellular.

To get further insight into the protein structure after freeze-drying, a SDS page was carried out, which shows the protein fingerprint after lyophilization and reveals major changes in protein composition and size. Disappearance of bands can be an evidence for protein fragmentation or changes in protein secondary structure, entailing a change in size. Figure IV-9 B illustrates the protein pattern of lyophilized and non-lyophilized cells, stained with Brilliant Blue G after separation in a 10% Tris-Glycine gel under reducing conditions. Lane A refers to non-lyophilized cells, lane B to cells freeze-dried in 10% HES and 5% HE without DMSO and lane C with 2% DMSO. The same protein amount (quantified as described above by a BCA assay) was loaded per lane. No differences in the overall protein distribution in all lanes are visible, but a closer look reveals a fading of certain bands after freeze-drying, highlighted in red boxes. For a better interpretation, the highlighted band intensities were normalized to the intensities before freeze-drying and presented as %-intensities. It is noticeable, that bands of lane C (2% DMSO) showed higher integrals than the ones of lane B (0% DMSO), as depicted in Figure IV-9 C and D. Preservation of certain proteins could be improved and some proteins show a higher recovery after freeze-drying in the presence of 2% DMSO. However, proteins are not identified and the performed SDS-PAGE gives just a first impression of the protein status after lyophilization and more specific investigations of protein survival after freeze-drying need to be conducted.

IV.3.3.5 EGF receptor and intracellular esterase activity after freeze-drying

EGF receptor activation plays an important role in proliferation, attachment, cell survival and inflammation [29]. Located on the cell membrane, the EGF receptor transduces extracellular signals directly to the nucleus and induces various cellular responses [30]. Factors such as EGF, TGF, epiregulin, amphiregulin and HB-EGF are ligands of the EGF receptor and play an important role in cellular communication, growth and survival of cells [31]. As a consequence the structural presence of the EGF receptor on the cell membrane before and after lyophilization was analyzed. Figure IV-10 A shows the presence of the EGF receptor in cells before and after freeze-drying adherent cells, stained with an AF-488 labelled anti EGFR antibody. Even though freeze-dried cells showed a slightly condensed cytoplasm, no difference in the intensity of the EGFR signal between non freeze-dried cells and cells freeze-dried with 0 or 2% DMSO was observed. To prove latter findings and to confirm that freeze-drying cells in suspension gives equal results compared to freeze-drying adherent cells, cells lyophilized in suspension were analyzed by flow cytometry (Figure IV-10 B). Again, no decrease in the EGF receptor signal is detectable after freeze-drying, verifying that no major structural damages of the EGF receptor epitope occurred during the freeze-drying process.

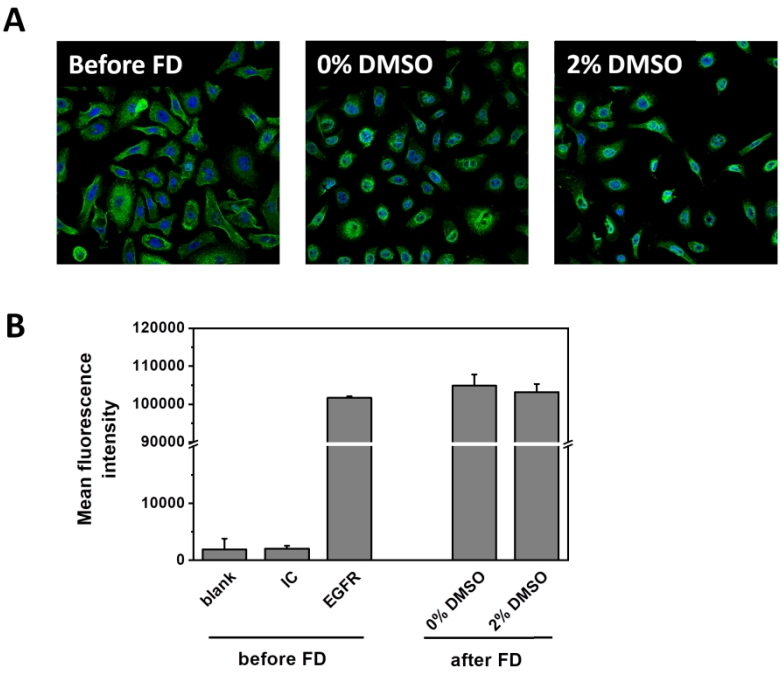


Figure IV-10. (A) Representative confocal microscopy pictures of cells before freeze-drying and freeze-dried adherent cells in 10% HES and 5% HE in HEPES buffer, with and without 2% DMSO. Cells were stained with a AF-488 labelled anti EGFR antibody. Green: EGF receptor, blue. DAPI. **(B)** Mean fluorescence intensity of cells before freeze-drying and freeze-dried cells in suspension after staining with a AF-488 labelled anti EGFR antibody. Analysis with flow cytometry. Blank: unstained cells. IC: Isotype control.

Another important aspect of the protein status after freeze-drying is not only structural integrity but also functionality of proteins. To evaluate whether intracellular proteins exhibit still intact functionality, the activity of intracellular esterases was determined. Calcein-acetoxymethyl ester (Calcein-AM) was used as a fluorescent precursor, which fluorescence is switched on after intracellular cleavage of the ester. Resulting Calcein is trapped and accumulated inside the cell and is therefore labelling metabolic active cells with intact intracellular esterases. Figure IV-11 A depicts adherent cells before and after freeze-drying, stained with Calcein-AM and analyzed with confocal microscopy. Cells prior to freeze-drying show strong intracellular Calcein staining, whereas freeze-dried cells exhibit only little fluorescence, indicating very low activity of intracellular esterases. Another explanation for these finding is insufficient entrapment of formed Calcein inside the cells and leakage through impaired cell membranes. Small molecules such as Calcein might easily exit cytoplasm even if the cell membrane exhibits only very little damage. Though, as we found that a higher amount of cells freeze-dried in presence of DMSO has intact membranes after freeze-drying, total loss of fluorescence cannot be explained by Calcein leave alone, but also indicates loss of protein activity. These microscopic findings could be verified by flow cytometry of cells in suspension (see Figure IV-11 B) and living cells showed a mean fluorescence intensity of greater than 100,000, whereas freeze-dried cells result in values of only around 3,000. For both methods only very little entrapped Calcein could be detected after freeze-drying.

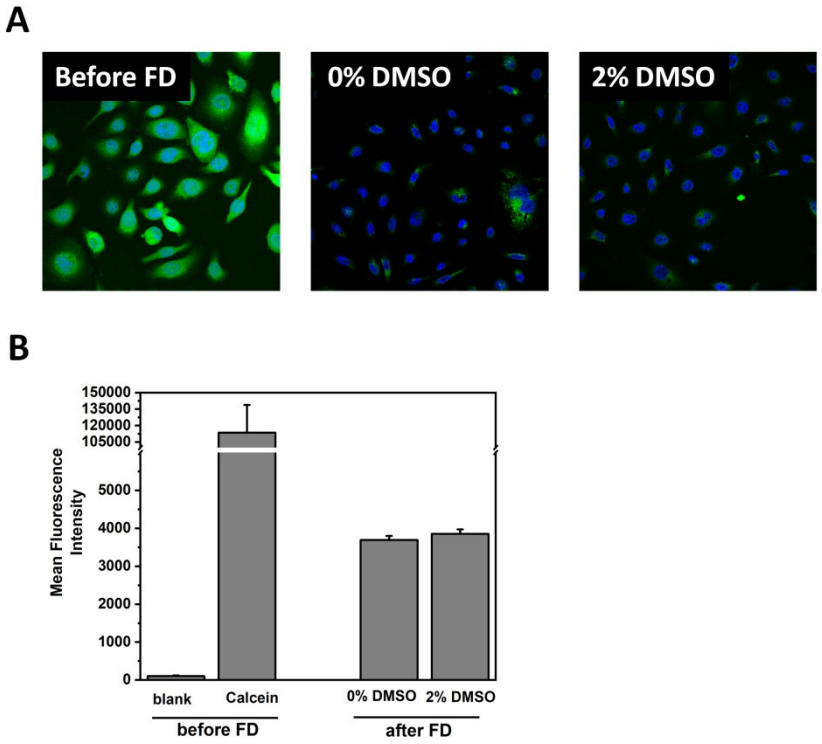


Figure IV-11. (A) Representative confocal microscopy pictures of cells before freeze-drying and freeze-dried cells in 10% HES and 5% HE in HEPES buffer with and without 2% DMSO. Cells were stained with Calcein-AM. Green: Calcein. Blue: DAPI. **(B)** Mean fluorescence intensity of cells before freeze-drying and freeze-dried cells in suspension after staining with Calcein-AM. Analysis with flow cytometry. Blank: unstained cells.

IV.4 Discussion

IV.4.1 Physicochemical properties

DMSO is widely applied for cryopreservation and long term storage of cells and tissues, though to our knowledge is has never been studied systematically for freeze-drying cells. Of course DMSO cannot be used solely as freeze-drying excipient, but as additional component in small concentrations, DMSO could be successful implemented as lyoprotectant. Herein, we present the lyophilization of formulations containing 10% HES, 5% HE and 0-4% (w/v) of DMSO, resulting in acceptable and elegant cakes with adequate physicochemical properties for storage.

We proved the hypothesis, that DMSO remains to a large extent in the lyophilized product and is depressing Tg in a concentration dependant manner. At this point it must be mentioned, that we did not address the question, whether DMSO can be removed to a larger extent in other formulations without HES or less solid content. For other organic solvents, such as TBA, it was reported, that lower content of organic solvent after freeze-drying was achieved with excipients leading to a crystalline matrix whereas the reverse was true for amorphous systems. Also different freezing conditions may influence on residual DMSO content, as a change in crystallinity can also influence solvent removal [32]. However, with a Tg of above 60°C for 2% DMSO, important requirements for storage at 2-8°C are fulfilled, but of course storage stability is not guaranteed

and further stability studies must be performed (see Chapter V). The high Tg of above 60°C is especially remarkable, as calculated on the resulting solid content, a DMSO concentration of 14.3% ± 0.3% (w/w) is obtained.

No macroscopic or microscopic collapse was detected via SEM for DMSO concentrations up to 2%. For 4% DMSO a coalescence of the cakes inner pores was observed, indicating a beginning collapse, matching with the low resulting Tg of 31.8 ± 1.0°C. HES as major part of our lyophilization formulation is the ideal counterpart for formulations containing DMSO as with its high molecular weight it increases Tg and therefore balances the Tg depressing properties of DMSO [17]. Nevertheless, it must be taken into account, that with DMSO the RM content must be as low as possible to avoid synergistic Tg depressing effect of water and DMSO.

Evaluating the macroscopic appearance of the resulting cakes, it is noticeable, that DMSO reduces cake cracking. According to Patel et al., cracking is caused by tensions building up in an amorphous system during drying, which is then relieved by rupture of the cake structure [22]. With remaining DMSO in the matrix during freeze-drying, DMSO might enable some movement throughout the cake and diminishes arising tensions and leading therefore to reduced cake cracking.

Referring to Yu and Quinn, in a binary DMSO water system DMSO is highly associated with water molecules in a ratio of 2 to 1 [9]. Together with the reduced SSA for samples containing higher amounts of DMSO, one would assume higher RM contents for samples with more DMSO, but interestingly the reverse was true. A possible explanation is that DMSO replaces water that is bound to excipients such as HES or cellular structures, leading to a higher amount of water accessible for sublimation and desorption during primary and secondary drying. Water might be removed more effectively in the drying steps, thus resulting in lower absolute RM contents. This theory is supported by the observation, that the drying process is accelerated with the addition of DMSO as free water can be removed easier. Thermocouple readouts indicate a reduced overall drying time, which is in accordance with the findings of the faster decrease of RM content of samples with 2% DMSO. However, to draw more valid conclusions about the drying time of samples with additional DMSO, more experiments should be performed and also process parameters of different lyophilization cycles must be compared.

IV.4.2 Analysis of cells

To our knowledge, until now no proliferating cells have been obtained after lyophilization, when differentiated eukaryotic cells of higher complexity were freeze-dried. The cell membrane plays a key role in preservation of cells during drying. Without intact cell membranes, compartmentation and organization of fundamental cellular organelles cannot be maintained which is incompatible

with viable cells. As a consequence, analysis of cell membrane integrity has been established as meaningful tool for estimating the success of freeze-drying cells and many researchers used (fluorescent) dyes to quantify the extent of membrane damage after lyophilization [33]–[36]. With the described lyophilization process, HaCaT keratinocytes do not proliferate after lyophilization, but we were able to show a notable increase in cell membrane integrity with the addition of 2% DMSO, measured by dye exclusion of trypan blue. Different sampling points during the lyophilization cycle showed that there is a certain residual moisture level, below which cells dehydrate and the cell membrane gets leaky. DMSO has the ability to stabilize cells and cell membranes below this critical residual moisture content, thus higher membrane integrity rates were achieved for a wide range of residual moisture contents. Moreover, also cellular recovery was enhanced with 2% DMSO after lyophilization, which elevates the total amount of membrane intact cells. Advances in membrane integrity are also reflected by the higher amount of total protein found in cells after freeze-drying. The effect of DMSO on cell membranes might be explained by the influence of DMSO on membrane fluidity by lateral expansion of lipids, thus enabling reorientation of the head groups, in a way making them more flexible and resistant to external forces [15]. Additionally, DMSO is able to alter freezing behaviour with reduced ice crystal formation, which was also reported by Kratochvílová et al., who showed a ten-fold decrease in the ice crystal size and crystallinity for 10% DMSO in frozen solutions [37].

Furthermore, DMSO can easily penetrate cell membranes and thereby access intracellular structures. Our findings suggest that DMSO on the one hand stabilizes the cell membrane and on the other hand also intracellular proteins. SDS-PAGE and subsequent protein analysis revealed higher concentrations for certain protein bands in the presence of DMSO. However, it is important to identify those proteins that are best preserved and to look for commonalities, such as cellular localisation or function, but also chemical and structural parameters.

To summarize, with the addition of DMSO as lyoprotectant, it was possible to obtain a higher amount of cells with intact cell membranes and proteins after freeze-drying. Beyond the already established use of DMSO as a powerful cryoprotectant, we could show that it has also promising lyoprotective effects in the field of lyophilization of therapeutic cells. Although it is not removed during freeze-drying, surprisingly lyophilizates with acceptable physicochemical properties were obtained. Lyophilization of eukaryotic cells is still a research area with limited success so far and the implementation of DMSO as lyoprotectant is only a small, but eventually important step towards a more widespread application of therapeutic cells. With a better preservation of cell membranes and cellular structures, DMSO might contribute to the still unsolved problem of freeze-drying cells of higher complexity.

IV.5 Acknowledgements

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IV.6 References

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IV.7 Supplementary material

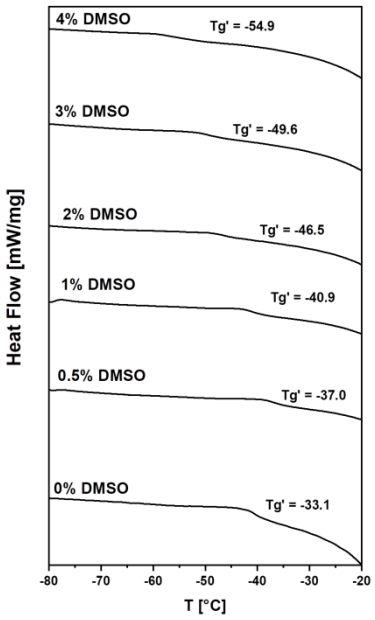

Figure IV-S.1. Thermograms of the maximally freeze-concentrated solution (Tg') of 10% HES and 5% HE in HEPES buffer containing between 0% and 4% (v/v) DMSO, respectively.

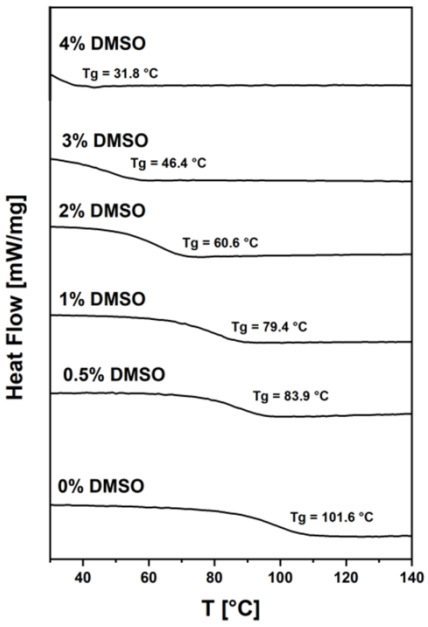

Figure IV-S.2. Thermograms of dry lyophilizates for formulations of 10% HES and 5% HE in HEPES, containing between 0% and 4% (v/v) DMSO, respectively.

Chapter V

Stability of freeze-dried HaCaT keratinocytes

V.1 Introduction

The most common reason for lyophilization of pharmaceutical products is improvement of shelf life stability and reduction of transport and storage costs [1]. Therefore sufficient storage stability must be guaranteed for a successful implementation of a lyophilized product. In 1994 M. Pikal described two main mechanisms which influence the stability of proteins during drying and subsequent storage: vitrification and water substitution. Excipients that stabilize the native conformation of proteins, e.g. by water replacement, lead to a thermodynamically more stable state, thus making chemical degradation reactions more unfavoured and less probable. Vitrification of excipients and solutes reduces movements within the product and chemical reactions are slowed down, so that degradation processes can be impeded [1]. Lyophilized samples should be therefore stored below their glass transition temperature Tg to maintain a glassy state and to avoid diffusion controlled chemical reactions initiated by increased mobility [1], [2]. However, even at storage temperatures below the Tg, molecular motions in the amorphous material occur and Hancock et al. suggested a storage of at least 50 K below glass transition temperature to avoid unwanted mobility within the product [3]. It seems advisable to choose an excipient composition that on the one hand replaces water in a dry state and on the other hand results in a sufficiently high Tg.

In the previous chapter (Chapter IV) we demonstrated that freeze-drying of HaCaT keratinocytes in the presence of DMSO led to an increase in cell membrane integrity. Despite the fact that a significant part of DMSO was not removed during freeze-drying, lyophilizates showed no indications of collapse and the glass transition temperature above 60°C suggests that samples can be stored at 2-8°C. However, storage stability needs to be proved experimentally and especially the sensitive cell membrane may be difficult to stabilize during storage. We therefore conducted a stability study of lyophilized HaCaT keratinocytes. Figure V-1 schematically depicts the setup of the performed stability study.

