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DIFFERENT INORGANIC NANOPARTICLES AS ENHANCERS FOR DERMAL DELIVERY OF PROTEINS IN PHARMACEUTICAL AND COSMETIC PRODUCTS

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ABSTRACT

Objectives: In last decades, dermal delivery of drugs has attracted a great interest.In this study, calcium phosphate nanoparticles (CaP-NPs), zinc oxide nanoparticles (ZnO-NPs) and some organic solventswere used as combination enhancers for dermal delivery of albumin.

Methods: Formulations contained albumin, solvent, CaP-NPs and/or ZnO-NPs. Permeation of albumin was determined by an *ex vivo* (animal skin) method. Distribution of the NPs in the skin layers was depicted by optical microscopic method.

Results: Among solvents, liquid paraffin (LP) showed the greatest increase in albumin permeation (total permeation of 384 mg). CaP-NPs and ZnO-NPs also increased the albumin permeation (19 and 22 mg, respectively).Combination of these NPs and LP further increased the albumin permeation (470 and 487 mg, respectively).

Conclusion: All solvents increased albumin permeation while LP showed a dramatic increase. CaP-NPs or ZnO-NPs also increased the permeation. Enhancing mechanism of the NPs was attributed to their deposition through skin layers shown by images. Using a combination of CaP-NPs and/or ZnO-NPs with LP caused synergistic permeations following by a saturation ata high dose of the NPs. The study suggests simultaneous use of proper doses of such inorganic NPs with LP to considerably enhance dermal delivery of albumin or other proteins.

Keywords: Albumin; Calcium phosphate nanoparticles; Organic solvents; Skin permeation, Zinc oxide nanoparticles.

INTRODUCTION

Dermal route of drug administration possesses several advantages. Its advantages over oral administration are decreased or total avoidance of first-pass drug metabolism, bypassing absorption steps, effects of pH and enzymes and transit time.Its advantages over injection and other routes are non-invasive and controlled delivery and termination[1].

The most important barrier for transdermal drug delivery is the skin's horny layer or *stratum corneum* (SC). This layer must be altered for penetration of drugs through the skin. This topic has been the subject of research for pharmaceutical scientists especially during the two recent decades[2]. Extensive research on chemical penetration enhancers (CPEs) has been performed which form the main strategy of formulation-design approaches for dermal and transdermal drug delivery[3]. It is now well known that formulation components can improve the quantity and rate of transdermal absorption of drugs[4]. Permeation of a drug through the skin in the presence of a CPE is related to physico-chemical characteristics of the CPE and the drug[5-7]. Therefore, skin permeation of drugs varies in the presence of different CPEs.Thus each pair of drug-CPE should be examined separately.In this case, CPEs should construct a situation to make new skin microstructures. More than two hundred CPEs have been shown to enhance skin permeation of drugs[3]mainly including aliphatic acids, fatty acids, esters, alcohols, oils and terpenes[1].

One group of the CPEs are hydrophobic nanoparticles (NPs) made from lipids or hydrophobic polymers.The drug is trapped inside these NPs. The polymeric NPs should be evaluated in terms of safety, biocompatibility and especially degradation kinetics. Therefore, they should be accurately designed to become suitable for use in medications[8-10], such as designing their preparation methods in order to obtain suitable NP size, surface charge, and degradation mechanisms. Lipid NPs have similar steps.

Another group is inorganic Nps. Among them, titanium dioxide NPs have been introduced as dermal enhancer and its physicochemical optimization as an enhancer was investigated[11].In this study inorganic NPs including calcium phosphate nanoparticles (CaP-NPs) and zinc oxide nanoparticles (ZnO-NPs) were used as enhancers. CaP with molecular weight (MW) of 310.176 g/mol is a safe and inexpensive natural chemical containing calcium and phosphate which are essential nutrients[12]. It has excellent biocompatibility because of

