Semisolid formulations based on solid-in-oil-in-water systems containing proteins

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In recent years, improvements have been made, through biotechnological processes, in the production and development of peptides capable of increasing collagen and elastin synthesis for anti-aging skin care. However, proteins have many limitations due to their structural, chemical and physical fragility to external aggressions, which may cause conformational changes, leading to loss of biological activity. Therefore, it is important to create delivery systems that protect these biomolecules from damage, allowing them to reach their target. This work aimed to develop a system able to carry bovine serum albumin (BSA), used as a model of a protein, and to incorporate this system in a semisolid formulation suitable for skin application. A microemulgel based on a solid-in-oil-in-water (S/O/W) microemulsion was prepared. Firstly, the association efficiency (AE) of lyophilised BSA-sucrose ester complex and the size of S/O nanodispersion were assessed; then, the characterization and stability evaluation of the final semisolid formulation through evaluation of pH, texture and rheological behaviour were performed. The average value of AE was 54.74% ± 2.17. It was possible to develop an S/O/W microemulsion, which allowed the subsequent development of an S/O/W microemulgel that assured suitable pH, texture and rheological characteristics for skin application.

Keywords: Microemulsion. Nanodispersion. Proteins. Peptides. Emulgel.

INTRODUCTION

Skin aging corresponds to a series of biochemical processes that lead to a gradual accumulation of internal and external damage. Generally, this damage becomes visible on the surface of the skin as time progresses. Because skin is the most important barrier against physical and chemical threats, the risk of developing diseases such as skin cancer increases as skin ages (Addor, 2018; Ziegler, Wiley, Velarde, 2015).

Intrinsic and extrinsic factors are at the origin of skin aging. Intrinsic changes in skin reveal a fine atrophy, with fine wrinkles, adipose tissue loss, low elasticity and predominant dryness that result from the natural process of long-term physiological alterations: alterations due to ethnic or genetic makeup, hormonal alterations, and similar biological processes. These types of factors are mainly responsible for oxidative processes like mitochondrial dysfunction, alteration in intracellular communication, genetic instability, cellular senescence and extracellular matrix deterioration. Reactive oxygen species (ROS) are the main substances responsible for these changes because ROS are secondary products of the mitochondrial aerobic processes (Ziegler, Wiley, Velarde, 2015).

In contrast, the aging resultant from high solar radiation exposure (a main extrinsic factor) produces skin with more deep wrinkles, increased thickness of the epidermis, loss

of shine, a rough surface and discoloration (Zhang, Duan, 2018). Other examples of extrinsic factors responsible for aging are exposure to cigarette smoke and pollution. Solar exposure is considered one of the primary causes of this phenomenon, which is also called photoaging.

Small peptide fragments are capable of inducing synthesis of extracellular matrix proteins by signalising the cells. This peptide-induced extracellular matrix-restoring mechanism naturally occurs in other tissues including the vasculature and also in wound healing. They act as 'matrikines' that consist of peptide fragments naturally produced during the processing of proteins, namely, collagen and elastin, which can act as signalling intermediates that stimulate cells to increase synthesis of those proteins (Aldag, Nogueira Teixeira, Leventhal, 2016). The KTTKS (Lys-Thr-Thr-Lys-Ser) pentapeptide is the carboxylic end of the collagen type I propeptide, which is cleaved during mature collagen production (Aldag, Nogueira Teixeira, Leventhal, 2016). The procollagen I-derived pentamer KTTKS stimulates the production of collagen types I and III and fibronectin *in vitro* in a dose- and time-dependent manner, with no effect on total protein synthesis or on the ratio of secreted proteins. However, by adding palmitoyl to the peptide fragment to overcome permeability issues, Pal-KTTKS caused visible improvement of fine lines and wrinkles in a 12-week clinical test when compared to placebo (Abu Samah, Heard, 2011; Aldag, Nogueira Teixeira, Leventhal, 2016).

Bioactive tetrapeptide GEKG (Gly-Glu-Lys-Gly) is also tough to be involved in these types of processes. It is included in a large number of extracellular proteins and, therefore, acts through 'matrikine' signalling (Aldag, Nogueira Teixeira, Leventhal, 2016; Schagen, 2017).