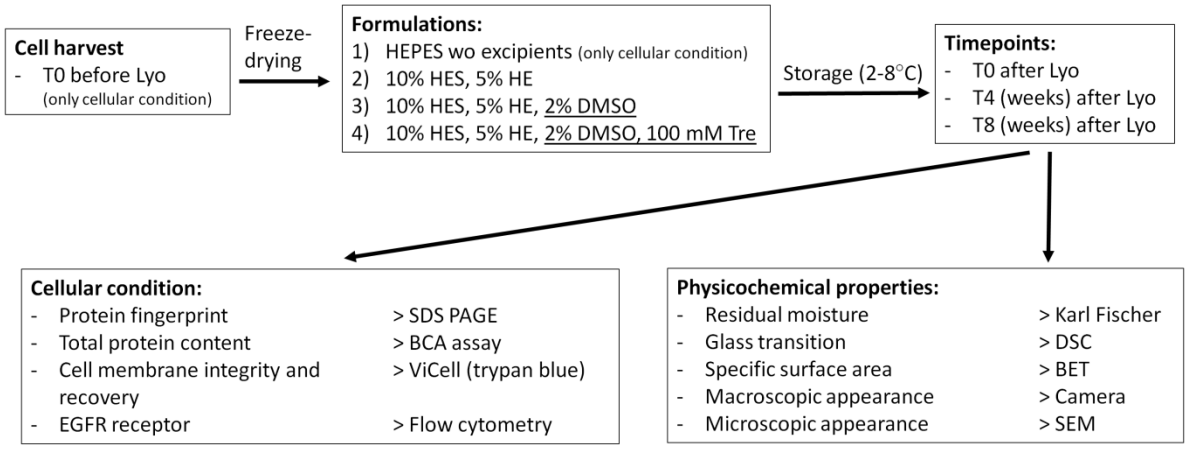

Figure V-1. Setup of 8 week stability study of lyophilized HaCaT keratinocytes.

Cells were freeze-dried in 10% HES, 5% HE with or without the addition of 2% DMSO. We further included samples with additional 100 mM trehalose (10% HES, 5% HE, 2% DMSO, 100 mM trehalose), since the glass forming characteristics of trehalose might contribute to a higher storage stability. For cellular analysis we also freeze-dried cells only in HEPES buffer without excipients which were used as negative control, but not assessed by physicochemical analysis. A storage temperature of 2-8°C was chosen to ensure sufficient temperature distance to the glass transition temperature and to enable convenient storage conditions. Samples were stored for 4 and 8 weeks and were analyzed regarding physicochemical and cellular properties.

V.2 Material and Methods

V.2.1 Material

Hydroxyethyl starch 200/0.5 (HES) was purchased from BOC Sciences (Shirley, NY, USA). Hydroxyectoine (HE), tris buffered saline (TBS: 0.05 M Tris buffered saline (0.138 M NaCl, 0.0027 M KCl), pH 8.0, at 25°C), formaldehyde (36% in water), sodium dodecyl sulphate (SDS), dulbeccoo's phosphate buffered saline (PBS: 0.2 g/l KCl, KH_2PO_4, 8 g/l NaCl, 1.15 g/l Na_2HPO_4 (anhydrous)) and polysorbate 20 was ordered by Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). HEPES and NaCl were ordered from VWR (VWR, Allison Park, PA, USA). HaCaT (Human adult low Calcium high Temperature) keratinocytes were provided from the DKFZ (Heidelberg, Germany).

V.2.2 Methods

Cell preparation: Cultivation of HaCaT keratinocytes was performed as described in Chapter III.

Sample preparation: For lyophilization, cells were resuspended in different formulations, all containing 10% (w/v) hydroxyethyl starch (HES) and 5% (w/v) hydroxyectoine (HE), dissolved in HEPES buffer (25 mM HEPES, 150 mM NaCl, pH 8.0). Formulations contained 0% or 2% (v/v) DMSO or 2% DMSO and 100 mM trehalose (see Figure V-1). Cells freeze-dried only in HEPES buffer were used as negative control for cellular analysis methods. One limiting factor for the setup of this stability study was the large number of cells that was required for all analytical methods. To minimize the number of cells, as little as possible cells were used for each analytical method. For the determination of cell membrane and cell recovery (ViCell analysis) 250,000 cells per vial and for all other cellular analysis methods 500,000 cells per vial were freeze-dried. We could show in Chapter III, that different cell concentrations do not influence cell survival and recovery during lyophilization. For the physicochemical analysis, placebo samples without cells were prepared and analyzed. Aliquots of 500 µl were filled in 10 R tubing vials (MGlas AG, Muennerstadt, Germany) and semi-stoppered with lyophilization stoppers (FluroTec® rubber stopper; West Pharmaceuticals, Eschweiler, Germany).

Lyophilisation: Freeze-drying was performed as described in chapter IV (Christ 2-6D laboratory scale freeze-dryer (Martin Christ, Osterode am Harz, Germany); Primary drying: ST at -35°C, 20 hours, vacuum 0.01 mbar; Secondary drying: ST at 20°C, 5 hours, vacuum 0.01 mbar). All samples were analyzed before and directly after lyophilization and after storage for 4 and 8 weeks at 2-8°C.

Sample analysis: Samples were analyzed regarding their physicochemical properties. Residual moisture content, glass transition temperature Tg, specific surface area, microscopic and macroscopic appearance were determined as described in Chapter IV. Regarding the cellular characteristics, we analyzed the protein fingerprint (SDS-PAGE), total protein content, cell membrane integrity and recovery rate, as well as presence of the EGF receptor. The conduction of these methods is also described in Chapter IV.

V.3 Results

V.3.1 Physicochemial properties

After 4 and 8 weeks of storage at 2-8°C, no major macroscopic changes were observed for any samples (see Figure V-2). Smaller cracks appeared for samples with 2% DMSO already after 4 week of storage, which were not present for samples with DMSO and additional trehalose. No collapse or shrinkage was observed throughout all formulations.

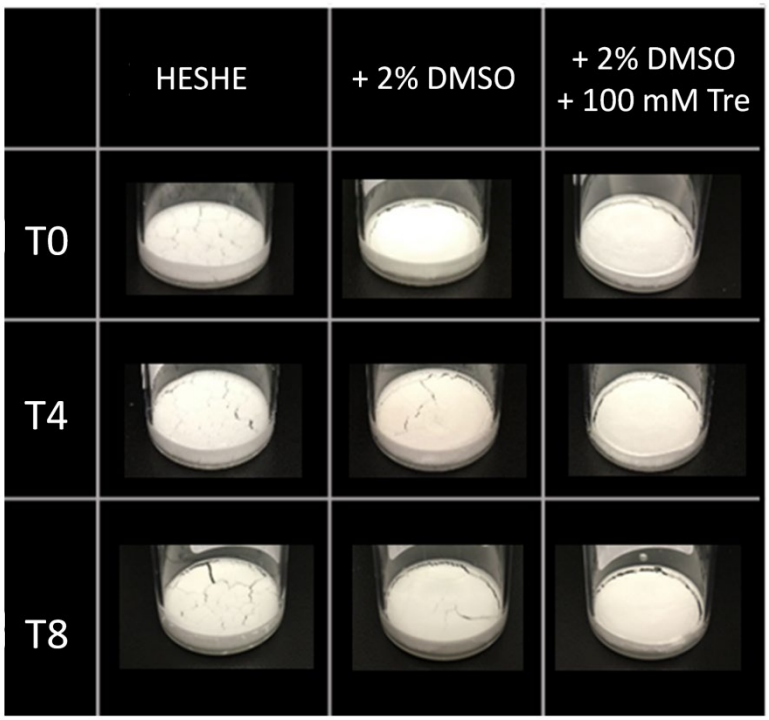

Figure V-2. Representative photographs of formulations containing 10% HES and 5% HE in HEPES buffer and 0 or 2% (v/v) DMSO, with or without 100 mM trehalose. Analysis after 0, 4 and 8 weeks storage at 2-8°C.

Also SEM pictures revealed no microcollapse as depicted in Figure V-3. Throughout the different formulations no differences in pore size and structure were visible after storage, neither occurred melting of the inner pores. These findings indicate that the lyophilized cakes were physically stable at storage temperatures of 2-8°C over a period of 8 weeks.

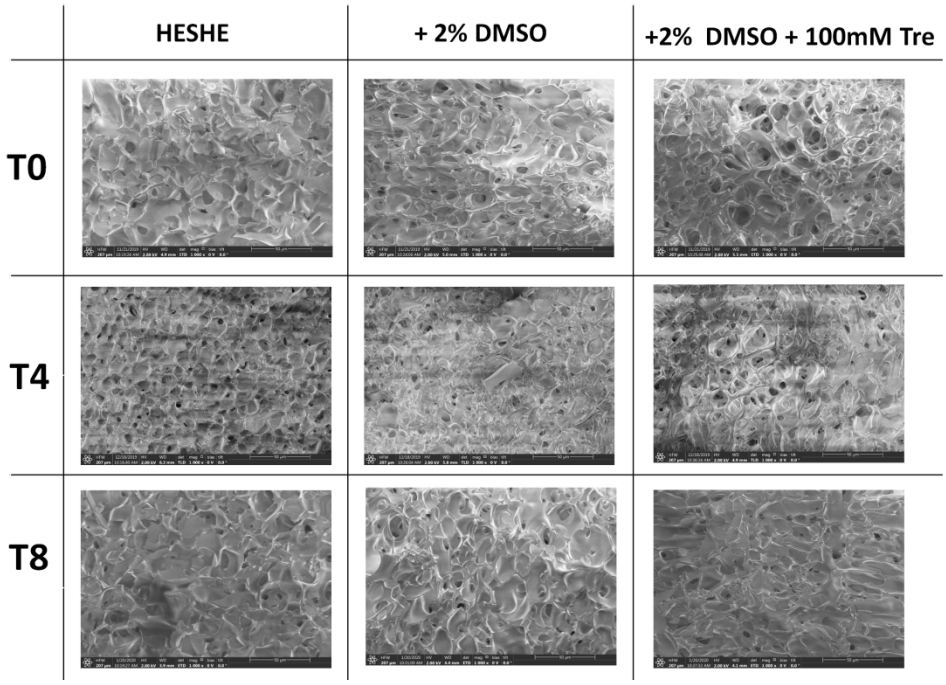

Figure V-3. SEM images of representative cake structures, containing 10% HES and 5% HE in HEPES buffer and 0 or 2% (v/v) DMSO, with or without 100 mM trehalose. Analysis after 0, 4 and 8 weeks storage at 2-8°C.

Figure V-4 A depicts residual moisture content and Tg of the lyophilized samples. Without DMSO, the RM content remains constant over the storage period, whereas samples with DMSO show an increase of RM content already after 4 weeks of storage. Though, we ascribe these findings to measuring artefacts, as RM values for samples with DMSO are in such a low range that even small inaccuracies during measurements result in noticeable deviations.

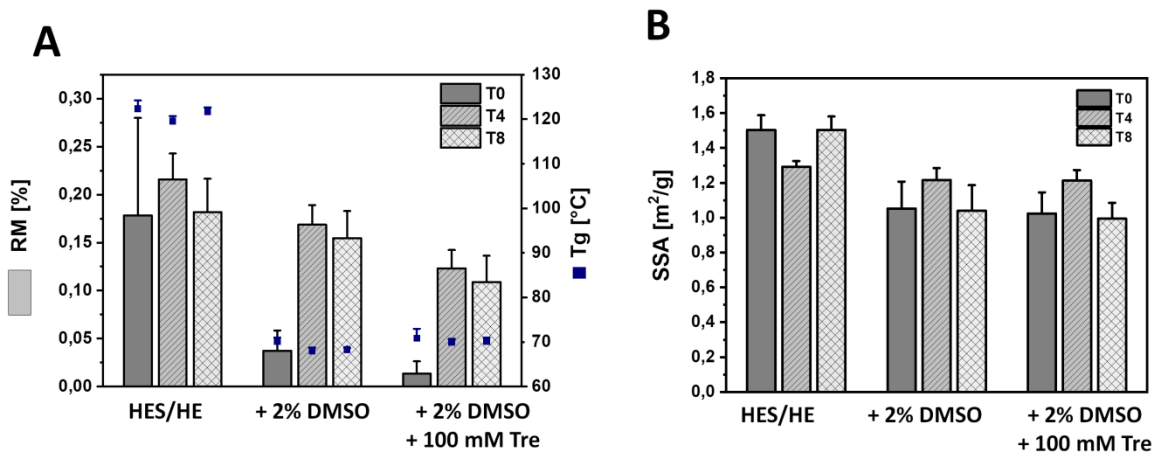

Figure V-4. A) Residual moisture content (squares) and Tg (circles). **B)** SSA. Formulations contained 10% HES and 5% HE in HEPES buffer and 0 or 2% (v/v) DMSO, with or without 100 mM trehalose. Analysis after 0 weeks (bars), 4 weeks (striped bars) and 8 weeks (plaid bars) storage at 2-8°C.

Also, the corresponding glass transition temperatures remain constant and a theoretical entry of water into the lyophilizates would be noticeable with a Tg depression. Tg values stay at constant levels for ~ 120°C without and ~ 70°C with DMSO. As described already in Chapter IV, the addition of DMSO led to a slight decrease in the specific surface area (Figure V-4 B). During the storage period, the SSA remains constant and only smaller variations without clear trend are noticeable. This is in agreement with the SEM pictures, where no change of the inner cake structure was observed. It can be concluded that from a physicochemical point of view, storage over a period of 8 weeks at 2-8°C does not influence cake appearance and structure, which was true for all formulations.

V.3.2 Cellular properties

The total protein content of the cells before and after freeze-drying and storage is depicted in Figure V-5 A. After lyophilization the protein content decreased almost to half of the starting value, throughout all formulations (see also Chapter IV). No further protein loss was measured after 4 and 8 weeks for all formulations with 10% HES and 5% HE. Only for cells dried in HEPES buffer a slight decrease in protein content was observed after 8 weeks of storage. SDS-PAGE analysis of the cell lysate revealed also no changes in protein pattern (Figure V-5 B-D). No new bands appeared or disappeared during the 8 week storage and the prominent protein bands were preserved. Interestingly the lane with cells freeze-dried only in HEPES buffer showed the lowest intensity, even the same amount of protein (11 µg per lane, calculated from the total protein content) was loaded. Also no differences for samples with additional DMSO or DMSO and trehalose were visible.

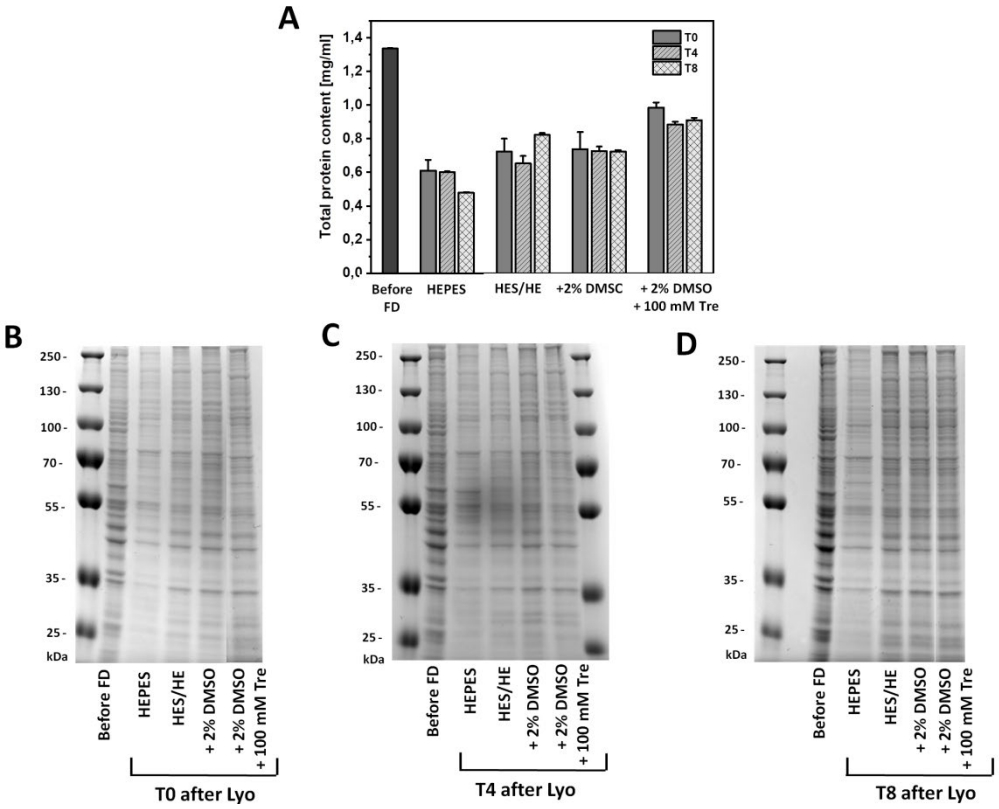

Figure V-5. A) Total protein content of lyophilized and non-lyophilized cells after cell lysis, determined with a BCA assay. Cells were freeze-dried in 10% HES and 5% HE in HEPES buffer and 0 or 2% (v/v) DMSO, with or without 100 mM trehalose. Analysis after 0 weeks (bars), 4 weeks (striped bars) and 8 weeks (plaid bars) storage at 2-8°C. **B-D)** Protein pattern of lyophilized and non-lyophilized cells after separation in a 10% Tris-Glycine gel under reducing conditions, 11 µg protein was loaded per lane. Proteins were stained with Brilliant Blue G. Cells were freeze-dried in 10% HES and 5% HE in HEPES buffer and 0 or 2% (v/v) DMSO or 100 mM trehalose. Analysis after **(B)** 0 weeks, **(C)** 4 weeks and **(D)** 8 weeks storage at 2-8°C.

The structural presence of the EGF receptor was analyzed by staining HaCaT keratinocytes with a fluorescent labelled anti EGF receptor antibody and subsequent quantification by flow cytometry. During the storage period no decrease in the mean fluorescent intensity of labelled HaCaT keratinocytes was observed (Figure V-6). In contrast, even a small increase in the EGF receptor signal was detected, with the strongest increase for cells freeze-dried in HEPES buffer. These findings were very surprising, however, this trend was already observed in a preceding short stability study at higher temperatures (data not shown). No increase was evident for the isotype control, which speaks against higher unspecific binding of the antibody. It is possible that some changes in the cellular surface structure or the EGF receptor epitope occurred during storage, which facilitated easier or more efficient binding of the antibody, thus increasing the signal. Nevertheless, it can be concluded that the EGF receptor epitope still exists after lyophilization and is stable also during storage.