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its chemical similarity to human hard tissues. CaP was used in nanoparticulate dispersed form as a carrier in biological systems, e.g. to transfer nucleic acids or other drugs[12-14]and beside that, CaP-NPs have been successfully used for transcutaneous vaccine delivery as carriers[14] which is the only study on the enhancing effect of CaP-NPs. Zinc is also a relatively inexpensive, biocompatible and non-toxic vital nutrient. It is proved that zinc has antioxidant and cytoprotective effects on skin keratinocytes in cell (HaCaT) culture[15,16]. In addition, ZnO with MW of 81.408 g/mol has been applied topically to heal wounds and for treatment of other skin disorders[15]. The skin distribution of zinc showed a peak in the epidermal layer decreasing toward the SC, but increased in the SC[17-19]. Similarly ZnO-NPs can not pass through the skin[20]. We have proved the enhancing effect of ZnO-NPs on skin penetration of ibuprofen and also on skin penetration of liquid enhancers in our previous works for the first time[21,22]. These findings highlight the lack of research on the enhancing effects ofthe CaP-NPs and/or ZnO-NPs in dermal drug delivery. Such NPs do not involve the problems mentioned above, in the third paragraph of introduction, about polymeric or lipid NPs. Here, drug was not confined within the inorganic NPs and the NPs were not used as drug carriers. Indeed, drug did not attach to or loaded into the NPs. Instead, the NPs were used simultaneously with the drug but separated from the drug in each formulation. In this order, the NPs positioned through the skin layers and therefore, could alter the skin microstructure helping the drug passing the skin. Such a method of using NPs as enhancer (simultaneous use with drug and without loading the drug), was successfully employed in our previous works[21,22]. Thus the NPs in this paper, have been used as penetration enhancers and not as carriers.

In this study, we decided to take advantage of another group of CPEs too, which are organic solvents. Solvents especially organic solvents have been widely studied and successfully used as CPEs in topical products. Among them, six solvents including sunflower oil (Su), olive oil (Ol), coconut oil (Co), liquid paraffin (LP), Dimethyl sulfoxide (DMSO) and Tetrahydrofuran (THF) were used in this study as CPEs[1,3,23,24]. The oils (Su, Ol and Co) were chosen becausein previous studies we found them as effective enhancers[21,22]. In addition, since they are natural and edible nutrient oils, they are completely safe.The rest of organic solvents (LP, DMSO and THF) were chosen becausethey are of the most famous enhancers used safely in tremendous FDA approved dermal products[1].

It seems that the enhancing mechanism of the inorganic NPs is to change the structure of the skin. Organic solvents were chosen because they have a mechanism different from the mechanism of the in organic NPs, which is related to their solvent properties. The reason of using such a combination of enhancers (inorganic NPs and solvents which have different mechanisms) was to obtain the maximum possible permeation of albumin and in fact, to understand whether the use of such combination would help in getting the highest permeation.

Fortunately the used inorganic NPs, zinc[7] and CaP, usually do not show toxicity [13,14]. Moreover, they can not practically and easily pass the skin[15,16,18,20]. As a result, they do not have the potential to cause toxicity. On the other hand, because the target organ is the skin bearing a permanent turnover, all materials accumulated in the skin (especially outer skin) will be removed along with the discarded skin within a few days.

Recently proteins have found extensive applications as therapeutic agents and, many protein- based active ingredients have been used in topical (dermal) pharmaceutical and cosmetic products for various purposes[25]. In this study, albumin was selected as the active ingredient and as a model for other therapeutic proteins. So the present research is an evaluation of the potential enhancing effect of the three types of enhancers, the solvents, CaP-NPs and/or ZnO-NPs on the dermal delivery of albumin. In contrast to the hydrophobic small molecule of ibuprofen (the drug studied in our previous article), albumin is a hydrophilic polypeptide with MW of 67 KDa leading to a different permeation condition.

On the other hand, unlike the proteins like collagen, most of the therapeutic proteins have a globular configuration like albumin. Therefore, albumin can be a worthy represent for other therapeutic proteins.

MATERIALS AND METHODS

CaP-NPs (nominal average particle size of 100 nm), ZnO-NPs (nominal average particle size of 100 nm), human serum albumin, Su, Ol, Co, LP, DMSO and THF, were purchased from Sigma-Aldrich company, USA.