Nanotechnology has been largely used in the formulation of cosmetics to improve their stability and functionality, as well as to guarantee higher chemical and physical stability of the active ingredients. The most important advantages of nanotechnology in cosmetics include an increase in the stability of cosmetic ingredients that may be destroyed due to oxidation and other causes, as this destruction is usually observed for substances such as vitamins, unsaturated fatty acids, antioxidants, proteins, among others. These systems also improve the efficiency of cosmetic ingredients and provide more control over these ingredients' release, achieving a long-term effect (Santos *et al.*, 2019). Additionally, nanotechnology can be applied to increase the absorption of cosmetic ingredients through the skin due to the reduced size of nanosystems and their physical and chemical properties (Nastiti *et al.*, 2017).

Nanodispersions are dispersions at the nanometric scale that can be used as systems for the administration of a variety of substances. For example, solid-in-oil nanodispersions constitute convenient and safe systems for the dermal administration of peptides and proteins (Martins *et al.*, 2016). These systems are based on coating proteins with a surfactant to form a protein–surfactant complex and enable proteins to retain their composition and structural integrity. In comparison to most aqueous systems, nanodispersion of the complex in the oil phase increases permeation through the skin without the need for physical enhancers or pretreatments (Kitaoka *et al.*, 2016). This complex is easily dispersed in non-aqueous solvents, such as isopropyl myristate (IM), which also increase the cutaneous permeation of formulations (Zidan *et al.*, 2017). Tahara *et al.* (2008) demonstrated in previous studies that solid-in-oil nanodispersions of hydrophilic drugs effectively enhanced the permeation of proteins into the skin.

MATERIAL AND METHODS

Material

Bovine serum albumin (BSA): (Sigma-Aldrich®, Spain); Chloroform: (VWR Prolabo Chemicals®, France); Gelifiant PFC: (Guinama®, Spain); Glycerine: (Acofarma®, Portugal); Imidazolidinil Urea: (Guinama®, Spain); Isopropyl myristate: (Fagron®, Spain); Nipagin: (Acofarma®, Portugal); Stearic acid: (Acofarma®, Portugal); Sucrodet Sucrose Ester SP30-C (E473): (Sisterna®, Netherlands); Triethanolamine: (Acofarma®, Portugal); Tween® 80: (Acofarma®, Portugal); Ultrapure water: Laboratory of Pharmaceutical Technology of FFUP (Portugal).

Methods

Bovine serum albumin (BSA) was used as a model of a protein to create the protein-surfactant complex. BSA

is widely used as a standard protein because of its size (MW: 66 kDa), stability, solubility in water and globular protein, and affordability and ease of accessibility.

Preparation of S/O nanodispersion

A BSA solution was prepared with a concentration of 10 mg/mL, dissolving 250 mg of BSA in 25 mL of ultrapure water. The sucrose ester (SP30-C) solution (5%, w/v) was prepared dissolving 2.5 g of this surfactant in 50 mL of chloroform. The preparation that resulted from mixing the two solutions was first homogenised by mechanical agitation using an Ultra-Turrax® to obtain a primary emulsion and second by ultrasonication to obtain the final water-in-chloroform nanoemulsion. The sucrose ester stays in the interface of the chloroform and aqueous solution of BSA. The homogenisation with Ultra-Turrax® was performed at 8000 rpm for 2 min. Ultrasonication was performed using a probe of 6 mm diameter at 70% of amplitude for 15 min, with a beaker containing the dispersion immersed in an ice bath to avoid high temperatures resulting from the process, which could deteriorate the protein (Leong, Martin, Ashokkumar, 2017). The lyophilisation of the water-in-chloroform nanoemulsion was performed over 24h, and the obtained final product consisted of a white powder, which contained BSA mixed with the SP30-C sucrose ester. Then, 2.2% of the lyophilised BSA-sucrose ester complex was dispersed in 25% of IM by ultrasonication for 10 min, submerging the beaker with the dispersion in an ice bath, again to avoid protein deterioration due to high temperatures. After this process, a solid-in-oil (S/O) nanodispersion was obtained.

Association efficiency

The association efficiency test was performed using a method that comprises the extraction of BSA that was not associated with the surfactant in the S/O dispersion. Water was added to the S/O dispersion and mixed for 1 min using an Ultra-Turrax®. Centrifugation was then performed for 30 min at 3000 x g (4302 rpm) using an Eppendorf™ 5804 Centrifuge.