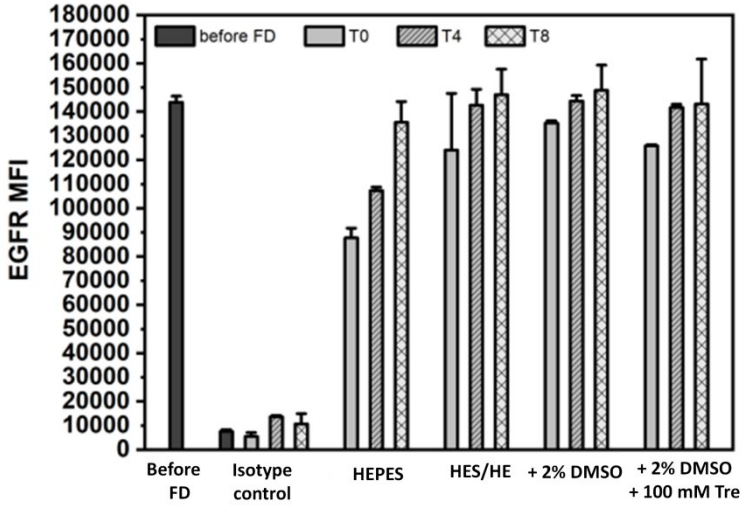

Figure V-6. Mean fluorescence intensity of cells before and after freeze-drying, reconstitution and subsequent staining with AF-488 labelled anti EGFR antibody. Cells were freeze-dried in 10% HES and 5% HE in HEPES buffer and 0 or 2% (v/v) DMSO, with or without 100 mM trehalose. Analysis after 0 weeks (bars), 4 weeks (striped bars) and 8 weeks (plaid bars) storage at 2-8°C.

Cell membrane integrity and recovery were analyzed with the automatic cell counter ViCell XR™ and no changes for any formulation were observed regarding total cell recovery (Figure V-7). However, storage time strongly affected cell membrane integrity. Cells freeze-dried in HEPES buffer exhibited membrane integrity values below 5% for all time points and also stable values over the storage time were observed for cells dried in 10% HES and 5% HE without DMSO. Directly after lyophilization, the addition of DMSO led to a cell membrane integrity rate of 32.2 ± 5.4% with 2% DMSO and 27.9 ± 0.3% with 2% DMSO and 100 mM trehalose. Surprisingly already after 4 weeks storage at 2-8°C, cell membrane integrity decreased by half and reached only 15.1 ± 1.3% for 2% DMSO and 17.2 ± 3.5% for 2% DMSO and 100 mM trehalose, which is in the same range of cells dried without DMSO. After 8 weeks of storage, cell membrane integrity values further decreased and are even below the range of cells dried in formulations without DMSO. These findings were quite unexpected, as all other measurements showed no major variances in the physicochemical or cellular properties. It appears that the addition of DMSO strongly increases cell membrane integrity after lyophilization, but unfortunately the effect cannot be maintained during storage.

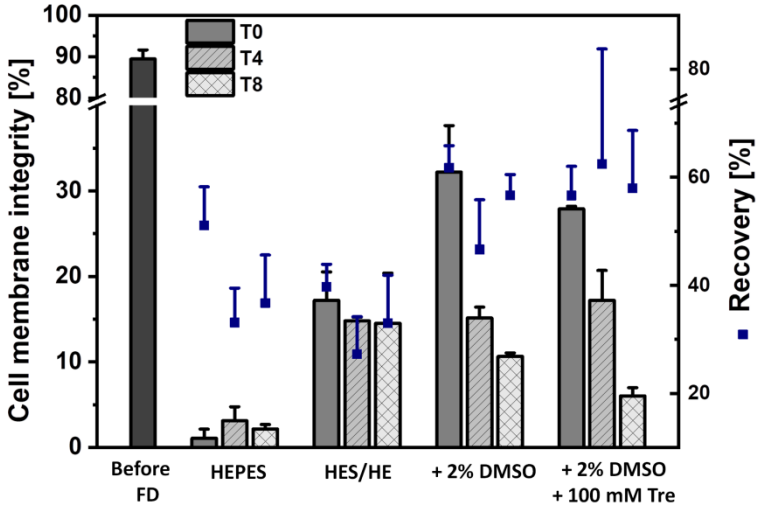

Figure V-7. Cell membrane integrity (bars) and recovery (squares) of cells after freeze-drying and reconstitution. Cells were freeze-dried in 10% HES and 5% HE in HEPES buffer and 0 or 2% (v/v) DMSO, with or without 100 mM trehalose. Analysis after 0 weeks (bars), 4 weeks (striped bars) and 8 weeks (plaid bars) storage at 2-8°C.

V.4 Discussion

We could prove that freeze-drying cells in presence of DMSO strongly enhances cell membrane integrity and protein survival. However, for a successful implementation of a lyophilized product, storage stability must be guaranteed, as one major advantage of freeze-drying is the improvement of storage conditions and longer shelf life stability [4]. On the basis of macroscopic and microscopic analysis, RM, Tg and SSA measurements, we demonstrated that the addition of DMSO leads to a physicochemical stable product over a storage period of 8 weeks at 2-8°C, without macroscopic or microscopic collapse. Also the total cell recovery, protein content and pattern did not reveal any degradation. However, a clear decrease of cell membrane integrity rates for samples with DMSO was noted during storage, whereas samples without DMSO remained at a stable level. Also the addition of 100 mM trehalose did not further stabilize the cell membrane. These findings point out that lyophilizates with DMSO are not as stable as supposed from the physicochemical analysis. Obviously changes within the products occured which could not be detected with the applied physicochemical analysis techniques.

GC-MS analysis of the lyophilized cake showed that DMSO is not removed during freeze-drying, but with this method no assessment of the distribution of DMSO within the cake can be made [5]. Considering some properties of DMSO, an even distribution throughout the entire cake matrix appears actually doubtful. DMSO is known to influence cell membrane integrity, easily permeates into cells and is able to access intracellular structures [6]. It is possible that this interaction and association with cellular components leads to an upconcentration of DMSO at the cell membrane or in the intracellular space. Assuming that the measured Tg is a cross section of the entire cake, areas with higher DMSO concentrations and even lower Tg values would not be detected with DSC measurements. Subsequently, if some “DMSO hotspots” have a much lower Tg, storage

temperature might exceed this Tg, thus enabling viscous flow and higher mobility within these areas [7]. As previously discussed, storage above Tg usually goes along with accelerated protein aggregation and chemical reactions [2]. Moreover, an enhanced mobility at certain areas within the cake might lead to membrane phase transitions, thus promoting cell rupture and membrane damage. To prove latter theory, we tried to show an upconcentration or association of DMSO within cellular structures in the lyophilized cake. With SEM-EDX (energy-dispersive X-ray), it was not possible to distinguish considerable between cells and the surrounding matrix. Also detection of intracellular DMSO by RP-HPLC was unsuccessful as no baseline separation between DMSO and (intra)cellular proteins could be achieved. So far, an uneven distribution of DMSO in lyophilized cakes could not be proved and remains a theory that needs to be confirmed. Furthermore we did not analyze if other excipients can enhance cell membrane stability of samples dried with 2% DMSO. Cells dried with 10% HES and 5% HE without DMSO showed stable physicochemical and cellular values without any decrease of cell membrane integrity rate during storage. However, the absolute cell membrane integrity rates are in general lower if cells are freeze-dried without additional DMSO.

V.5 References

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Chapter VI

(-)-Epigallocatechingallate as lyoprotectant for freeze-drying HaCaT keratinocytes

VI.1 Introduction

The polyphenol (-)-Epigallocatechingallate (EGCG) is the main catechin of green tea (*Camellia sinensis)*. It is a flavonoid, substituted with three adjacent hydroxyl groups at the B ring, two hydroxyl groups in ortho position at the A ring and a galloyl moiety conjugated to the C ring (depicted in Figure VI-1).

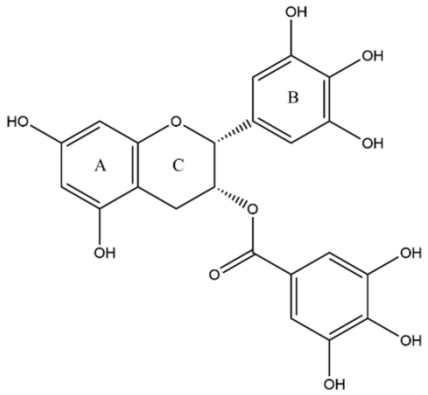


Figure VI-1. Chemical structure of (-)-Epigallocatechingallate.

Flavonoids and in particular EGCG are known for various biochemical characteristics, such as antiviral, antibiotic or anticarcinogenic effects, which make EGCG a promising substance with therapeutic potential against a broad range of disorders like arteriosclerosis, HIV, diabetes or metabolic syndrome [1]–[5].

Especially its antioxidative capacities are in the focus of research and EGCG was shown to be a potent radical scavenger and inhibitor of lipid peroxidation. Chelation of metal ions with subsequent suppression of the formation of metal induced radicals as well as scavenging reactive nitrogen species contribute to the strong antioxidative effect [6]. The o-dihydroxy catechol structure of the B ring is to a large part responsible for the antioxidative activity, but also other hydroxyl groups and the galloyl moiety can serve as hydrogen atom donor or quench free radicals by single electron transfer. Thus the lipid peroxidation chain reaction can be interrupted and the formed radicals or radical cations are stabilized by the phenolic moieties [7]–[9]. Metal catalysed oxidation reactions can be stopped by oxidation of the phenolic compound, which is also able to complex metal cations such as Cu^{2+}, or Fe^{2+} [4].

Besides antioxidative properties, tea catechins show antibacterial and antiviral effects, which is ascribed to the interplay of EGCG and biological membranes. Ikigai et al. reported a strong interaction of EGCG with bacterial membranes and showed that the addition of EGCG induces leakage and damage of bacterial cells or model liposomes [10]. Kumazawa could confirm an interaction of EGCG with lipid bilayers by solid state ^{31}P and ^{2}H NMR and related it to the

manifold hydroxyl groups of the B ring and the galloyl moiety [2]. This galloyl group is described to enhance partition in biological membranes and to promote a structure change within the membranes [11].

Both, antioxidative properties as well as the interference with the cell membrane make EGCG an interesting excipient to stabilize cells during freeze-thawing and freeze-drying. Natan et al. used EGCG to freeze-dry mononuclear cells with a post rehydration viability of 88-91% and they hypothesised that both the antioxidant activity as well as the interplay of EGCG with the cell membrane contributed to a successful stabilization of the cells. However, the authors mentioned that EGCG might also inhibit cell growth and proliferation which they attributed also the same mechanism that stabilizes cell membranes during drying [3].

Another drawback of EGCG is its low stability and rapid degradation, dependant on pH, temperature and oxygen concentration. At neutral or basic pH, EGCG rapidly degrades within few hours, which is even accelerated in presence of higher amounts of dissolved oxygen [12], [13]. EGCG is oxidised by hydrogen peroxide or peroxy radicals, but also the influence of trace amounts of ubiquitous metal ions are discussed [14].

It is obvious that EGCG is a compound with manifold characteristics and we want to investigate its qualities as lyoprotectant for freeze-drying HaCaT keratinocytes. It is further in our interest to face the question of the mode of action and to further elucidate the relevant structures of EGCG.

VI.2 Materials and methods

VI.2.1 Materials

Hydroxyethyl starch 200/0.5 (HES) was purchased from BOC Sciences (Shirley, NY, USA). (-)-Epigallocatechingallate (EGCG), (-)-Epicatechingallate (ECG), (-)-Epigallocatechin (EGC), gallic acid (GA), hydroxyectoine (HE), dulbeccoo's phosphate buffered saline (PBS: 0.2 g/l KCl, KH_2PO_4, 8 g/l NaCl, 1.15 g/l Na_2HPO_4 (anhydrous)), hypotaurine, taurine, ascorbic acid, gluthatione, L-cysteine, alpha-tocopherol, curcumine, dithioerythritol, trifluoracetic acid, acetonitrile and L-glutamine were ordered by Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). HEPES and NaCl were used from VWR (VWR, Allison Park, PA, USA). HaCaT (Human adult low Calcium high Temperature) keratinocytes were provided from the DKFZ (Heidelberg, Germany).

VI.2.2 Cell and sample preparation

Cell preparation: Cultivation of HaCaT keratinocytes was performed as described in Chapter III.

Sample preparation: Cells were lyophilized in EpiLife/S7 without excipients or in formulations containing 10% (w/v) hydroxyethyl starch (HES) and 5% (w/v) hydroxyectoine (HE), dissolved in EpiLife/S7 or in HEPES buffer (25 mM HEPES, 150 mM NaCl, pH 8.0). EGCG, ECG, EGC, GA and antioxidants were added in varying concentrations.

For freeze-drying cells in suspension, cells were resuspended in a concentration of 0.5 – $1 \cdot 10^6$ cells/ml in respective formulations. Aliquots of 500 µl were filled in 10 R tubing vials (MGlas AG, Muennerstadt, Germany) and semi-stoppered with lyophilization stoppers (FluroTec® rubber stopper; West Pharmaceuticals, Eschweiler, Germany). In earlier experiments, this cell concentration showed to be adequate to obtain sufficient cell and protein content for further cellular analysis.

For lyophilization of adherent cells, cells were seeded in a concentration of 4000, 7000 or 10000 cells/cm^2 in a 6-well plate (TPP products, Trasadingen, Switzerland) and were cultivated for 24 hours at 37 °C, 5% CO_2. Prior to freeze-drying, growth medium was aspirated and cells were washed once with PBS. For lyophilization, 1 ml of respective formulation were added per well and cells were freeze-dried directly in the well plate.

Lyophilization: Freeze-drying was performed as described in chapter III (Christ 2-6D laboratory scale freeze-dryer (Martin Christ, Osterode am Harz, Germany); Primary drying: ST at -20°C, 7 hours, vacuum 0.25 mbar; Secondary drying: ST at 20°C, 4 hours, vacuum 0.25 mbar).

VI.2.3 Analysis of cells

Reconstitution: Freeze-dried samples were reconstituted in the 1.5 x volume of the originally lyophilized volume with EpiLife/S7 (37°C). For example 500 µl freeze-dried volume was reconstituted with 750 µl EpiLife/S7.

Cell membrane integrity and total cell recovery: Cell membrane integrity and total cell recovery was determined as described in Chapter III.

Calcein-AM and EH staining: After lyophilization of adherent cells, cells were reconstituted with 1.5 ml EpiLife/S7 (37°C). Cell staining and the determination of the percentage of EH or Calcein positive stained cells is described in Chapter III. Additionally, the staining intensity of the cells that were labelled as EH or Calcein positive was analyzed. This intensity is the average grey value within the particle selection. It is calculated as the grey values of all pixels in the selection, divided by the total pixel number [15]. The intensity gives information about the amount of converted Calcein and bound EH and can therefore give further information about the extent of membrane damage or esterase activity.

Cell migration: A would healing/migration assay (Culture-Insert 2 Well in µ-Dish 35 mm, from ibidi GmbH, Gräfelfing, Germany) was used to investigate the influence of EGCG on cell migration and proliferation (see Figure VI-2).

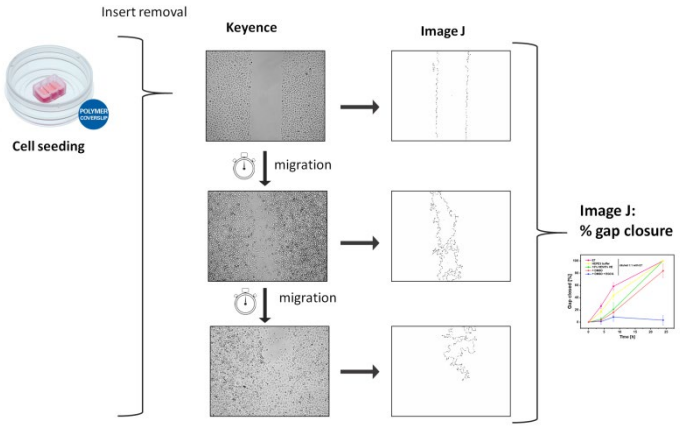


Figure VI-2. Ibidi wound healing/migration assay. Analysis with the software Fiji (Fiji is just ImageJ).

35000 cells were seeded per well and incubated for 24 hours at 37°C, 5% CO_2. Culture inserts were carefully removed with sterilized tweezers and cells were washed twice with PBS. Aliquots of 1 ml of respective solution were added per culture dish. Six pictures of different parts of the gaps were taken for each time point with a Keyence BZ8100 Fluorescence microscope (Keyence, Osaka, Japan) equipped with a Nikon SPlan Fluor 10x/0.45 objective (Nikon, Japan). The cell free area was analyzed with the software Fiji (*Fiji Is Just ImageJ*) and the gap closure calculated according the following equation:

$$Gap\ closure\ [\%] = (1 - \frac{cell\ free\ area\ at\ t(x\ h)}{cell\ free\ area\ at\ t(0\ h)}) * 100$$

Figure VI-3 shows the macro applied for all pictures to determine and calculate the cell free area.

```
run("8-bit");
run("Bandpass Filter...", "filter_large=40 filter_small=3 suppress=None tolerance=5
autoscale saturate");
run("Sharpen");
run("Find Edges");
run("Find Edges");
setAutoThreshold("Default");
//run("Threshold...");
setThreshold(0, 120);
//setThreshold(0, 120);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Find Edges");
run("Invert LUT");
run("Find Edges");'
run("Analyze Particles...", "size=10000-Infinity pixel show=Outlines display
summarize");
```

Figure VI-3. Macro applied to analyse cell number and staining intensity. Data processed with Fiji.

VI.2.4 EGCG degradation

Visualization: Between 0 and 0.9 mg/ml EGCG was dissolved in EpiLife/S7 and stored at RT for 24 hours. Pictures were taken with a Nikon D5300 camera (Nikon Corporation, Tokyo, Japan) in front of a white panel.

Absorbance spectrum: 0.5 mg/ml EGCG was dissolved in EpiLife/S7 and stored for 0 – 72 hours at RT. The absorbance spectrum was measured spectrophotometrically between 200 and 500 nm, using the NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, USA).

HPLC analysis: 0.5 mg/ml EGCG was dissolved in EpiLife/S7 or HEPES buffer (25 mM HEPES, 150 mM NaCl, pH 8.0) and stored for 0 – 48 hours at 2-8°C. Samples were analyzed by reversed-phase high-performance liquid chromatography (RP HPLC). A Dionex Ultimate 3000 system (Thermo Fisher, Dreieich, Germany) was used and aliquots of 5 µl were injected on a Betasil C8 column (125 x 4.5 µm, Thermo Fisher, Dreieich, Germany). Detection was performed at 280 nm wavelength. Eluent A consisted of 10% (w/v) acetonitrile and 0.1% (w/v) trifluoracetic acid in HPW. Eluent B consisted of 0.1% (w/v) trifluoracetic acid in acetonitrile. A gradient from 0 – 18% B in 18 min with a flow of 0.5 ml/min was applied; the column oven temperature was set to 37 ºC and the autosampler temperature to 4°C. Recovery was calculated by dividing the area of the peak after storage by the area of the peak directly after sample preparation, multiplied by the factor 100.

VI.3 Results

VI.3.1 EGCG as lyoprotectant

In a first experiment, the concentration of 0.945 mg/ml EGCG (which was used by Natan et al., [3]) was added to a formulation containing 10% HES, 5% HE, formulated in EpiLife/S7. Cells were freeze-dried in suspension and Figure VI-4 A shows that with the addition of EGCG, cell membrane integrity increases from 23.1 ± 7.4% without EGCG to 69.5 ± 5.7% with the addition of EGCG. Also total cell recovery rises from 35.5 ± 6.7% without EGCG to 74.3 ± 11.3% with EGCG. Figure VI-4 B depicts an exemplary cut out of pictures taken with the ViCell™ XR. Red circles highlight cells with impaired cell membranes, stained with trypan blue, whereas green circles indicate intact cells without trypan blue dyeing. Cells with intact and impaired cell membrane can be clearly distinguished and cells freeze-dried with EGCG show a brighter cytoplasm with no trypan blue staining.