Preparation of formulations

Formulations numbered 1-22 were prepared.Their constituents are listed in Table 1. Every formulation contained 500 mg albumin and 2 mL of deionized water (DW) or one of the solvents mentioned above.Such a dose for the solvents, indeed is the maximum solvent concentration used in different topical preparations as enhancer. The maximum dose was chosen to obtain a maximum enhancing action[3].Then 200 mg CaP-NPs or ZnO-NPs was added to formulations 2, 3 and 10-22. Such a dose for NPs was their optimum concen-tration as topical enhancer, obtained in our previous work[21]. Then the whole formulation was mixed with a mechanical overhead mixer (Heidolph, RZR 2020, Germany) for 15 minutes to make a paste.

Permeation test

Dermal penetration of albumin was determined by an *ex vivo* method using a two chamber (donor and receiver) diffusion cell. The cell had an effective diffusion area of 10 cm^2 between the two chambers. This method is supported by the fact that SC, the main site of enhancer action, shows similar behavior *in vivo* and *in vitro* (*ex vivo*)[26]. Thirty mL of phosphate buffered saline (PBS), pH 7.4, was poured into the receiver chamber as the medium. A piece of mouse full skin was cut from mouse back. The skin was placed and fixed between the two chambers. The whole amount of each formulation paste (listed in the Table 1) was placed and spread on the skin

Table 1 : *Formulations 1-22 and their constituents (mg or mL).*

Formulation Number	Albumin (500 mg)	Solvent (2 mL)	CaP-NPs (200 mg)	ZnO-NPs (200 mg)
1	✔	DW	----	----
2	✔	DW	✔	----
3	✔	DW	----	✔
4	✔	Su	----	----
5	✔	Ol	----	----
6	✔	Co	----	----
7	✔	LP	----	----
8	✔	DMSO	----	----
9	✔	THF	----	----
10	✔	Su	✔	----
11	✔	Ol	✔	----
12	✔	Co	✔	----
13	✔	LP	✔	----
14	✔	DMSO	✔	----
15	✔	THF	✔	----
16	✔	Su	✔	----
17	✔	Ol	✔	----
18	✔	Co	----	✔
19	✔	LP	----	✔
20	✔	DMSO	----	✔
21	✔	THF	----	✔
22	✔	LP	✔	✔

by a clean swab. Then the cap was placed and fixed. After that, the cell was placed in a shaker-incubator (Heidolph incubator 1000, Heidolph co., Germany) with a temperature of 32°C for 1.5 hours[3,27,28]. Choosing an exposure time of 1.5 hours was because the topical products usually do not remain for a long time on the skin. After that, 3 mL of PBS was taken out and analyzed for the concentration of albumin using a UV-VIS spectrophotometer (Perkin-Elmer-Lambda 25, USA) at 278 nm[29]. The total amount of albumin (in 30 mL) was calculated and reported as the dermal permeated albumin (mg). These amounts were plotted versus their related formulation numbers in Figures 1-4.

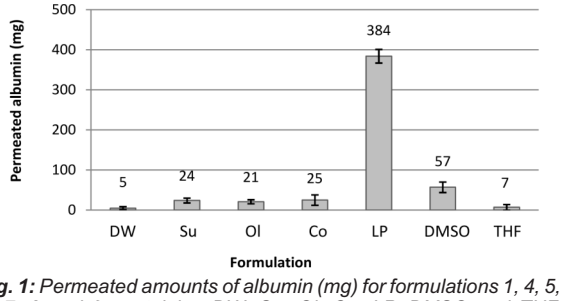

Fig. 1: *Permeated amounts of albumin (mg) for formulations 1, 4, 5, 6, 7, 8 and 9 containing DW, Su, Ol, Co, LP, DMSO and THF, respectively. (n=3)*