After centrifugation, the amount of free BSA, which was in the aqueous phase, was determined in triplicate (n=3) by the BCA (bicinchoninic acid) quantification method. The BCA protein assay detects protein concentrations from 20 to 2000 µg/ml.. The working solution has an apple-green colour that turns purple at 37°C after 30 min in the presence of protein (Pierce, 2010). The BCA colour was determined at the wavelength of 562 nm using the Jasco® V-650 UV-VIS Spectrophotometer.

The S/O nanodispersion association efficiency (%) was calculated as follows (Pierce, 2010):

Association efficiency (AE)% = (([BSA]$_{total}$ - [BSA]$_{free}$)/ [BSA]$_{total}$) × 100

Preparation of S/O/W microemulsion

The S/O nanodispersion was emulsified first in ultrapure water containing 5% Tween® 80 using an Ultra-Turrax® for 5 min to obtain a primary emulsion and second with ultrasonication for 10 min to obtain the final S/O/W microemulsion.

Preparation of S/O/W microemulgel

The S/O/W microemulgel was prepared by gelification of the aqueous external phase of the S/O/W microemulsion. The gel formation started as the gelling agent PFC® was added, with a final concentration of 0.5%, to the S/O/W microemulsion. To obtain a stable gel it is important to guarantee a final pH of 7. Therefore, triethanolamine was slowly added until the desired pH was achieved. Imidazolidinyl urea was also added in the concentration of 0.5% as a preservative.

Texture analysis

For texture analysis, a Stable Micro Systems TA-XT2i texture analyser was used in the compression mode to perform a penetration test using a 13-mm cylindrical probe. Tests were done in triplicate (n=3) at room temperature after 8, 15, 30 and 60 days of the preparation of the semisolid formulations, with a load cell of 5 kg, a trigger force of 0.05 N, a penetration depth of 5 mm and

a test speed of 3 mm.s^{-1}. Texture analysis was conducted by analysing adhesiveness and firmness of semisolid formulations stored at 20°C and 34°C for 60 days.

The statistical analysis was managed with GraphPad Prism® software by performing a multiple *t*-test for each parameter. For that, the Holm–Sidak method was used to determine statistical significance, and each row was individually analysed without assuming a consistent standard deviation.

Rheology analysis

The rheological behaviour of semisolid formulations stored at 20°C and 34°C for 60 days was evaluated using a Thermo HAAKE™ Viscotester™ 550 concentric cylinder viscometer. The analysis was performed at 20°C using shear rates from 1 s^{-1} up to 500 s^{-1} and then from 500 s^{-1} down to 1 s^{-1}.

RESULTS

Association efficiency

The association efficiency mean value of 54.74% (± 2.17) showed that BSA is more than half-associated with the sucrose-ester. Some of the BSA was potentially not released from the complex during the process of centrifugation. The possibility of BSA being destroyed during lyophilisation can also be considered.

Texture analysis

As described before, texture analysis was conducted by analysing firmness and adhesiveness of semisolid formulations stored at 20°C and 34°C for 60 days, respectively represented in Figures 1 and 2.

The texture analysis of the S/O/W microemulgel at 20°C and 34°C showed a decrease of firmness with time. The firmness of the S/O/W microemulgel was not considerably different by changing the temperature of storage from 20°C to 34°C except after 60 days of storage, when firmness was 0.201 ± 0.008 N at 34°C and 0.227 ± 0.005 N at 20°C ($P \leq 0.05$) (Figure 1).