As EGCG is a very potent molecule with many unknown effects and also for the purpose of cost reduction, different concentrations of EGCG (0 – 1.5 mg/ml) were evaluated (Figure VI-4 C). No effect was seen for low concentration (0.2 mg/ml), whereas for higher concentration a dose

dependant increase in cell membrane integrity and recovery is noticeable. Above 0.5 mg/ml EGCG no further increase was observed, with a cell membrane integrity of 63.6 ± 4.7%. Therefore, for further experiments the lowest active concentration of 0.5 mg/ml EGCG was used.

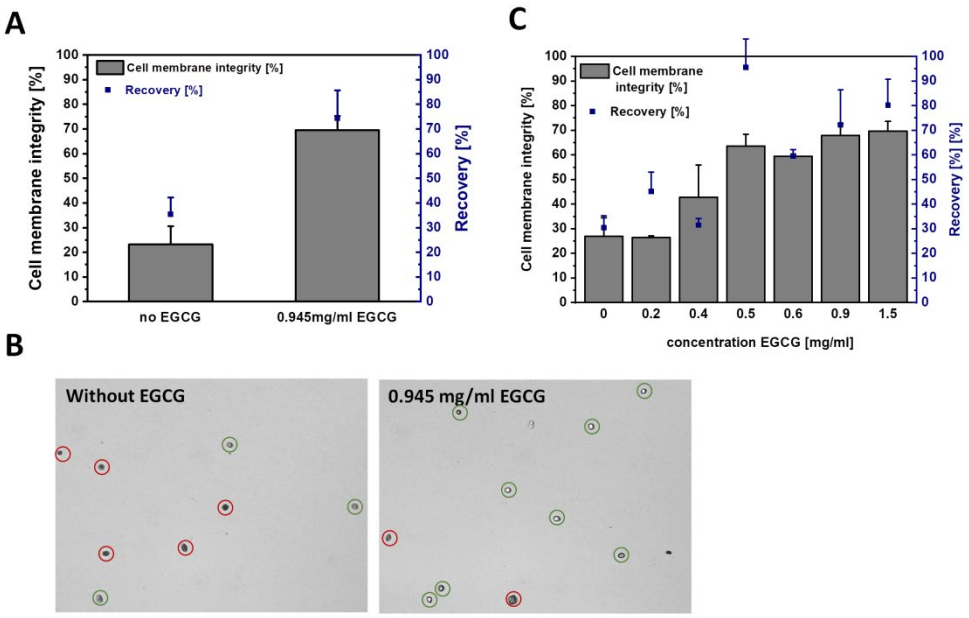


Figure VI-4. (A, C) Cell membrane integrity (bars) and recovery (squares) of cells freeze-dried in formulations containing 10% HES and 5% HE in EpiLife/S7 and between 0 and 1.5 mg/ml EGCG, respectively. **(B)** Representative pictures of ViCell XR™ after freeze-drying cells in 10% HES and 5% HE in EpiLife/S7, without and with 0.945 mg/ml EGCG (green: intact cell membrane, red: impaired cell membrane).

VI.3.2 Calcein-AM and EH staining

EGCG does not only increase membrane integrity of cells in suspension, but also protects membranes and intracellular proteins of freeze-dried adherent cells, measured with Ethidiumhomodimer-1 (EH) and Calcein-Acetoxymethylester (Calcein-AM) staining. Calcein-AM is a fluorescent precursor, which fluorescence is switched on after intracellular cleavage of the ester. Resulting Calcein is trapped and accumulated intracellularly and is therefore labelling metabolic active cells with intact intracellular esterases. EH is a membrane impermeable high affinity nucleic acid dye, which emits red fluorescence if bound to nucleic acids and is therefore labelling cells without intact cell membranes. EH together with of Calcein-AM is a widely established pair of fluorescent dyes to distinguish between living and dead cells.

Cells were analyzed regarding percentage and staining intensity of EH or Calcein positive stained cells. Cells were freeze-dried only in cell culture medium without excipients (EpiLife/S7) or in 10% HES and 5% HE in EpiLife/S7 with 0 – 0.5 mg/ml EGCG. We further investigated the influence of different cell densities during drying and incubation times of the reconstituted cells after freeze-drying. Cells were seeded and freeze-dried in densities ranging from 4000 - 10000 cells/cm^2. After reconstitution cells were incubated for 24 or 48 hours in cell culture medium

EpiLife/S7 prior to cell staining. The results clearly indicate that EGCG enhances cell membrane integrity and protects intracellular esterases (Figure VI-5). Without any excipients, above 80% of all cells are positive stained with EH (except cells with a density of 10000 cells/cm^2 after 48 hours). For most samples, the addition of 10% HES and 5% HE entails little reduction of EH staining, proving a slight membrane stabilization by 10% HES and 5% HE. With the addition of 0.25 and 0.5 mg/ml EGCG the number of EH positive cells significantly decrease to almost 0%. 0.1 mg/ml EGCG yields in higher amounts of EH positive stained cells, which is in accordance with the previous findings, that higher concentrations of EGCG are required to assure a stabilizing effect. Also higher Calcein staining is measured in the presence of EGCG which is true for all cell densities and time points. Without excipients, only around 20% of all cells show an intracellular Calcein signal, which rises to 40 - 60% with EGCG, whereas the addition of 10% HES/5% HE has only little influence on the intracellular Calcein staining.

The obtained results suggest no difference between different cell densities, whereas the incubation time indicates an influence on cellular conditions. EH staining is comparable for both time points, whereas the overall values for Calcein positive stained cells are lower after 48 hours incubation (~ 40%) than for 24 hours (~ 60%). Apparently, a longer cultivation time of the cells after lyophilization does not induce cellular repair mechanisms. The reverse was true and cells further reduce their metabolic activity with longer incubation times.

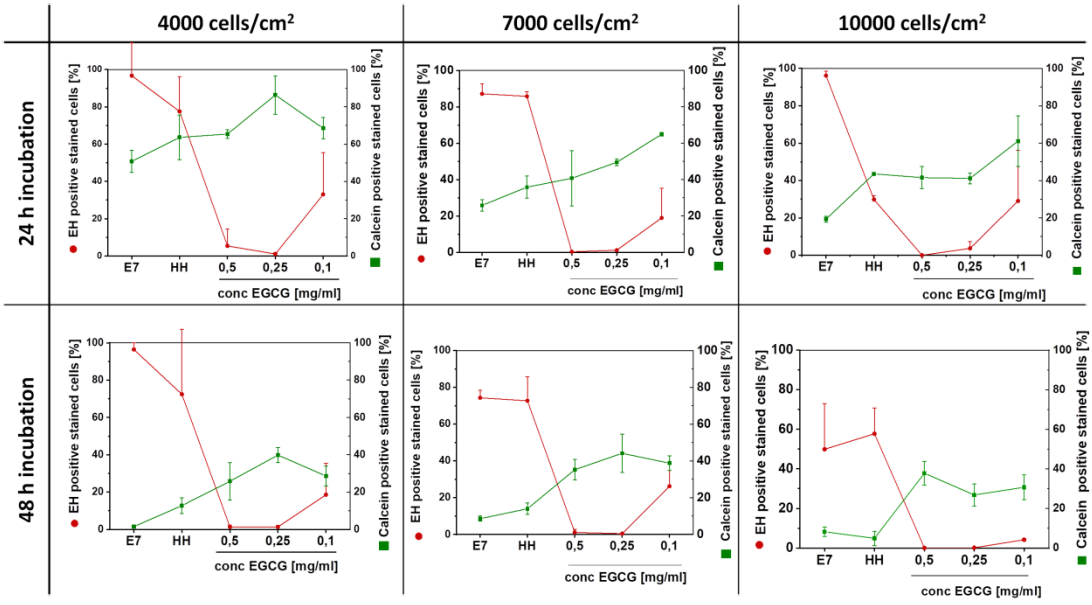


Figure VI-5. Percentage of EH (red circles) and Calcein (green squares) positive stained cells after freeze-drying and reconstitution. Cells were freeze-dried in EpiLife/S7 (E7) or 10% HES, 5% HE in EpiLife/S7 (HH) with 0 – 0.5 mg/ml EGCG. Cells were seeded in densities of 4000, 7000 and 10000 cells/cm^2 and stained 24 and 48 hours after reconstitution and incubation in EpiLife/S7. Pictures were analyzed with the software Fiji.

Further we analyzed staining intensity of the cells that were identified as EH or Calcein positive (see Figure VI-6). The intensity of cell staining can provide information on the total amount of formed Calcein and bound EH, which can further specify the extent of cell damage.

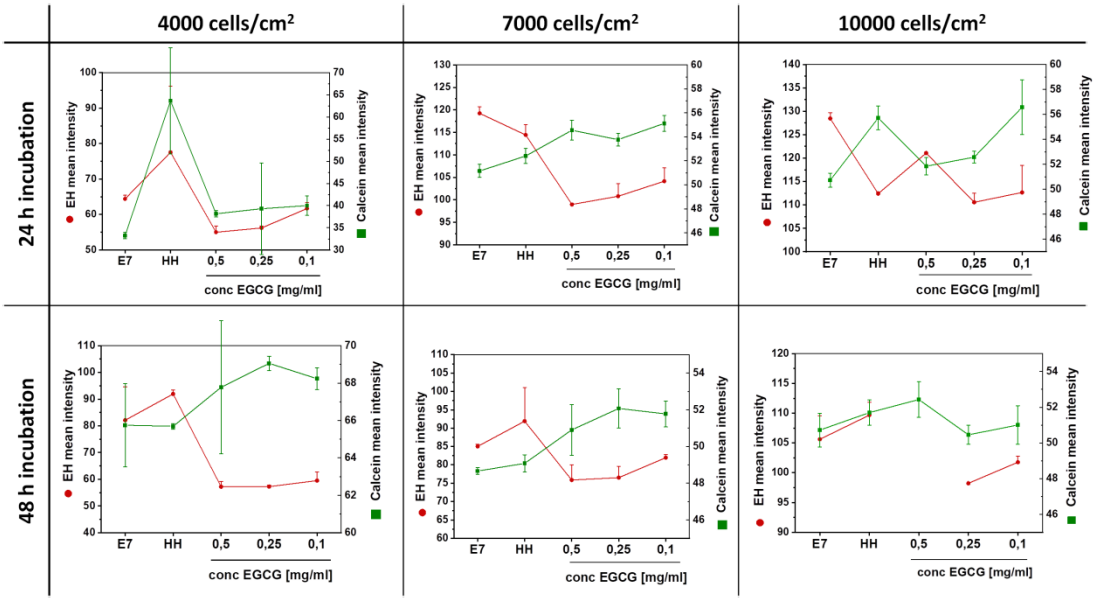


Figure VI-6. Mean intensity of cells positive stained with EH (red circles) and Calcein (green squares) after freeze-drying and reconstitution. Cells were freeze-dried in EpiLife/S7 (E7) or 10% HES, 5% HE in EpiLife/S7 (HH) with 0 – 0.5 mg/ml EGCG. Cells were seeded in densities 4000, 7000 and 10000 cells/cm² and stained 24 and 48 hours after reconstitution and incubation in EpiLife/S7. Pictures were analyzed with the software Fiji.

The results for the intensity measurements mostly correlate with previous findings. After 48 hours of incubation and for all seeding densities, it can be observed that EGCG enhances also Calcein staining intensity. This is also true for 7000 cells/cm² after 24 hours of incubation, whereas for 4000 and 10000 cells/cm² the highest intensity was calculated for samples without EGCG. EH intensity decreases with the use of EGCG for all cell densities and time points. The low EH staining intensity of the remaining stained cells shows that only very little EH is bound to nucleic acids of the cells that were detected as “EH-positive”. These findings confirm that EGCG has a positive influence on both cell membrane integrity and intracellular protein activity of freeze-dried adherent cells.

VI.3.3 Mode of action I – structure?

Once the effect of EGCG on cell membrane and protein survival is proved, the question about the underlying stabilizing mechanism arises. As a potent antioxidant, EGCG might reduce oxidative stress during freezing and subsequent drying, but also an interplay of EGCG with the polar head groups of the cell membrane is discussed. EGCG was even shown to enter cytoplasm by simple incubation, which could facilitate stabilization of intracellular proteins [16].

Many researchers discussed the underlying molecular events that were leading to the stabilizing properties of EGCG and compared EGCG with other catechins (see Figure VI-7). (-)-Epicatechingallate (ECG) differs only in one hydroxyl group from EGCG, whereas (-)-Epigallocatechin (EGC) is missing the entire galloyl moiety. Caturla et al. stated that the galloyl moiety is required for sufficient interaction with biological membranes and to prevent lipids from oxidation [11]. Thus EGCG and ECG are supposed to be more effective than EGC, which is also in accordance with the findings of C. Rice-Evans, who explains the higher activity of EGCG and ECG with a higher affinity to lipid peroxidases due to the higher hydrophobicity [7].

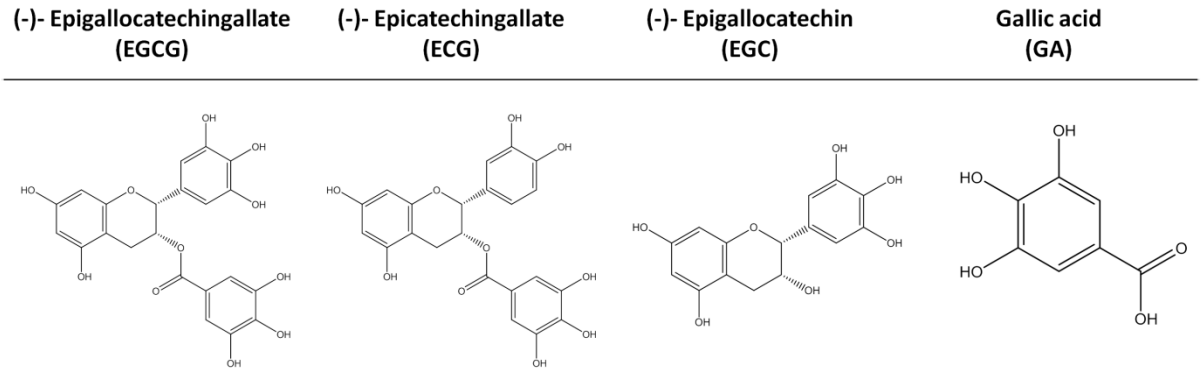


Figure VI-7. Chemical structures of EGCG, ECG, EGC and GA.

To further investigate EGCGs mode of action, we compared EGCG with these catechins and gallic acid (GA), which can also be found in green tea. We added ECG, EGC and GA in equimolar concentrations to EGCG to a formulation containing 10% HES, 5% HE in EpiLife/S7 (see Table VI-1).

Catechin	Concentration [mg/ml]	Concentration [mM]
(−)-Epigallocatechingallate	0.95	2.1
(−)-Epigallocatechin	0.63	2.1
(−)-Epicatechingallate	0.91	2.1
Gallic acid	0.35	2.1

Table VI-1. Concentration of (-)-Epigallocatechingallate and structurally related molecules.

The results are illustrated in Figure VI-8 and it is clear that EGCG led to the highest cell membrane integrity and recovery rate.

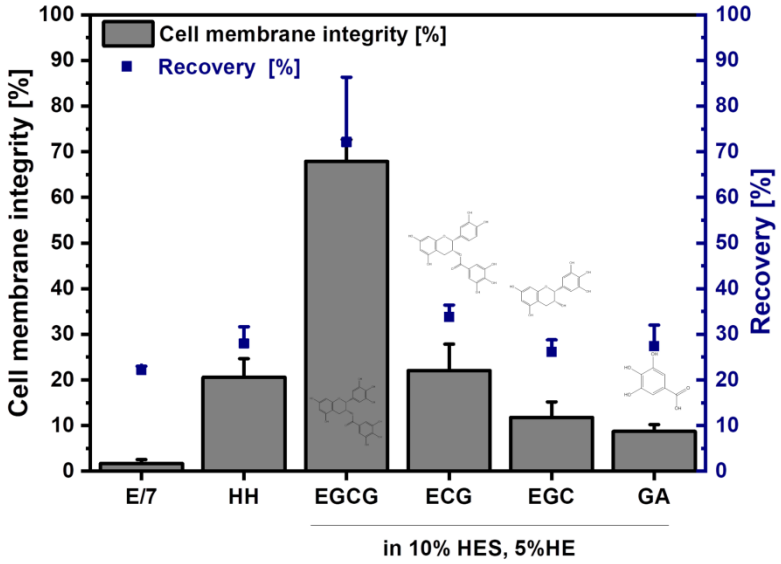


Figure VI-8. Cell membrane integrity (bars) and recovery (squares) of cells freeze-dried in formulations containing 10% HES and 5% HE in EpiLife/S7 and 0.945 mg/ml EGCG or structure related catechins in equimolar amounts.

The addition of ECG resulted in a membrane integrity of 22.1 ± 5.8%, whereas EGC and gallic acid showed membrane integrity rates only around 10%, which is even below 20.6 1 ± 4.0% for cells dried without any additional catechin. This supports the hypothesis that the galloyl group is an important structural characteristic. However, neither ECG nor gallic acid reached the protection level of EGCG, indicating that both characteristics, three hydroxyl groups of the B ring as well as the galloyl moiety, are required for successful stabilization and that not only a single structural element can protect the cell membrane.

As a next step we addressed the question, if it is possible to enhance the biological activity by combining the necessary structures, such as the galloyl group with the three hydroxyl groups of the B ring. We combined either EGC or ECG with gallic acid in equimolar concentrations, calculated as 0.5 mg/ml EGCG (see Table VI-2).

Catechin	Concentration [mg/ml]	Concentration [mM]
(−)-Epigallocatechingallate	0.5	1.09
(−)-Epigallocatechin + Gallic acid	0.33 (EGC) + 0.19 (GA)	1.09 (ECG) + 1.09 (GA)
(−)-Epicatechingallate + Gallic acid	0.48 (ECG) + 0.19 (GA)	1.09 (EGC) + 1.09 (GA)

Table VI-2. Combinations and concentration of EGCG, EGC + GA and ECG + GA.

The combination of ECG or EGC with gallic acid did not further stabilize cells after freeze-drying(see Figure VI-9). The reverse was true, as a lower cell membrane integrity was observed compared to cells freeze-dried only in 10% HES and 5% HE. Apparently both structural elements of EGCG, the 3 adjacent hydroxyl groups of the B ring and the galloyl moiety, need to be combined in one molecule to achieve sufficient stabilization of cells in the dry state.