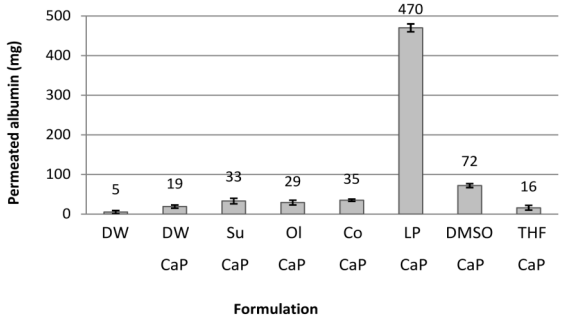

Fig. 2: *Permeated amounts of albumin (mg) for formulations1, 2, 10, 11, 12, 13, 14 and 15 containing DW, DW, Su, Ol, Co, LP, DMSO and THF, respectively. Formulations 2 and 10-15 each also contained CaP-NPs. (n=3)*

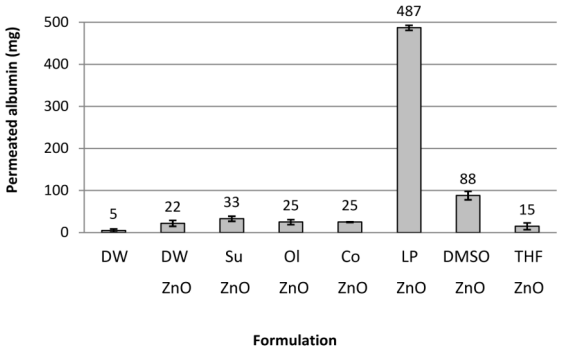

Fig. 3: *Permeated amounts of albumin (mg) for formulations 1, 3, 16, 17, 18, 19, 20 and 21 containing DW, DW, Su, Ol, Co, LP, DMSO and THF, respectively. Formulations 3 and 16-21 each also contained ZnO-NPs. (n=3)*

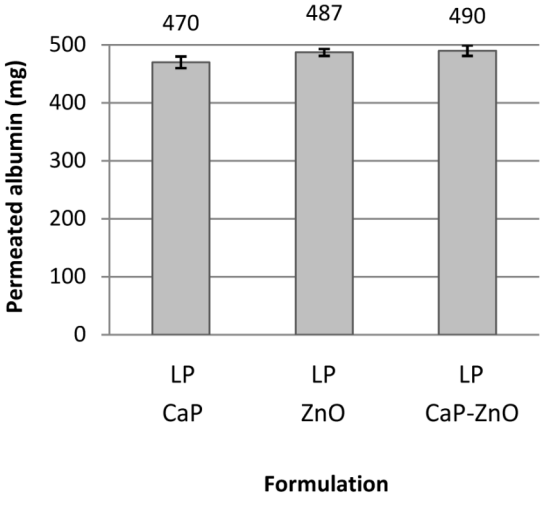

Fig. 4:*Permeated amounts of albumin (mg) for formulations 13, 19 and 22 containing LP. Formulation 13 also contained CaP-NPs.Formulation 19 also contained ZnO-NPs. Formulation 22 also contained both CaP-NPs and ZnO-NPs. (n=3)*

Microscopic imaging

Formulations 2 and 3 (but without albumin) were applied on the hair-removed mice skin pieces according to the conditions mentioned above for the permeation test. Then the skin pieces were washed with saline and formalin and imaged by light microscopy to obtain histomicrographs of the skins showing the skin distribution of CaP-NPs and ZnO-NPs (Figure 5)[30].

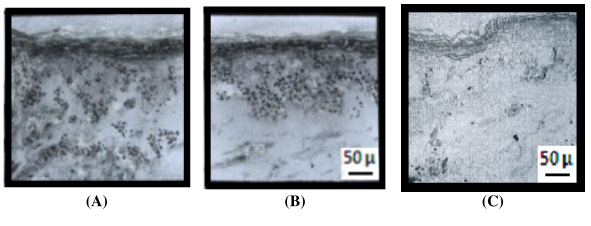

Fig. 5: *Histomicrographs of mice skin treated by (A):CaP-NPs, (B):ZnO-NPs and (C): untreated skin,obtained by light microscopy.*

Statistical analysis

Each formulation was tested 3 times for permeation and the reported data (permeated amounts of albumin) are the related mean ±SD (n=3). One-way analysis of variance (ANOVA) was used for comparing the differences. SPSS for Windows (release 11.5.0) was employed for statistical analysis. The p-value <0.05 was considered to be significant.