The adhesiveness of the S/O/W microemulgel decreased with time at both temperatures of storage. After 30 days, the adhesiveness was significantly lower at 34°C (-1.1095 ± 0.049 N.mm) than at 20°C (-1.408 ± 0.007 N.mm). After 60 days, the decrease of adhesiveness was even more significant at 34°C (-0.997 ± 0.015 N.mm) when compared to storage at 20°C -1.277 ± 0.030 N.mm ($P \leq 0.05$) (Figure 2). The decrease in both firmness and adhesiveness may be indicative of system instability because these characteristics were more pronounced in the case of the preparation stored at 34°C.
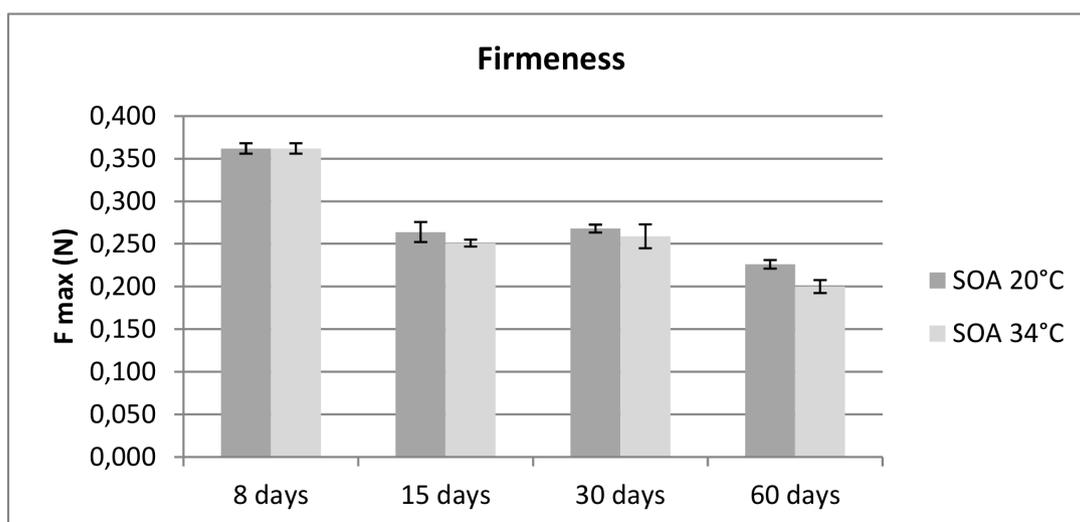
FIGURE 1 - Comparison of the firmness of the S/O/W microemulgel determined at 8, 15, 30, 60 days of storage at 20°C and 34°C.
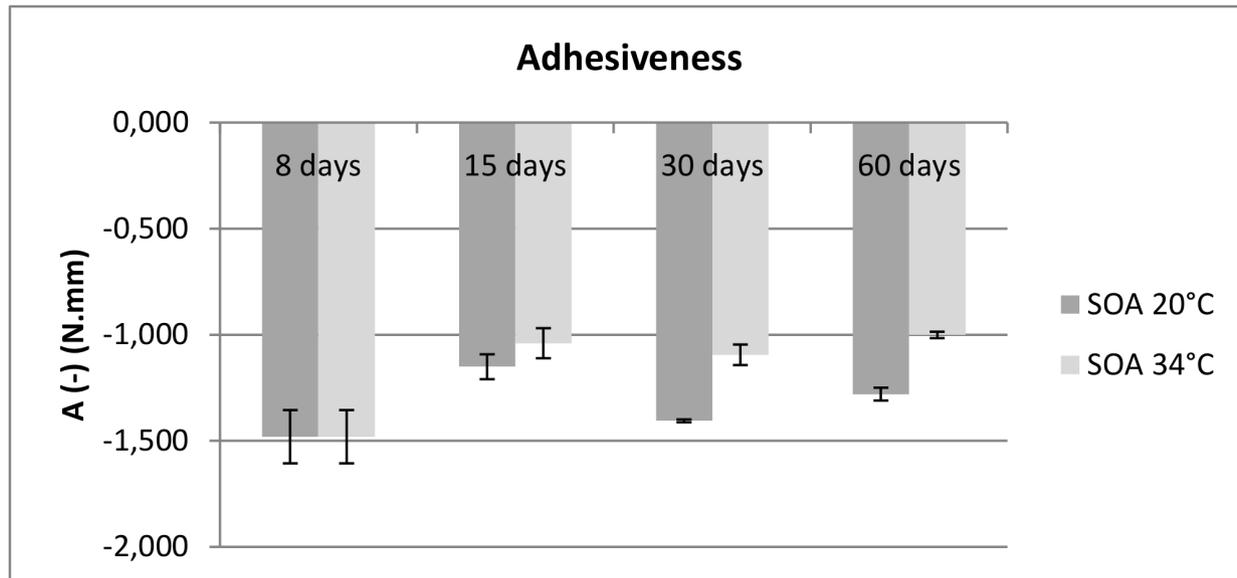
FIGURE 2 - Comparison of the adhesiveness of the S/O/W microemulgel determined at 8, 15, 30, 60 days of storage at 20°C and 34°C.

Rheology analysis

The S/O/W microemulgel stored at 20°C showed a decrease of viscosity with time, presenting a shear stress of 567 Pa after 8 days and 336.5 Pa after 60 days, at a maximum shear rate of 500 s^{-1} (Figure 3).