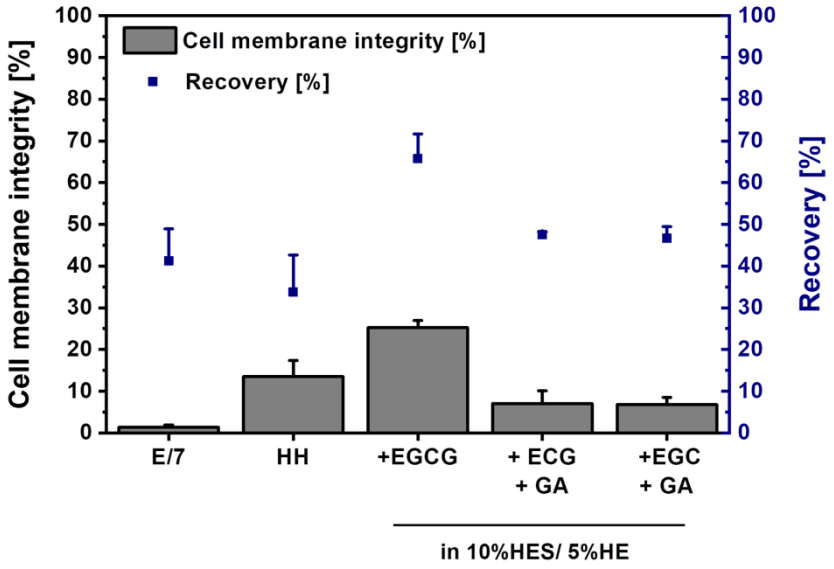


Figure VI-9. Cell membrane integrity (bars) and recovery (squares) of cells freeze-dried in formulations containing 10% HES and 5% HE in EpiLife/S7 and EGCG or combinations EGC and GA or ECG and GA.

VI.3.4 Mode of action II – antioxidants?

Since structurally similar molecules did not stabilize the cells during lyophilization in a similar manner than EGCG, we tried to reveal the role of the antioxidant activity. If EGCG is preventing oxidative stress and thus protecting the cells from further damage, also other antioxidants should show at least comparable effects. We therefore chose eight non enzymatic antioxidants which were already described in literature to show positive effects during cryopreservation or lyophilization of cells (see Table VI-3).

However, most of the chosen antioxidants were used for the cryopreservation of semen, as spermatozoa is known to be highly susceptible for lipid peroxidation and the formation of ROS [17].

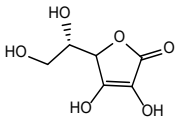
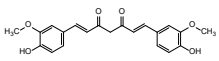
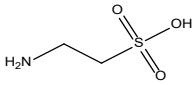
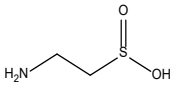
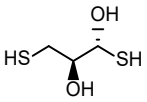
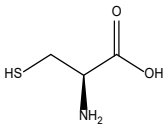
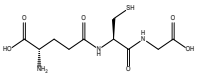
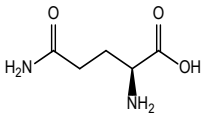
Excipient	Structure	Application	Cell type	Used conc. in literature	Applied conc.	Ref.
Ascorbic acid		Cryo-preservation, freeze-drying	human hemato-poietic cells, Pseudomonas chlororaphis Murine Germ Cells	80 µg/ml, 1 g/l, 0.1, 0.5, 1 mM	50 µM	[18], [19], [20]
Curcumin		Cryo-preservation	Bull semen, boar semen	0.5 mM, 0.25 – 0.5 mmol/l	100 µM	[21] [22]
Taurine		Cryo-preservation	Bull semen, rabbit semen, human hematopoietic ells	2 mM, 0.5 mM, 25 µg/ml	2 mM	[18] [17] [23]
Hypo-taurine		Cryo-preservation	Rabbit semen, Murine Germ Cells	0.5 mM 3.5, 7, 14 mM	7 mM	[20], [23]
Dithio-erithritol		Cryo-preservation	Bull semen	0.5 mM	1 mM	[21]
L-Cysteine		Cryo-preservation	Bull semen	2 mM	10 mM	[17]
Glutha-thione		Cryo-preservation	Porcine adipose-derived stem cells Murine Germ Cells	100 µM 50 - 200 µM	100 mM	[20], [24]
L-Glutamine		Cryo-preservation	Boar semen	20 – 80 mM	20 mM	[25]

Table VI-3. Antioxidants used for freeze-drying of HaCaT keratinocytes.

Antioxidants were added additionally to 10% HES and 5% HE in EpiLife/S7 and the results are depicted in Figure VI-10. None of the tested antioxidants showed stabilizing effects on cell membrane or total cell recovery; furthermore, except for hypotaurine, taurine and ascorbic acid, even lower membrane integrity values were achieved. We concluded from these results that antioxidants have no positive influence on cell membrane integrity of HaCaT keratinocytes during freeze-drying and it is unlikely, that the effect of EGCG can be attributed to its antioxidative properties.

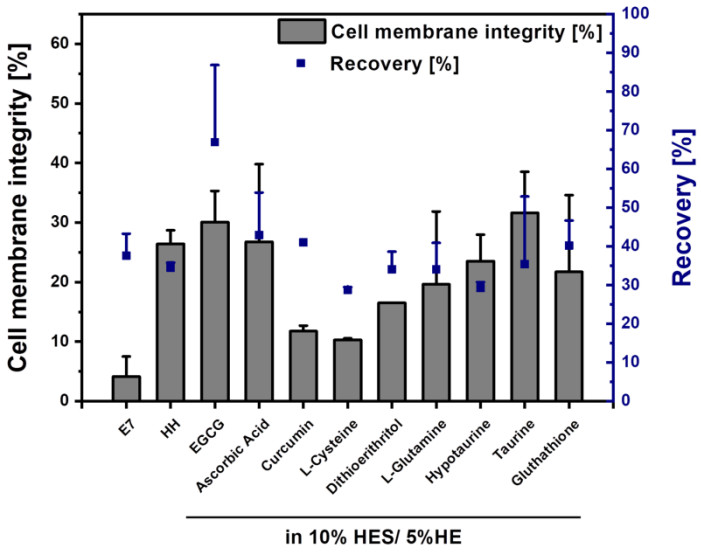


Figure VI-10. Cell membrane integrity (bars) and recovery (squares) of cells freeze-dried in EpiLife/S7 (E7) or formulations containing 10% HES and 5% HE in EpiLife/S7 (HH) and different antioxidants.

VI.3.5 Stability of EGCG

The stability and degradation of EGCG and other catechins is already described in literature [12]–[14], [26]–[29]. Autooxidation, dimerisation and epimerisation were found to be the major reactions down the degradation pathway and Gallocatechin, Epicatechin, Theasinensin A and another dimer named P2 were identified amongst the oxidation products [26]. The degradation follows a first order degradation kinetic in a temperature and pH dependant manner, EGCG being more stable at lower pH values [27], [12].

Thereupon we investigated the stability of EGCG, formulated in EpiLife/S7, but also in HEPES buffer (25 mM, 150 mM NaCl) at different pH values. Formulated in EpiLife/S7 (pH 7.4), a colour change was observed after storage for 24 hours at room temperature, which was more intense with higher concentration of EGCG (Figure VI-11 A).

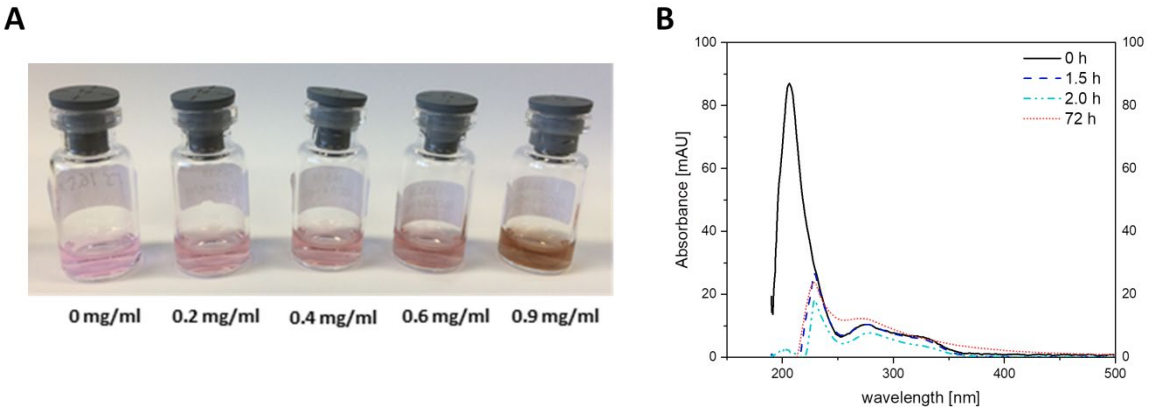


Figure VI-11. **(A)** Macroscopic changes of different concentration of EGCG in EpiLife/S7 after storage for 24 hours at RT. **(B)** Absorbance spectrum of 0.5 mg/ml EGCG in EpiLife/S7 after 0 (line), 1.5 (dashed), 2.0 (dashed-dotted) and 72 (dotted) hours of storage at RT.

Also the absorbance spectrum of EGCG shows a drastic decrease of the maximum at 200 nm already after 1.5 hour at RT, with no further reduction after longer incubation times (see Figure VI-11 B).

For a more detailed analysis of the degradation kinetics, the decrease of EGCG was determined by RP-HPLC. Directly after preparation in EpiLife/S7 at pH 7.4, EGCG elutes after 8.5 minutes, however, already some degradation products are visible (Figure VI-12 A). Preparation at pH 5 shows only one peak and no degradation products (data not shown). After 8 and 24 hours, the main peak area decreases and more hydrophobic or hydrophilic degradation products with shorter and longer retention time appear. Figure VI-12 B and C show the recovery of 0.5 mg/ml EGCG in EpiLife/S7 and HEPES buffer and it is remarkable that EGCG rapidly degrades at higher pH. EGCG is more stable when formulated in HEPES buffer than in EpiLife/S7, even if the pH is adjusted to the same value. At pH 7.4, formulated in EpiLife/S7, the recovery of EGCG after 10 hours at 4°C is only around 50%, whereas storage in HEPES buffer (pH 7.4) resulted in 70% recovery. No degradation was observed for pH 5 and 6 in HEPES after 24 hours whereas in EpiLife/S7 only at pH 5.0 stability over 24 hours was recorded. To summarize, a rapid degradation of EGCG, with faster degradation at higher pH values could be confirmed. Also EpiLife/S7 proved to be the less favourable basis compared to HEPES buffer. To avoid degradation, EGCG should be therefore formulated either in HEPES buffer at pH 6.0, or in EpiLife/S7 at pH 5.0. If formulation under these conditions is not possible, a rapid degradation of EGCG must be considered when results are interpreted.

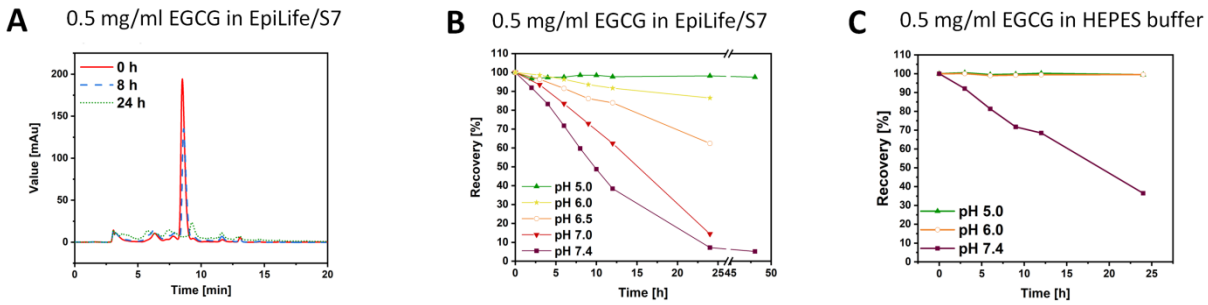


Figure VI-12. (A) HPLC chromatogram of 0.5 mg/ml EGCG in EpiLife/S7 (pH 7.4) after 0 (line), 8 (dashed) and 24 (dotted) hours incubation at 4°C. **(B)** Recovery of 0.5 mg/ml EGCG in EpiLife S7 at different pH values after 0 – 48 hours incubation at 4°C (triangle up: pH 5.0, stars: pH 6.0, circles: pH 6.5, triangles down: pH 7.0, squares: pH 7.4). **(C)** Recovery of 0.5 mg/ml EGCG in HEPES buffer (25 mM HEPES, 150 mM NaCl) at different pH values after 0 – 48 hours incubation at 4°C (triangles up: pH 5.0, circles: pH 6.0, squares: pH 7.4).

VI.3.6 Migration inhibition

EGCG is a highly bioactive component, which has also unwanted properties, such as a proliferation inhibition of T-cells, which was described by Wu et al [30]. We therefore analyzed the migrative behaviour of cells after addition of freeze-dried and reconstituted formulations containing EGCG to identify any interference of EGCG with the growth properties of adherent

HaCaT keratinocytes. Formulations with 10% HES, 5% HE in HEPES buffer with 0.5 mg/ml EGCG were freeze-dried. The lyophilizates were reconstituted in E7:HPW in a ratio of 1:1. Previous experiments showed (data not shown) that on the one hand reconstitution with 50% HPW is required to reduce the osmotic pressure of the resulting formulations. On the other hand reconstitution with 50% EpiLife/S7 is necessary to provide inevitable nutrients for cells to proliferate and migrate. Reconstituted formulations were added to culture dishes and cellular migration was monitored until complete gap closure. Figure VI-13 represents the course of the gap closure after 0, 5, 22 and 26 hours of incubation with respective formulation.

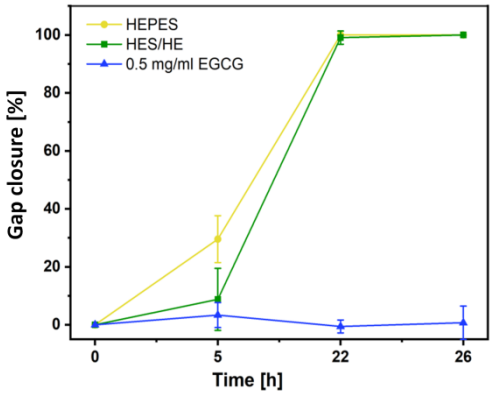


Figure VI-13. IBIDI migration assay: time dependent gap closure of HaCaT keratinocytes after addition of different freeze-dried and reconstituted formulations (circles: HEPES buffer, squares: 10% HES, 5% HE in HEPES buffer, triangles up: 10% HES, 5% HE, 0.5 mg/ml EGCG in HEPES). All formulation were reconstituted with EpiLife/S7:HPW in a ratio of 1:1.

With the addition of freeze-dried and reconstituted formulations of HEPES buffer or 10% HES/5% HE, cells migrate into the gap with total gap closure after 22 hours. However, the addition of EGCG completely inhibits cell growth and migration and no cell movement was observed even after 26 hours of incubation.

VI.4 Discussion

We were able to show, that EGCG sufficiently stabilizes HaCaT keratinocytes during freeze-drying and enhances cell membrane integrity, total cell recovery and intracellular esterase activity. However, the mode of action of the stabilizing effect of EGCG is still not elucidated. Structure related molecules showed no effect, nor did other non enzymatic antioxidants. This implies that ROS events and oxidative stress are not mainly responsible for cell or membrane damage during freezing or drying. Also no single parts of the structure such as the galloyl group, three adjacent hydroxyl groups at the B-Ring, or two ortho hydroxyl group at the A-Ring could sufficiently stabilize the cells and structure related molecules did not result in comparable effects. Only EGCG, combining all characteristics, was able to protect cell membranes and intracellular

proteins. Apparently a combination of all structure elements is required for a sufficient interaction and thus stabilization of the cell membrane and also intracellular structures.

Discussing EGCG as promising lyoprotectant, its rapid degradation is one point that needs to be taken into account. We could prove that depending on pH and buffer, EGCG rapidly degrades within few hours, with a faster decrease in cell culture medium EpiLife/S7 and at neutral or basic pH. However, many effects of EGCG were determined under cell culture conditions at neutral pH and a rapid degradation of EGCG must be assumed. Many researchers therefore attribute the effect of EGCG rather to its degradation products instead of intact EGCG [26]. Some oxidation products are already elucidated and were proved to be responsible for a wide range of pharmacological and biochemical effects [14]. Theasinensin A is a major product of EGCG degradation and suppressed the antibiotic resistance of methicillin-resistant *Staphylococcus aureus*. Furthermore it is discussed if EGCG or Theasinensin A is responsible for the antibacterial properties of green tea [29]. This is another possible explanation, why structural similar molecules did not show any activity, as they might have a different degradation pathway and do not lead to active degradation products. Furthermore, to make meaningful comparisons between reports on the effects of EGCG, it is therefore advisable to have a closer look at the formulation buffer and pH.

As a potent excipient, EGCG shows also unwanted properties, such as the observed migration inhibition of cells, which may also correlate with the influence on cell membrane integrity during freeze-drying as both effects can be attributes to an interaction with lipid bilayers. On the one hand this interaction might lead to a more flexible membrane that can withstand stress during desiccation. On the other hand a strong interaction with membranes can turn into the opposite and induce membrane damage and disintegration. It is reported that EGCG can interrupt liposomal membranes and induce rapid leakage of Carboxyfluorescein [10]. These effects seem to be dose dependant and low concentrations (~ 1 nM) were shown to protect liposomes from Calcein leakage, whereas higher concentrations promote membrane disruption [31]. Uesato et al. performed cytotoxicity assays to determine the toxicity of tea catechins on HCT 116 or Hep G2 cell and concluded IC_{50} values of 70 – 200 µM with MTT assays and 8 – 60 µM with clonogenic assays after 1 day of incubation [32]. Of course our applied concentration (~ 1 mM) exceeds these reported toxic concentrations, however, the exposure of cells to EGCG before they are frozen stretches only over a very limited time frame, as cells were directly frozen without any incubation time. To avoid detrimental effects of longer incubation times, a fast handling after the addition of EGCG is therefore required. Even if the exposure time of EGCG to the cells is too short to lead to toxic side effects, we agree with Natan et al. that before analyzing the cells and cell growth after lyophilization, cells should be washed to remove excess EGCG and to avoid unwanted effects [3].

To sum up, we were able to show that EGCG is a potent lyoprotectant, which effectively enhances membrane integrity, total cell recovery and protein activity after freeze-drying of HaCaT keratinocytes. However, the rapid degradation and the growth inhibiting properties of EGCG are drawbacks that need to be considered, if EGCG is applied as lyoprotectant. The potential toxicity and degradation requires a fast handling upon EGCG addition and after lyophilization we recommend a subsequent washing step to remove excess EGCG. Still many questions remain unsolved, such as the mode of action and interaction of EGCG or its degradation products with lipid bilayers. According to our findings, it is unlikely that the antioxidant activities of EGCG are responsible for the protective behaviour of EGCG and we propose that all active structural elements are necessary to stabilize and interact with the cell membrane during freeze-drying.