RESULTS

Figure 1 shows the dermal permeated albumin (mg) for formulations 1 and 4-9.Permeated albumin in the presence of DW (without enhancer) (formulation 1) was almost negligible (5±4 mg). Using oils, Su, Ol and Co (formulations 4-6), almost led to more ($p<0.05$) permeations of albumin (24±6, 21±5 and 25±13 mg, respectively).No statistical difference was seen between permeated albumin related to these three solvents ($p>0.05$).Using LP (formulation 7) led to a dramatic increase in permeated albumin (384±17 mg). Such an increase is about 76 times more than that for DW and 19 times more than those for oils (in average) ($p<0.05$).Using DMSO (formulation 8) resulted in significantly more permeated albumin (57±13 mg) compared to DW and oils ($p<0.05$).The last formulation of the Figure 1 (formulation 9) showed that THF led to a very low permeated albumin which did not make a difference when compared with that for DW($p>0.05$).Thus LP was the most effective CPE among the solvents.

Figure 2 represents the permeated albumin for formulations 1, 2 and 10-15. Formulation 1 is repeated in this figure for better comparison.Using CaP-NPs and DW (formulation 2) resulted in more permeated albumin (19±4 mg) compared to DW alone ($p<0.05$).Similarly, using CaP-NPs and each one of the solvents (formulations 10-15) resulted in more permeated albumin(33±7, 29±6, 35±3, 470±10, 72±5 and 16±6 mg, respectively) compared to those for each solvent alone ($p<0.05$ for each pair).Among such increases, the increased caused by CaP-NPs and LP (about 86 mg increase) was much more than the increases caused by CaP-NPs and other solvents ($p<0.05$ for every pairof formulations). Thus the most effective combination of CPEs was simultaneous use of CaP-NPs and LP (formulation 13) (resulted from data of the Figures 1 and 2). That led to a permeated albumin of 470±10 mg.Indeed CaP-NPs and LP generated a synergistic enhancing effect.

Figure 3 shows the permeated albumin for formulations 1, 3 and 16-21. Formulation 1 is repeated in this figure for better comparison. Using ZnO-NPs and DW (formulation 3) resulted in more permeated albumin (22±7 mg) compared to DW alone ($p<0.05$).Similarly, usingZnO-NPs and every one of the solvents (formulations 16-21) resulted in more permeated albumin (33±6, 25±6, 25±1, 487±6,88±10 and 15±8mg, respectively) compared to those for every solvent alone ($p<0.05$ for each pair). Among such increases, the increase caused by ZnO-NPs and LP (about 103 mg increase) was much more than the increases caused by ZnO-NPs and other solvents ($p<0.05$ for every pair of formulations). This data is even higher than that for CaP-NPs ($p<0.05$). Thus the most effective combination of CPEs, was simultaneous use of ZnO-NPs and LP (formulation 19) (resulted from data of the Figures 1-3). That led to a permeated albumin of 487±6 mg. Indeed a similar synergistic enhancing effect was occurred by combination use of ZnO-NPs and LP.

Figure 4 presents the permeated albumin for formulations 13, 19 and 22.Formulation 13 and 19 are repeated in this figure for better comparison.According to this figure, simultaneous use of ZnO-NPs and LP (formulation 19) resulted in more permeated albumin (487±6 mg) compared to that for CaP-NPs and LP ($p<0.05$).The last formulation of the Figure 4 (formulation 22) shows that simultaneous use of CaP-NPs, ZnO-NPs and LP resulted in more permeated albumin (490±9 mg) compared to CaP-NPs and LP($p<0.05$), but did not statistically resulted in more permeated albumin compared to ZnO-NPs and LP ($p>0.05$). Therefore, CPE combination (formulation 19) consisting of ZnO-NPs and LP and CPE combination (formulation 22) consisting of CaP-NPs, ZnO-NPs and LP resulted in highest permeated amounts of albumin compared to all other formulations of the study.

Figure 5 shows the skin distribution of the two kinds of NPs in the absence of albumin or any other ingredient. The CaP-NPs were distributed in SC, epidermis and dermis. While ZnO-NPs positioned in SC and epidermis.