The S/O/W microemulgel stored at 34°C also exhibited a decrease of viscosity over time, showing a shear stress of 567 Pa after 8 days and 400.8 Pa after 60 days, at a maximum shear rate of 500 s^{-1}. However, the decrease at 34°C was surprisingly less pronounced than at 20°C.

Both S/O/W microemulgels at 20°C and 34°C revealed a plastic behaviour, as this formulation presented a decrease of viscosity with the increase of shear rate and a slight yield stress (Figure 4).

Non-Newtonian behaviours of the semisolid formulation stored at both temperatures were proven by the Herschel–Bulkley rheological model (Table I). The Herschel–Bulkley model agrees with the experimentally measured data over a whole range of tested shear rates, revealing the best approximation of all models tested (Table I).

All formulations showed a flow index (*n*) value below 1, which confirms shear-thinning behaviour. The formulation stored at 34°C had a higher viscosity after 60 days compared to the formulation stored at 20°C, and as a consequence, the stress required for the sample stored at 34ºC to begin to flow should be higher.
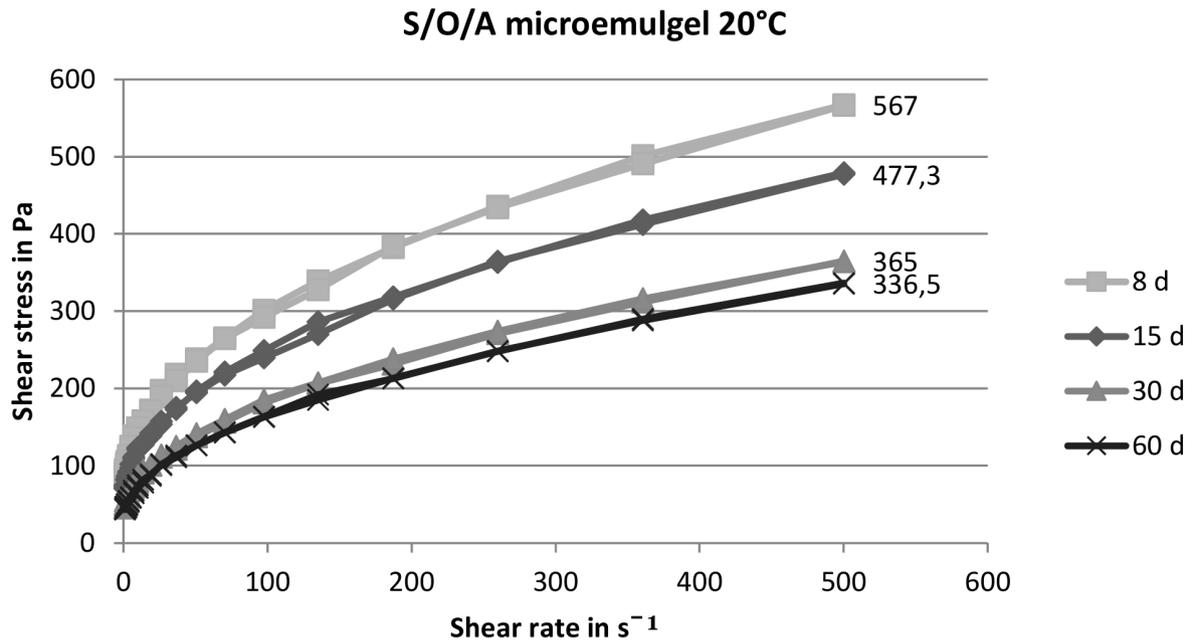
FIGURE 3- Rheograms of the S/O/W microemulgel stored at 20°C for 60 days.
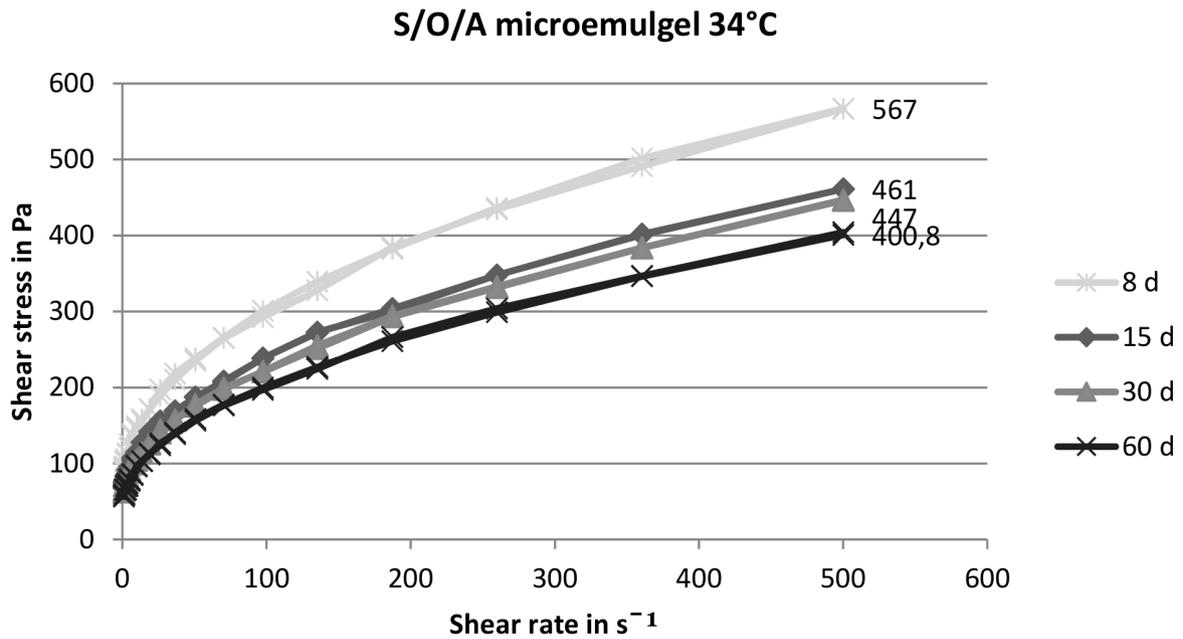
FIGURE 4 - Rheograms of the S/O/W microemulgel stored at 34°C for 60 days.