VI.5 References

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Chapter VII

Multiplex analysis, *in vitro* and *in vivo* activity of freeze-dried keratinocytes

VII.1 Introduction

Wound healing is a dynamic event with a carefully balanced interplay of various cell types, growth factors and cytokines. Especially keratinocytes secrete a variety of different proteins that are involved in inflammation, proliferation and remodelling processes. *In silico*, *in vitro* and *in vivo* models have been established to improve the understanding of wound healing processes which subsequent may lead to new treatment options of burn wounds and other difficult skin lesions [1]. The apparent advantages of *in vitro* experiments are a cheap and easy implementation with fast readouts. A well established 2D *in vitro* model is the scratch assay or migration assay in which keratinocytes or other cell types are cultivated in a culture dish until they form a confluent cell layer. Usually the cell layer gets wounded with a sterile pipette tip and the migration of cells into the cell free gap is monitored. This is a particularly simple method that shows a good correlation with *in vivo* cell migration [2]. However, drawbacks are a limited reproducibility of a uniform scratch with a defined size, which requires experience and training. Also the surface coating of the culture dish can get impaired during scratching, leading to altered migration behaviour [3]. Further improvements are culture inserts with a gap holder of defined size that can be removed after the surrounding cells form a confluent cell layer. A well defined gap offers controlled and comparable experimental conditions. In general, 2D migration assays present a fast and reproducible way to access first information about the migrative behaviour of cells in a certain environment. However, just one single cell type is observed, which gives only information about a small cut-out of the complex procedure during wound healing. Co-cultures can overcome this problem and are used to study the interaction of multiple cell types (for example keratinocytes and fibroblasts). A so called boyden chamber, also referred as trans-well migration assay or chemotaxis assay, can be used to measure cell migration through a semi permeable membrane. Cells are plated on this membrane which is submerged in a well that contains medium or other chemoattractants and subsequently the migrative behaviour of the cells through the membrane can be assessed [4].

Ex vivo or skin explants provide a 3D model that reflects the microenvironment and cell-cell interactions during wound healing and mimics to a large extent *in vivo* conditions and the environment of normal skin. However, missing innervations of the skin and limited comparability of different body sites are drawbacks of *ex vivo* models [1], [5].

The gold standard are still i*n vivo* models, which represent the real (patho-)physiology of wounds and provide the best opportunity to study wound healing processes and new treatment options. Human studies have the highest clinical relevance, however, it may be difficult to find volunteers with comparable wounds and often ethical concerns limit the use of human wound models. As a result, animal models emerged as widespread alternative, even if the animal skin differs in some

parts from the human skin. Rodents, rabbits or pigs are commonly used for wound models, each of them exhibiting separate advantages and disadvantages. Rodents and rabbits are cheap, readily available and a wide range of genetically engineered phenotypes exist [1]. However, they show limited comparability with human skin, since they have a thinner epidermis, a contractile wound healing phenotype and denser hair than human skin. Additionally their skin is only loosely connected to the subcutaneous tissue where human skin is tightly attached to it [6], [1]. Pigs share numerous similarities with the human organism and also the porcine skin shows best accordance and high anatomical and physiological comparability with human skin [7]. Pigs have a relatively thick epidermis, similar structured collagen and skin that is adherent to the underlying structures. However, despite little structural differences, high costs and large experimental setups are restrictions of porcine animal models [1], [6], [8]. In general, ethical concerns and the resulting administrative expenses need to be considered if *in vivo* animal models are conducted.

In the present chapter we will focus on the *in vitro* and *in vivo* activity of lyophilized keratinocytes in wound models. As a first step, we analyzed the structural presence of important proteins that are involved in the wound healing process. A multiplex-assay enables simultaneous detection and quantification of a large number of secreted proteins, based on ELISA technology. Subsequently a 2D migration assay was performed to measure cell migration after addition of lyophilized keratinocytes. Finally, to overcome previously described drawbacks of *in vitro* assays, an *in vivo* porcine wound model was implemented to compare the wound healing efficacy of freeze-dried cells compared with placebo.

VII.2 Materials and Methods

VII.2.1 Materials

Hydroxyethyl starch 200/0.5 (HES) was purchased from BOC Sciences (Shirley, NY, USA). Hydroxyectoine (HE), tris buffered saline (TBS: 0.05 M Tris buffered saline (0.138 M NaCl, 0.0027 M KCl), pH 8.0 at 25°C), sodium dodecyl sulphate (SDS), dulbeccoo's phosphate buffered saline (PBS: 0.2 g/l KCl, KH_2PO_4, 8 g/l NaCl, 1.15 g/l Na_2HPO_4 (anhydrous)), deoxycholic acid, trichloracetic acid and polysorbate 20 was supplied by Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). HEPES and NaCl were ordered from VWR (VWR, Allison Park, PA, USA). HaCaT (Human adult low Calcium high Temperature) keratinocytes were provided from the DKFZ (Heidelberg, Germany). HEKs (Human Epidermal Keratinocytes), isolated from human adult skin of a 28 year old Caucasian woman, were generously provided from QRSkin GmbH (Würzburg, Germany).

VII.2.2 Multiplex analysis and *in vitro* experiments

Cell preparation: Cultivation of HaCaT keratinocytes was performed as described in Chapter III.

Sample preparation: For lyophilization of cells in suspension, cells were resuspended in different formulations, containing 10% (w/v) hydroxyethyl starch (HES) and 5% (w/v) hydroxyectoine (HE), dissolved in HEPES buffer (25 mM HEPES, 150 mM NaCl, pH 8.0). 2% (v/v) DMSO or 0.5 mg/ml EGCG were added and cells were freeze-dried in a concentration of 0.5 – $1 \cdot 10^6$ cells/ml. Aliquots of 500 µl were filled in 10 R tubing vials (MGlas AG, Muennerstadt, Germany) and semi-stoppered with lyophilization stoppers (FluroTec® rubber stopper; West Pharmaceuticals, Eschweiler, Germany).

For lyophilization of adherent cells, cells were grown in a 6-well plate (TPP products, Trasadingen, Switzerland) for 24 hours. Prior to freeze-drying, growth medium was aspirated and cells were washed once with PBS. For lyophilization, 1.5 ml per of formulations containing 10% HES and 5% HE with 2% DMSO or 0.5 mg/ml EGCG were added per well and cells were freeze-dried directly in the well plate.

Lyophilization: Freeze-drying was performed as described in chapter IV (Christ 2-6D laboratory scale freeze-dryer (Martin Christ, Osterode am Harz, Germany); Primary drying: ST at -30°C, 20 hours, vacuum 0.01 mbar; Secondary drying: ST at 20°C, 5 hours, vacuum 0.01 mbar).

Cell migration: A migration assay was performed as described in Chapter VI.

Lysis of cells: 300000 cells were plated per well of a 6-well plate (TPP products, Trasadingen, Switzerland) and were incubated for 24 hours at 37°C, 5% CO_2. Cells were freeze-dried as described above and reconstituted with 2 ml HEPES buffer (37°C). The supernatant was collected for further upconcentration and cells were washed three times with ice cold PBS. Reagent based lysis was performed with 50 µl of lysis buffer (RIPA buffer (Sigma Aldrich, St. Louis, MO, USA) with phosphatase inhibitor PhosSTOP and protease inhibitor cOmplete™ ULTRA tablets (prepared according manufacturers instruction, dilution to 1x concentration, both from Roche Diagnostics (Rotkreuz, Switzerland)) for 30 minutes on an orbital shaker on ice. Subsequently cells were scraped, transferred into Eppendorf reaction tubes (Greiner AG, Kremsmünster, Austria) and centrifuged for 10 minutes at 16000 g at 4°C. To determine the protein concentration, a BCA assay of the cell lysate was performed according the manufacturer's instructions, using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Protein upconcentration: The supernatant after reconstitution of lyophilized adherent cells was collected as described above and centrifuged (10 minutes at 10 g) to remove cell debris. Aliquots of 1400 µl were incubated for 10 minutes at RT with 140 µl of 0.15% deoxycholic acid. Aliquots of 70 µl trichloracetic acid were added and protein precipitation was performed for 1 hour on ice. Precipitated proteins were centrifuged (4 minutes at 16 g at 4°C) and washed 3 times with cold

acetone (-20°C). Excess acetone was removed in a heating block at 95°C and the resulting cell pellet was dissolved in 50 µl PBS. The protein concentration was determined by a BCA assay, according the manufacturer's instructions, using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Gel electrophoresis: Aliquots of 15 µg of protein lysate and upconcentrated supernatant (for upconcentrated HEPES supernatant only 2 µg) were mixed with loading buffer (4x Laemmli Loading buffer, VWR, Allison Park, PA, USA) and purified water to a volume of 30 µl and heated for 5 minutes at 95°C. Aliquots of 25 µl of latter mixture were loaded in each well of a 10% tris-glycine gel (Novex™ WedgeWell, Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). Aliquots of 5 µl of PageRuler™ Plus Prestained Protein Ladder (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) were loaded in the first and the last well. The gel was run for 90 minutes at 100 V in 1× Rotiphorese® running buffer (Carl Roth, Karlsruhe, Germany). After the run, the gel was rinsed twice in deionised water and stained for 1 hour in Brilliant Blue G solution (Sigma Aldrich, St. Louis, MO, USA). Subsequently the gel was destained (destaining solution: 50% HPW, 40% methanol, 10% acetic acid) for 24 hours. Pictures were taken with a Biorad Chemidoc (Bio-Rad Laboratories, Hercules, CA, USA).

Multiplex analysis: A protein cell lysate was prepared as described above from lyophilized adherent cells and was used for the analysis of 12 specific growth factors and cytokines. Quantification was performed with a customised Luminex® Screening kit (R&D Systems, Abingdon, UK) in the BioPlex 200 System (Bio-Rad, Watford, UK) according the manufacturer's instructions. Luminex® assays are ELISA based systems for simultaneous and quantitative measurement of multiple cytokines, growth factors or other proteins. Protein recovery was calculated as the measured concentration of each protein after lyophilization divided by the protein concentration before lyophilization, multiplied by the factor 100.

VII.2.3 *In vivo* experiments

Cell preparation: HEKs were cultivated in EpiLife/S7 in presence of 10 µM Y-27632, a rho kinase inhibitor (derived by MedChemExpress, Monmouth Junction, NY, USA), which was shown to increase proliferation rate of primary keratinocytes [9]. Cells were grown on flasks coated with collagen 1 (Cellcoat, Greiner AG, Kremsmünster, Austria).

Sample preparation: $2.5 \cdot 10^6$ cells/ml were resuspended in a formulation containing 10% HES and 2% DMSO in HEPES buffer (25 mM, 150 mM NaCl, pH 8) and aliquots of 1 ml were filled in washed and autoclaved (121°C, 15 min) glass dishes of 30 mm diameter. Since HE was under patent protection, 5% HE was excluded from this formulation upon request of our cooperation partner [10]. Placebo samples were prepared as control.

Lyophilization: A FTS Lyostar™ 3 freeze-dryer (SP Scientific Stone Ridge NY USA) was used. After an equilibrium step of 30 minutes at -5°C, samples were cooled with a constant freezing rate of 1 K/min down to -50°C. Temperature was hold for 90 minutes at -50°C and primary drying was carried out at -35°C with a vacuum set at 20 mTorr for 20 hours. Secondary drying was performed at 20°C and 20 mTorr for 5 hours with a heating ramp of 0.1 K/min until 0°C and 0.2 K/min until 20°C. The product temperature was recorded with thermocouples and chamber pressure was controlled with a pirani and a capacitance manometer.

Porcine in-vivo experiments: The Animal Care and Use Committee (Veterinary University Vienna, Austrian Ministry of Science and Research) approved animal experiments, which were conducted at the medicinal university of Graz at the Research Unit for Safety in Health. The animals were anesthetized with 0.5 mg/kg Midazolam (Dormicum®), 2 mg/kg Azaperon (Stresnil®), 0.1 mg/kg Butorphanol (Butomidor®) and 10 mg/kg Ketamin (Ketasol®). Propofol (2-5 mg/kg) and 1-2% Isofluran were used to ensure sufficient anaesthesia. After the treatment, fentanyl patches were used to provide analgesia. As described above, samples were freeze-dried in glass petri dishes (diameter 30 mm, filling volume 1 ml), to obtain flat, intact samples that can be removed from the petri dish to get dissolved directly on the wound bed (see Figure VII-1 B). Under anaesthesia and analgesia the prepared lyophilizates with cells and placebo samples were applied directly on 3 x 3 cm sized dermatome wounds of 1.2 mm depth arranged on the flank of a domestic pig (*suus domesticus*), see Figure VII-1 A. Epicite^{hydro} (QRSkin GmbH, Würzburg, Germany), a wound dressing composed of hydrated cellulose was added as secondary wound cover. Seven applications for lyophilizates containing cells and eight for placebo samples on two different pigs were performed. After 7 days the animals were sacrificed and photo documentation of the wounds for further analysis were performed.

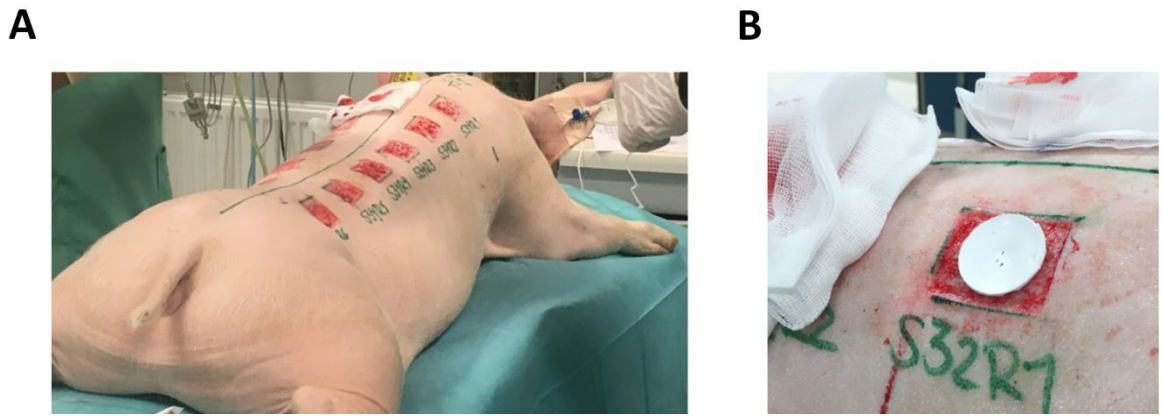

Figure VII-1. **(A)** Dermatome wounds of an anesthetised pig. **(B)** Application of lyophilizates directly on the dermatome wound.

Microscopic analysis of wounds and reepithelialisation assessment: Histology and histomorphometry was performed by TPL pathlab (Freiburg, Germany). After sacrification, 8 mm

punch biopsies of each wound were carefully taken through the dressing, close to center, keeping it in place. Afterwards, the remaining wound dressing was removed and the entire wounds with a size of 3 x 3 cm surrounded with 1 cm of healthy tissue were excised above the fascia. Tissue samples were fixed for 48-72 hours with 4% formaldehyde, embedded in paraffin, and sectioned at a nominal thickness of 3 µm. Sections were stained with routine haematoxylin and eosin (HE).

Histopathological analysis was performed blindly by the study pathologist. Estimation of the global percent reepithelialisation and formation of new epidermal and dermal tissue was performed by estimation of the percentage of reepithelialisation of 16 to 21 immediately consecutive 100x microscopic fields covering the entire wound section of the wounds of each placebo and cell samples. Morphometry of reepithelialisation was performed and also the area of new dermal and epidermal tissue was determined as depicted in Figure VII-2. Measurements included wound width at section level, sums of lengths of epithelial gaps or sums of lengths of newly formed epithelium and percent reepithelialisation as the ratio of total length of newly formed epithelium divided by the wound length.

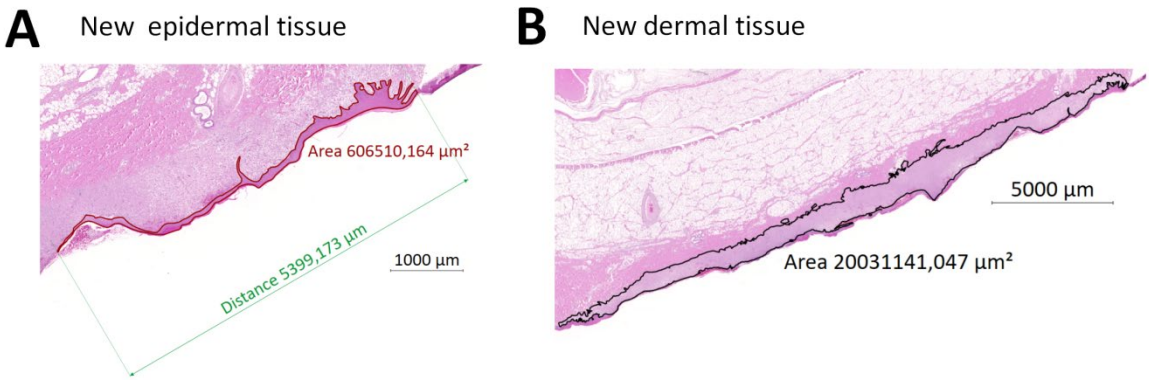

Figure VII-2. (A) Histopathological analysis of new epidermal tissue. **(B)** Histopathological analysis of new dermal tissue.

VII.3 Results

VII.3.1 Multiplex analysis

For a more detailed analysis of the presence of intracellular proteins, the most promising formulations were further analyzed. Cells were therefore freeze-dried in formulations containing 10% HES, 5% HE with 2% DMSO or 0.5 mg/ml EGCG. Cells before freeze-drying and cells freeze-dried only in HEPES buffer without excipients were used as control. The concentration of 12 different cytokines and growth factors after lyophilization and cell lysis was measured with the multiplex technology (Table VII-1).

	Growth factor/cytokine	Effect/function
1	Fibroblast growth factor (FGF) acidic	Mitogenic effects [11]
2	Growth regulated peptide (GRO) alpha	Keratinocyte proliferation, migration and angiogenesis [12]
3	Interleukin 1 (IL-1) alpha	Inflammatory response, activation of immune cells [13]
4	Interleukin 6 (IL-6)	Keratinocyte proliferation, reorganisation of keratin intermediate filaments [14]
5	Urokinase	Breakdown of extracellular matrix, tissue remodelling [15]
6	Epidermal Growth factor (EGF)	Keratinocyte migration [16]
7	Fibroblast growth factor (FGF) basic	Mitogenic effects, keratinocyte proliferation [11]
8	Interleukin 1 (IL-1) beta	Inflammatory response, activation of immune cells [13]
9	Interleukin 8 (IL-8)	Cell migration, chemoattractant to keratinocytes [17]
10	Interleukin 18 (IL-18)	Inflammatory response [18]
11	Tissue Inhibitor of Metalloproteinases 1 (TIMP-1)	Counterpart of MMPs, regulation of collagen degradation [19]
12	Vascular endothelial growth factor A (VEGF A)	Angiogenesis, granulation tissue formation [11]

Table VII-1. Analyzed growth factors and cytokines and their function in wound healing and tissue repair.