TABLE I - Herschel-Bulkley model parameters of S/O/W microemulgels after 8 and 60 days of storage at 20°C and 34°C

Herschel-Bulkley Model	S/O/W microemulgel 20°C	S/O/W microemulgel 34°C
8 days	30.601	30.601 K
	0.452	0.452 n
	58.439	59.439 τ_0
	0.9996	0.9996 R^2
60 days	13.307	16.169 K
	0.505	0.498 n
	29.335	43.070 τ_0
	0.9997	0.9998 R^2

CONCLUSIONS

In the present study, a complex BSA-sucrose ester was prepared via the ultrasound method, and subsequent lyophilisation of the ester into a stable and fine powder of the complex was employed to maintain the physical and chemical characteristics of the protein. The complex BSA-sucrose ester was then dispersed in an oil, and the obtained solid-in-oil nanodispersion was emulsified in water, originating a solid-in-oil-in-water (S/O/W) microemulsion. The association efficiency of the BSA-sucrose ester complex showed that BSA is more than half-associated with the sucrose ester, but some of the BSA may have been destroyed during the lyophilisation process. Later, a semisolid formulation, namely, an S/O/W microemulgel, was obtained by gelling the external phase of the S/O/W microemulsion and then storing the microemulgel at 20°C and 34°C for 60 days. After that, this formulation was characterised in terms of texture and rheology. It was found that the S/O/W microemulgel presented a plastic behaviour without thixotropy. However, the S/O/W microemulgel revealed slight destabilisation mainly caused by the apparent partial deterioration of the polymeric network of the gel. The evaluation of different concentrations of the gelling agent could determine the optimal concentration of this polymer to achieve the best textural and rheological characteristics.

The use of proteins in semisolid formulations creates several difficulties, and therefore, nano- and microsystems seem to be suitable solutions to improve protein bioavailability, distribution and safety. As a result, in the present study, a semisolid formulation based on a solid-in-oil-in-water microemulsion showed future potential for delivering peptides and proteins. This approach can be integral to anti-aging skin treatments, but it may also be useful to the vascular system and in wound healing.

In future approaches, it is important to use different methodologies to characterise the release capacity of the protein from the surfactant complex. It is also crucial to evaluate skin permeation of the complex and the complex's cytotoxicity using human skin equivalents. One further necessity, because biological activity belongs to the protein and not to the complex itself, is to verify the cosmetic effects of the anti-aging peptides or proteins when these compounds are delivered as a surfactant complex.

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