A drastic decrease of more than 50% in the concentration of most analyzed protein after lyophilization was observed (see Figure VII-3). Cells dried only in HEPES buffer showed the strongest decrease and the lowest concentration after freeze-drying, confirming previous findings that cells and cellular components are highly damaged without stabilizing excipients. The addition of 10% HES and 5% HE led to higher recovery rates for most analyzed proteins, however no clear trend is detected for the addition of 2% DMSO or 0.5 mg/ml EGCG, indicating no overall benefit for the addition of these excipients. For FGF acidic, IL-6 and Urokinase a slight decrease after DMSO and EGCG addition was measured, whereas DMSO led to higher amounts of detected GRO alpha and IL-18. EGCG on the one hand led to higher concentrations of IL-1 alpha and FGF basic, whereas the recovery of GRO alpha, IL-1 beta and IL-18 is reduced with the addition of EGCG. However, differences are only very little, and no valid conclusions can be drawn.

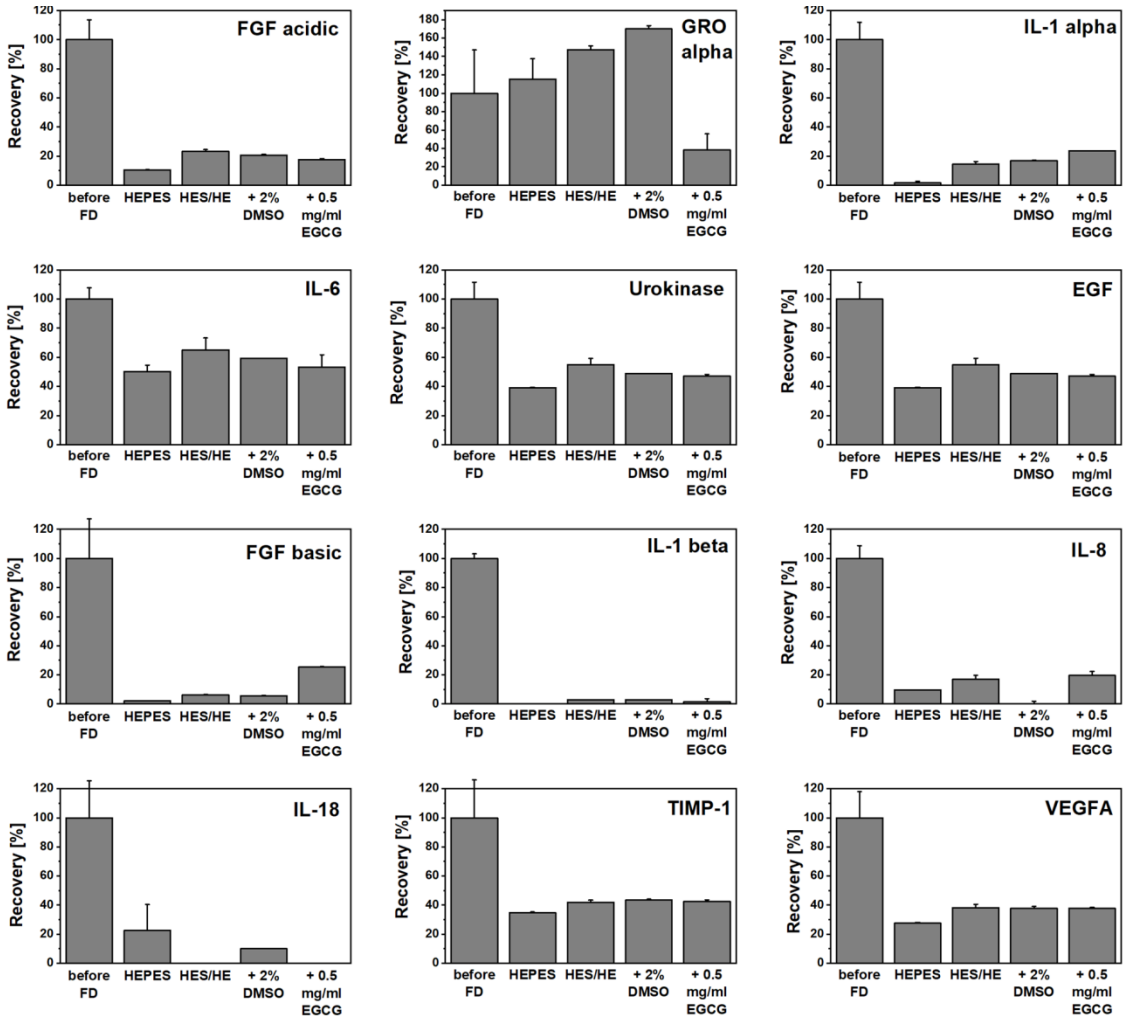

Figure VII-3. Multiplex analysis of 12 different cytokines and growth factors of cell lysates before and after lyophilization of adherent cells. Cells were freeze-dried in HEPES buffer, 10% HES, 5% HE with or without 2% DMSO or 0.5 mg/ml EGCG.

VII.3.2 *In vitro* Migration assay

We performed migration assays to measure any positive or negative impact of lyophilized cells on the migrative behaviour of adherent cells. To avoid additional strain on the lyophilized cells, to assure that no proteins are washed out and to mimic *in vitro* conditions, we added the reconstituted lyophilizates without additional washing step. HaCaT keratinocytes were freeze-dried in HEPES buffer without excipients or in 10% HES and 5% HE with and without 2% DMSO. EGCG was not included, since it led to a complete growth inhibition, which was already described in Chapter VI. Cells were reconstituted in E7:HPW in a ratio of 1:1. Previous experiments showed (data not shown) that on the one hand reconstitution with 50% HPW is required to reduce the osmotic pressure of the resulting formulations. On the other hand reconstitution with 50% EpiLife/S7 is necessary to provide inevitable nutrients for cells to proliferate and migrate. Placebo samples without cells were freeze-dried and reconstituted accordingly.

Figure VII-4 depicts the time course of the gap closure after addition of respective formulations. It is notable that no difference for samples freeze-dried in HEPES buffer can be observed, whereas cells dried in 10% HES and 5% HE with or without 2% DMSO lead to a decelerated gap closure compared to placebo samples. Obviously, lyophilized cells did not enhance cellular migration, but impeded the migrative behaviour. We speculate that this might be due to a physical inhibition of cell growth by the lyophilized cells. After addition to the culture dish, the cells settled down into the cell free gap and physically hindered the surrounding cells to migrate into the gap. This may be the reason why cells lyophilized in HEPES buffer show no inhibiting effect, since only a small number of originally implemented cells can be retrieved after lyophilization without excipients. Thus the total number of cells that sink into the gap is limited.

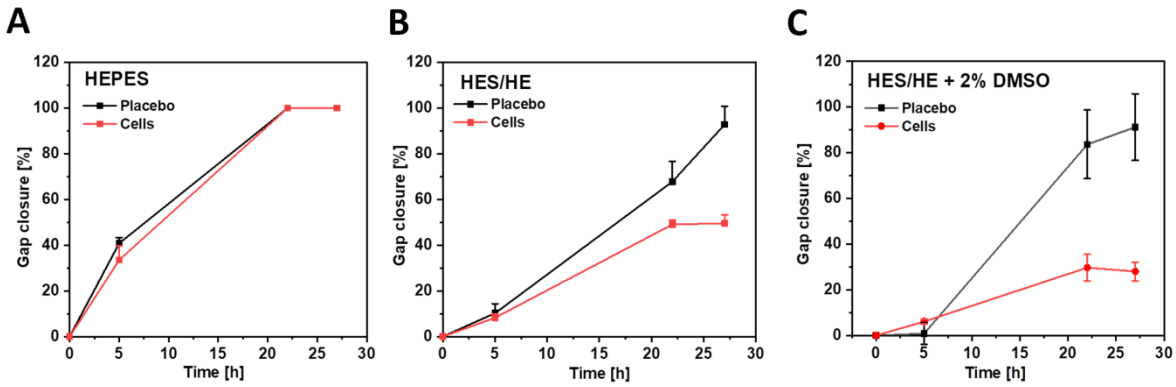

Figure VII-4. IBIDI migration assay: time dependent gap closure of HaCaT keratinocytes after addition of freeze-dried and reconstituted formulations with (red) and without cells (placebo, black). Freeze-dried in **(A)** HEPES buffer, **(B)** 10% HES, 5% HE in HEPES buffer, **(C)** 10% HES, 5% HE, 2% DMSO in HEPES buffer. All formulations were reconstituted in 50% EpiLife/S7 and 50% HPW.

Because of this potential limitation, we separated cells and cell supernatant after lyophilization and analyzed them separately. After reconstitution, samples were centrifuged and the resulting cell pellet was resuspended in 50% HEPES buffer and 50% EpiLife/S7 and was compared with the supernatant and a reconstituted placebo sample. Cell migration and gap closure were measured after the addition of respective formulations (Figure VII-5). As before, no difference for samples dried only in HEPES buffer was observed, whereas the cell pellet for samples dried with excipients led to delayed cell growth and migration. Interestingly, a comparison of the supernatant and placebo samples showed that the gap closure is accomplished faster, if cells were dried in presence of HES/HE and/or DMSO, which implies that the supernatant induces cell migration. One explanation is that proteins that leaked from cytoplasm are stabilized by the excipients and are still intact after lyophilization, thus inducing cell migration. The supernatant of samples dried only in HEPES buffer showed no effect, because without excipients proteins are not stabilized, degrade and lose their activity.

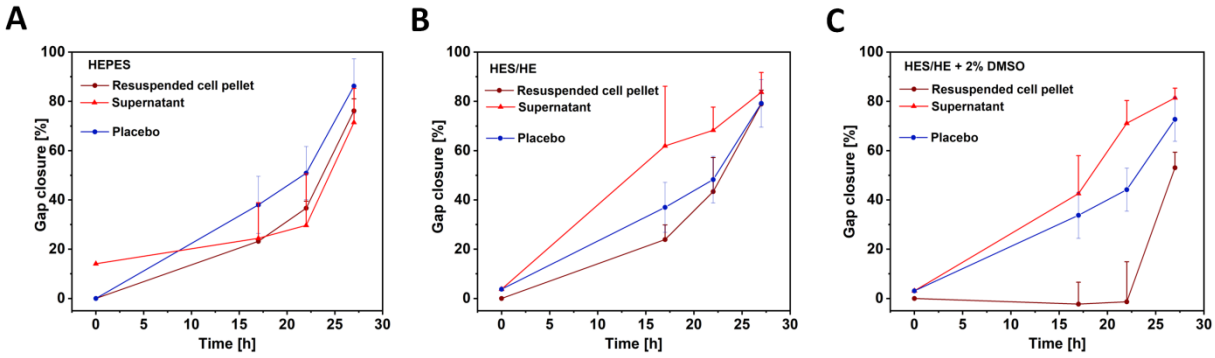

Figure VII-5. IBIDI migration assay: time dependent gap closure of HaCaT keratinocytes after addition of freeze-dried and reconstituted formulations. Freeze-dried in **(A)** HEPES buffer, **(B)** 10% HES, 5% HE in HEPES buffer, **(C)** 10% HES, 5% HE, 2% DMSO in HEPES buffer. All formulations were reconstituted with 50% EpiLife/S7 and 50% HPW and cell samples were centrifuged and separated into cell pellet and supernatant. The cell pellet was resuspended in 50% EpiLife/S7 and 50% HPW. Cell pellet (dark red circles), supernatant (red triangle) and placebo (blue square).

To summarize, without stabilizing excipients on the one hand cells get highly damaged, in a way that migration is less strongly inhibited by physical hindrance. On the other hand also proteins that leaked from the ruptured cells are not stabilized and degrade. This could be the explanation, why no significant difference between pellet, supernatant and placebo samples can be detected for samples dried in HEPES buffer, whereas for samples with excipients the cell pellet inhibits and the supernatant induces cell migration.

To analyse if the supernatant contains active ingredients, we upconcentrated the supernatant of reconstituted cells and analyzed the protein pattern. Figure VII-6 shows a SDS-PAGE with subsequent staining of the protein bands for the supernatant and the corresponding cell lysate of cells that were dried in HEPES buffer or 10% HES and 5% HE with and without 2% DMSO.

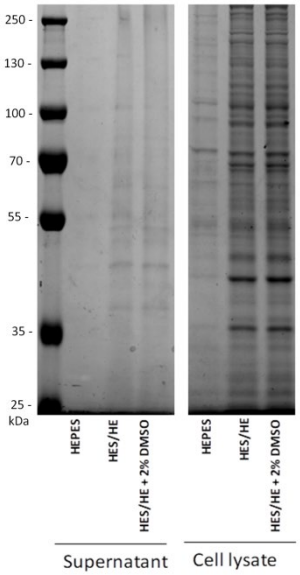

Figure VII-6. Protein pattern of lyophilized cells and the upconcentrated supernatant after reconstitution. Proteins were separated in a 10% Tris-Glycine gel under reducing conditions and stained with Brilliant Blue G. Cells were freeze-dried in HEPES buffer, 10% HES and 5% HE in HEPES buffer with and without 2% DMSO.

Clearly, the supernatant contains a broad range of proteins that show a similar pattern than the proteins from the cell lysate. We suppose that these proteins contributed to the accelerated gap closure and migration induction of adherent cells after addition of the supernatant of lyophilized cells. Only for the supernatant of cells dried in HEPES buffer, almost no proteins were recovered after upconcentration and subsequently also only few bands were detected, which supports the theory that proteins degrade without stabilizing excipients.

The performed migration assays showed that the addition of lyophilized cells has a negative influence on cell migration and proliferation of adherent cells. However, distinction between cell pellet and supernatant revealed a migration inhibition only for the cell pellet but not for the supernatant. Moreover, the supernatant seem to slightly induce cell migration and SDS-PAGE could prove that a variety of proteins can be detected in the supernatant, which might be the reason for enhanced cell migration and proliferation.

VII.3.3 *In vivo* experiments

To draw valid conclusions regarding the effect of lyophilized keratinocytes, *in vivo* studies represent the gold standard. We therefore tested lyophilized cells on a porcine wound model and measured reepithelialisation and formation of new epidermal and dermal tissue after application of freeze-dried keratinocytes compared to placebo samples. HaCaT keratinocytes were used as model cell line for all preceding experiments. However, to avoid any interference of the genetically modified HaCaT cell line with the porcine skin cells, isolated primary keratinocytes from an adult donor were used for *in vivo* experiments.

It was our aim to develop lyophilizates that can be applied directly on the wound bed without preceding reconstitution or handling step. This requires stable lyophilizates that can be easily removed from their primary container to get applied directly on the wound. To fulfil these requirements, we freeze-dried cells in glass dishes with a diameter of 30 mm and a filling volume of 1 ml, resulting in a theoretical filling height of 1.4 mm (Figure VII-7 A). A stable and intact cake without any cracks is essential to remove it from the glass dish in one entire piece. As described in Chapter IV, the addition of 2% DMSO reduced cake cracking and enables therefore optimum handling (Figure VII-7 B). With the addition of DMSO we could obtain elegant cakes without cracks that are stable enough to allow easy removal and handling of the lyophilized product (Figure VII-7 C). The lyophilizates were applied directly on the dermatome wounds and covered with a secondary wound dressing (Figure VII-7 D and E).

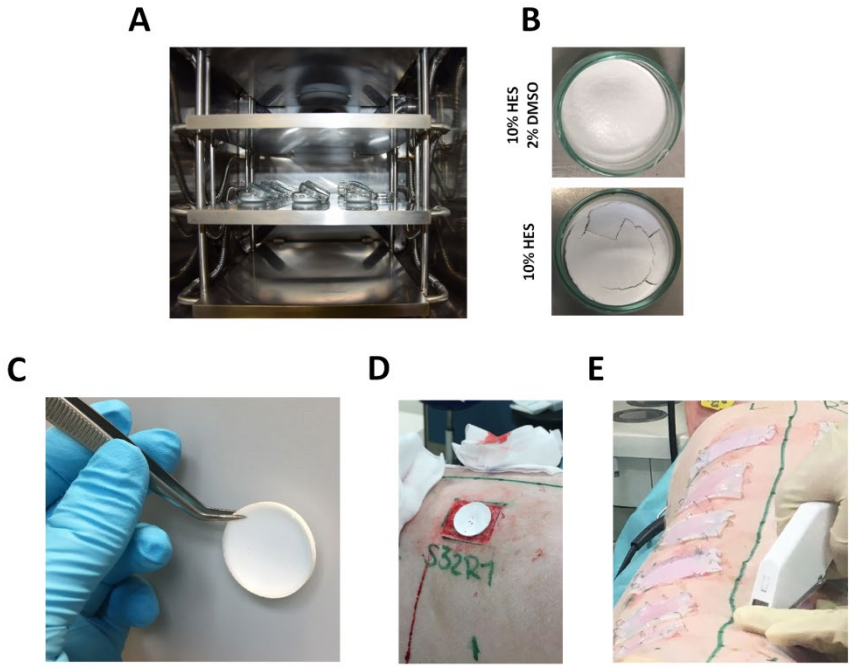

Figure VII-7. (A) Freeze-drying samples in glass dishes. **(B)** Representative images of freeze-dried samples containing 10% HES with or without 2% DMSO. **(C)** Handling of a lyophilized sample. **(D)** Application of the lyophilized sample directly on the wound bed. **(E)** Coverage of the wound bed with the secondary dressing Epicite^{hydro}.

Figure VII-8 shows the wounds directly after wounding and after 7 days of treatment.

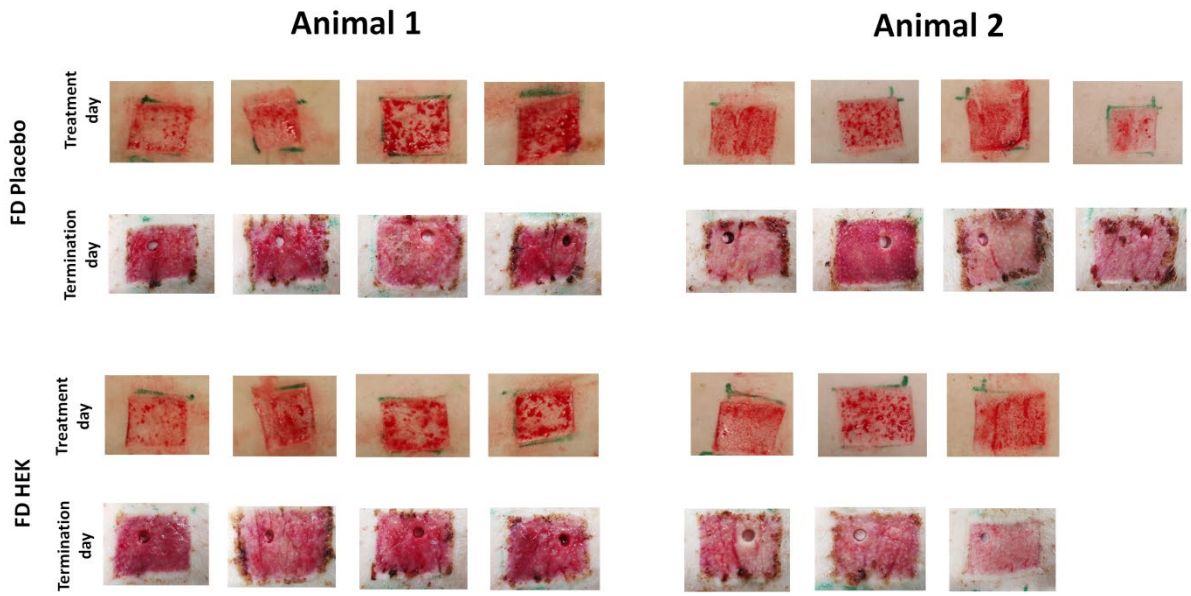

Figure VII-8. Dermatome wounds directly after wounding and after 7 days of treatment with placebo samples or lyophilized HEKs.

A biopsy was taken from each wound and analyzed regarding reepithelialisation and area of new epidermal or dermal tissue (see Figure VII-9). The treatment with lyophilized cells enhances reepithelialisation and increases also the newly formed dermal and epidermal skin area. The differences in the area of new epidermal tissue are rather small, whereas the area of new dermal

tissue could be increased by 45 % and reepithelialisation by 26% with the treatment of freeze-dried keratinocytes compared to placebo samples. These results demonstrate that the healing process could be accelerated after the addition of lyophilized primary keratinocytes. When the indifferent results from the *in vitro* experiments and protein analysis are considered, these are especially remarkable findings. However, only one single formulation (10% HES, 2% DMSO in HEPES buffer) was tested. Future experiments may reveal difference between cells dried with additional EGCG or without DMSO, if there are any.

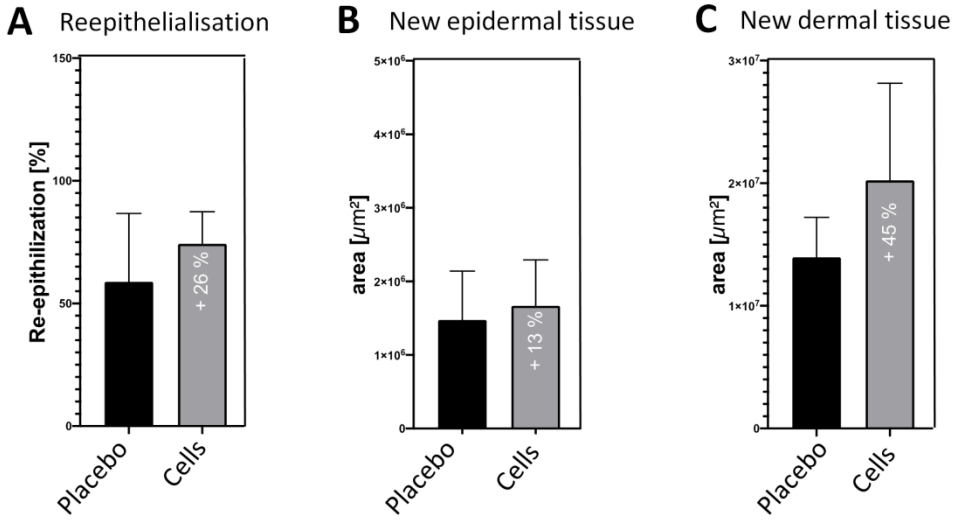

Figure VII-9. *In vivo* porcine wound model after application of lyophilized primary keratinocytes (black bars) and placebo samples (grey bars) after 7 days of treatment. Histopathological and microscopic analysis of **(A)** Reepithelialisation, **(B)** new epidermal tissue and **(C)** new dermal tissue. Cells were freeze-dried in 10% HES and 2% DMSO in HEPES buffer.

VII.4 Discussion

In comparison with confluent sheets of cultured epithelial autografts, keratinocytes applied in cell suspensions offer simple handling and drastic cost reduction and many researchers reported successful treatment of (burn) wounds with sprayed keratinocyte suspensions [20]–[23]. However, keratinocytes must be freshly isolated, thus requiring complex preparation and immediate use. It was our aim to overcome these problems and to stabilize keratinocytes in a dry state in a way that they can be transported and stored until use, without losing the simplicity of keratinocyte suspensions. In this chapter we investigated if lyophilized keratinocytes can be used as therapeutic agent for (burn) wounds and studied the effect of freeze-dried keratinocytes to accelerate cell migration and wound healing. Table VII-2 summarises the formulations that were used for the described multiplex analysis, *in vitro* and *in vivo* experiments.

Formulation	Multiplex analysis	Migration assay	Porcine wound model
HEPES buffer (25 mM, 150 mM NaCl, pH 8.0)	✓	✓	
10% HES, 5% HE in HEPES buffer	✓	✓	
10% HES, 5% HE, 2% DMSO in HEPES buffer	✓	✓	
10% HES, 2% DMSO in HEPES buffer			✓
10% HES, 5% HE, 0.5 mg/ml EGCG in HEPES buffer	✓	✓ (Chapter VI)	

Table VII-2. Formulations that were used for freeze-drying cells and subsequent analysis of intracellular proteins (Multiplex assay), *in vitro* and *in vivo* experiments.

As a first step we analyzed a variety of proteins that are involved in the wound healing process and in keratinocyte migration and proliferation. With the multiplex technology we were able to simultaneously quantify twelve different proteins in the cell lysates of freeze-dried HaCaT keratinocytes and compared it with the concentrations prior to lyophilization. The majority of intracellular proteins could not be recovered after lyophilization and a decrease of more than 50% for most proteins was observed. However, proteins were still detectable in a significant amount. The lowest concentrations were found for cells dried in HEPES buffer, whereas no clear difference between the protein concentration of cells dried in 10% HES, 5% HE and with or without 2% DMSO or 0.5 mg/ml EGCG was measured.

Scratch assays are widespread accepted *in vitro* assays that measure cell migration and provide first information about a potential activity of the tested substance on cell migration and also wound healing [2]. To overcome the drawbacks of a self created scratch, we used culture dishes, which create a defined, reproducible cell gap after removal of a culture insert. Our experiments showed a growth inhibition by the lyophilized cells (dried in 10% HES, 5% HE, with or without 2% DMSO), whereas the corresponding cell supernatant led to a slight induction of cell migration. A performed SDS-PAGE detected a significant amount of proteins in the upconcentrated supernatant of freeze-dried cells, showing the same protein pattern than lyophilized cells. We assume that these proteins in the supernatant are responsible for the migration induction of the supernatant.

A migration assay can provide only preliminary information about wound healing. Therefore i*n vivo* experiments and wound models are still the gold standard for making statements about the

effectiveness of treatment options. In particular porcine wound models correlate strongly with the human skin and are especially valuable in terms of their significance. To assess the effect of our lyophilized products, we therefore performed porcine *in vivo* experiments with artificially wounded pigs. Furthermore, we developed a new wound cover which can be applied directly on the wound bed without preceding reconstitution step. This is especially worth mentioning, as lyophilizates usually require complex handling and preparation prior to use. Cells and placebo samples were freeze-dried in glass dishes and stable lyophilizates were obtained that can be removed in an entire piece from the glass dish and placed on the wound bed. This facilitates handling of lyophilized products and is an important step to a more widespread application of freeze-dried pharmaceuticals. In the performed *in vivo* experiment, lyophilized primary cells showed accelerated reepithelialisation and formation of more epidermal and dermal tissue, compared to placebo samples. Despite limited recovery of intracellular proteins and a very limited growth induction in *in vitro* assays, we could show a remarkable *in vivo* activity which proves our concept that keratinocytes can be stabilized during freeze-drying in a way that their ability to enhance wound recovery is preserved. We hope that freeze-dried cells and in particular keratinocytes will contribute to a more efficient treatment of burn wounds and other skin lesions.

VII.5 References

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Chapter VIII

Summary of the thesis

The scope of this thesis is described in **Chapter I** and was to develop a lyophilization process to freeze-dry human keratinocytes as novel treatment option for burn trauma and other skin lesions. It was our aim to obtain cells with intact relevant proteins and sufficient *in vitro* and *in vivo* activity, without the particular requirement to achieve proliferation or "living cells".

Chapter II contains the general introduction of the thesis with the objective to present the current status in the field of (freeze-) drying cells. Until now, no general lyophilization process exists and formulation and process parameters need to be experimentally defined for every cell type. Trehalose is inevitably the most common excipient when it comes to (freeze-) drying, however, trehalose cannot enter the cytoplasm and must be technically loaded intracellularly to take full advantage of its stabilizing properties. This was achieved by endocytotic or receptor mediated uptake, gene transfection or pore formation, all resulting in varying intercellular trehalose concentrations. Besides trehalose, other sugars, macromolecules, polyols and antioxidants dominate the broad field of excipients. Furthermore, the success of stabilizing cells in the dry state varies for different cell types. For microorganisms it was possible to obtain cells with the ability to form colonies and to proliferate even after lyophilization and reconstitution. Spermatozoa without intact cell membranes or motility were shown to direct embryonic development, followed by live offspring of mice and even foals. Platelets were able to maintain their physiological functionality and could elicit a response to aggregation agonists after freeze-drying. The most complex and therefore most difficult cells to stabilize are eukaryotic cells and only stem cells were shown to proliferate after lyophilization.

The following chapter (**Chapter III**) describes the specification of basic lyophilization parameters that were applied for future experiments. It was shown that rapid freezing drastically reduces cell membrane integrity as well as recovery rate and that an intermediate freezing rate (1 K/min) is preferred. Reconstitution with 1.5 x volume of cell culture medium or HPW was identified as best rehydration conditions, whereas pre-hydration with water vapour showed detrimental effects. No correlation between cell concentration and membrane integrity rate was detected and also growth density, cellular doubling time and passage number showed no major influence on cell recovery and membrane integrity after freeze-drying. Furthermore we were able to replace the multi component cell culture medium EpiLife as basis for the freeze-drying formulation by 25 mM HEPES buffer, containing 150 mM NaCl (pH 8). All defined parameters are summarized in Table VIII-1 and were applied for further studies.

Parameter	Applied condition
Calcium	No additional Calcium
Cell density	0.5 – 1·10^6 cells/ml
Freezing rate	1 K/min
Rehydration	1.5 x volume, 37°C, EpiLife/S7 or HPW
	no pre-hydration
Basis buffer	25 mM HEPES with 150 mM NaCl (pH 8)
	EpiLife/S7

Table VIII-1. Applied cultivation, lyophilization and reconstitution conditions for freeze-drying HaCaT keratinocytes.

In **Chapter IV, V** and **VI** we analyzed different excipients and their effect on freeze-drying cells after addition to the lyophilization formulation. **Chapter IV** focused on DMSO, which is commonly known as cryoprotectant. Freeze-drying of HaCaT keratinocytes in the presence of DMSO led to an increase in cell membrane integrity from 25.3 ± 2.7% without DMSO to 41.4 ± 4.3% with 2% DMSO, which could be maintained for a wide range of residual moisture contents. Visualization of the cell membrane confirmed these findings and DMSO also increased the total protein content of cells after freeze-drying. Despite that a significant part of DMSO was not removed during freeze-drying, no macroscopic or microscopic collapse was observed by SEM or BET measurements. A Tg above 60°C for 2% DMSO indicates that samples can still be stored at temperatures of 2-8 °C. However, the following stability study (**Chapter V**) revealed a drastic decrease in cell membrane integrity for samples with 2% DMSO within the observed storage time of 8 weeks at 2-8°C. To our surprise, the lyophilizates were stable from a physicochemical point of view, since no changes in Tg, RM, SSA or SEM were detected. Obviously changes within the lyophilizates occurred which could not be detected with the applied physicochemical techniques.

(-)-Epigallocatechingallate (EGCG) is a potent antioxidant and the most abundant catechin that is found especially in green tea (*Camellia sinensis)*. Its potential to stabilize HaCaT keratinocytes during freeze-drying was studied in **Chapter VI**. The addition of 0.5 mg/ml EGCG to the lyophilization formulation led to an increase in cell membrane integrity from 23.1 ± 7.4% without EGCG to 69.5 ± 5.7% with EGCG. This was confirmed by reduced staining of cells with Ethidiumhomodimer-1 and furthermore the intracellular esterase activity was enhanced (measured with the addition of Calcein-AM). However, the mode of action remained uncertain and neither structural similar molecules nor the use of other antioxidants led to comparable results. Furthermore we could confirm a rapid degradation of EGCG at higher or neutral pH values and also observed a growth inhibition of HaCaT keratinocytes in presence of 0.5 mg/ml EGCG. These are limitations of EGCG that must be considered if it is used as lyoprotectant.

Chapter III – VI aimed to optimize a lyophilization formulation and process to enhance the condition of keratinocytes after lyophilization. Cell membrane integrity and the (structural) presence and activity of proteins were analyzed. Though, no answers regarding functionality or effect of the lyophilized cells as therapeutic option were made. **Chapter VII** therefore addresses the question whether lyophilized keratinocytes contain essential growth factors and cytokines that are important for wound healing processes. Multiplex analysis of twelve relevant proteins confirmed that up to 50% of cellular proteins could be retained intracellularly after lyophilization. Furthermore *in vitro* and *in viv*o activity of freeze-dried keratinocytes was studied. 2D migration assays were performed to measure the migration induction of lyophilized cells on adherent keratinocytes, but only little effect was observed. However, *in vivo* experiments and wound models are the gold standard for making statements about the effectiveness of treatment options. In particular porcine wound models correlate strongly with the human skin and are therefore especially valuable in terms of their significance. To assess the ability of our lyophilized products to improve wound healing, we performed pivotal porcine *in vivo* experiments with artificially wounded pigs. We could report accelerated reepithelialisation and formation of new epidermal and dermal tissue for lyophilized primary keratinocytes compared to placebo samples. These findings confirm that even if cells are severely damaged they still exhibit important activity in an *in vivo* wound healing model. It can be therefore concluded that it is not necessary to obtain in particular viable or proliferating cells after lyophilization, as it is sufficient to preserve relevant proteins and to maintain cell membrane integrity to a high extent. Furthermore, we established lyophilizates that can be applied and dissolved directly on the wound bed. This is especially worth mentioning, as lyophilizates usually require complex handling and preparation prior to use. The established lyophilizates enable therefore facilitated handling, which is an important step to a more widespread application of freeze-dried pharmaceuticals.

Taking together the conclusions of all chapters, we screened numerous excipients and determined DMSO and EGCG as promising compounds to stabilize HaCaT keratinocytes in the dry state, even if they have certain drawbacks that need to be considered. A lyophilization process was established that produces lyophilizates that were successfully tested on an *in vivo* porcine wound model and which can be applied directly on the wound bed without prior handling step. However, we want to emphasize that freeze-drying cells is a challenging field of science and that no proliferating cells were obtained with the described lyophilization process and further research is required to eventually achieve this ambitious goal.

Chapter IX

Appendix

IX.1 List of Abbreviations

2,3-DPG	2,3-diphosphoglycerate
AD	Air Drying
ATP	Adenosintriphosphate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CA	California
Calcein-AM	Calcein-Acetoxymethyl Ester
CAM	Calcein-Acetoxymethylester
DAPI	4′, 6-Diamidino-2-Phenylindole
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
E. coli	Escherichia coli
ECG	(-)-Epicatechingallate
EDTA	Ethylenediaminetetraacetic Acid
EGCG	(-)-Epigallocatechingallate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EH	Ethidiumhomodimer-1
EpiLife/S7 or E7	Epilife medium with Supplement S7
FBS	Fetal Bovine Serum
FD	Freeze-drying
FGF	Fibroblast Growth Factor
FIJI:	Fiji Is Just ImageJ
FPE	Fluid Phase Endocytosis
g H_2O/g dw	gram water per gram dry weight
GA	Gallic acid
GC-MS	Gas Chromatography-Mass Spectrometry
HaCaT	Human Adult Low Calcium High Temperature
EGC	(-)-Epigallocatechin
GRO	Growth Regulated Peptide
HB-EGF	Heparin-binding EGF-like growth factor
HE	Hydroxyectoine
HEK	Human Epidermal Keratinocytes

HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HES	Hydroxyethyl Starch
HPC	Hematopoetic Progenitor Cell
HPLC	High Performance Liqid Chromatography
HPW	Highly Purified Water
HSA	Human Serum Albumin
HSC	Hematopoetic Stem Cell
HSP	Heat Shock Protein
ICSI	Intracytoplasmic Sperm Injection
IL	Interleukin
kDa	kilo Dalton
LAB	Lactid Acid Bacteria
Lb	Lactobacillus
LD_{50}	Median Lethal Dose
LEA	Late Embryogenesis Abundant proteins
LM	Liquid Nitrogen
MA	Massachusetts
Mb	Megabases
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MMP	Matrix Metalloproteinase
MNC	Mononuclear Cell
MO	Missouri
MPN	Most Probable Number
MSC	Mesenchymal Stem Cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular Weight
NFSM	Non Fat Skim Milk
NY	New York
OD	Optical density
PA	Pennsylvania
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PVP	Polyvinylpyrrolidon

RBC	Red Blood Cell
RIPA	Radioimmunoprecipitation Assay
RM	Residual Moisture
ROS	Reactive Oxygen Species
RP-HPLC	Reversed Phase-HPLC
RT	Room Temperature
SD	Spin-drying
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
SSA	Specific Surface Area
TBA	tert- Butanol
TBST	Tris Buffered Saline with 0.1% Polysorbate 20 (Tween 20)
Tg	Glass transition temperature
Tg’	Glass transition temperature of the maximally freeze concentrated solution
Tm	Phase transition temperature of biological membranes
Tre	Trehalose
VD	Vacuum-drying
VEGF	Vascular Endothelial Growth Factor

IX.2 List of publications and presentations associated with this thesis

IX.2.1 Publications

C. Müller, A. L. Wagner, U. Rockinger, G. Winter, F. Bracher

"Development of a convenient method for the determination of dimethyl sulfoxide in lyophilised pharmaceuticals by static headspace gas chromatography-mass spectrometry"

Analytical methods, pp. 4–7, 2019.

U. Rockinger, M. Funk, G. Winter

"Current approaches of preservation of cells during (freeze-) drying"

J Pharm Sci., accepted manuscript

U. Rockinger, C. Müller, F. Bracher, M. Funk, G. Winter

"DMSO as new, counterintuitive excipient for freeze-drying human keratinocytes"

Eur J Pharm Sci., vol. 160, 2021.

IX.2.2 Poster Presentations

U. Rockinger, M. Funk, G. Winter

"Lyophilization of human keratinocytes as a novel treatment for burn traumata"

Short Course and Conference, 2018, Garmisch-Partenkirchen, Germany

U. Rockinger, M. Funk, G. Winter

"Lyophilization of human keratinocytes for a novel treatment of burn traumata"

11th Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology (PBP) World Meeting, 2018, Granada, Spain