DISCUSSION

Data presented in the results proved that albumin necessarily needed CPEs for dermal permeation. Because the albumin is a hydrophilic high MW compound and a globular protein[25,31] hindering it to pass the skin.Lipids of the SC limit the dermal permeation of such molecules.Organic solvents (and their fatty acid content) can dissolve and soften the lipids and therefore enhance the permeation of substances[3]like albumin. But results showed that THF did not affect the albumin permeation. This can be apparently because THF is almost polar and highly volatile and it has low ability to dissolve albumin and also the lipids[26]. Su is an unsaturated mixture of mostly oleic acid (omega-9) and linoleic acid (omega-6) group of oils and about 10% terpenes[31]. Ol is mainly composed of the mixed triglyceride esters of oleic acid (55-83%) and palmitic acid[31]. Co consists of 91% saturated fatty acids[31]. Results showed that these oils, Su, Ol and Co, slightly increased the permeated albumin which can be because of their lack of ability to dissolve albumin and weak ability to dissolve the lipids. Besides, albumin can bind fatty acids[25] which may help albumin permeation in the presence of these solvents. Results showed that DMSO increased the permeated albumin more than the oils. This can be because of its slight ability to dissolve albumin and its strong ability to dissolve the lipids, although it is volatile[1].According to the results, among solvents used in this study, LP increased the permeated albumin significantly more than other solvents. This can be because LP strongly dissolves and softens the lipids much more than the other solvents[3], and possesses a lower viscosity than the oils. In addition, LP contains

hydrophobic hydrocarbon chains penetrating and depositing between the hydrophobic chains of SC lipids and decreases the consistency of the SC lipids[26]leading to a high permeation of albumin.Therefore, LP caused a discriminating permeated albumin although it cannot dissolve albumin. Thus LP was chosen as the best solvent (CPE) for the permeation of albumin in this study. Results showed that simultaneous use of CaP-NPs and every one of the solvents, led to more permeated albumin compared to every solvent alone. It was shown in the results that such increases, caused by CaP-NPs, were proportional to the permeated albumin caused by every solvent alone. This indicates that the mechanism of CaP-NPs enhancing action was to strengthen the effect of every solvent. According to the results, among the combinations, combination of CaP-NPs and LP increased the permeated albumin more than other combinations (CaP-NPs and other solvents) leading to an increase equal to 86 mg of albumin.

Results revealed that like CaP-NPs, simultaneous use of ZnO-NPs and every solvent, led to more permeated albumin compared to every one of the solvents alone. The characteristics of this effect were similar to those mentioned for CaP-NPs in the previous paragraph, e.g. the combination of ZnO-NPs and LP increased the permeated albumin more than other combinations (ZnO-NPs and other solvents). This increase was equal to 103 mg albumin which introduced ZnO-NPs and LP as the most effective CPE combination for albumin in this study.Results also showed that even using both CaP-NPs and ZnO-NPs with LP (a triple combination) did not significantly increase the permeated albumin more than the combination of ZnO-NPs and LP. As the conclusion, two combinations were found to be the most effective including combination of two enhancers of ZnO-NPs and LP, and combination of three enhancers of CaP-NPs, ZnO-NPs and LP.

Such enhancing effect of CaP-NPs and ZnO-NPs can be referred to their nanometer size and their almost crystalline state.Also according to the microscopic images (Figure 5), it can also be referred to their penetration into the skin and being retained in the skin layers for a period of time. This phenomenon disorders the microstructure of the skin layers and thus makes the skin permeable to albumin[18]. On the other hand, same phenomenon makes the skin more permeable to the solvents and then the solvents help the permeation of albumin.

Zinc has been shown to have no interactions with most pharmaceutically active molecules[14]but ZnO-NPs can interact with the components of the skin layers[17-19]. The latter characteristic of ZnO-NPs can help them to alter the order and consistency of the skin structure which strengthens its enhancing effect.

Albumin can bind low MW cations (e.g. Ca^{2+}, Na^{+} and K^{+}) with a relatively high affinity[25,31].Therefore, the binding of albumin to the CaP-NPs could not help the albumin permeation to be more than what caused by ZnO-NPs.

According to the results, using both CaP-NPs and ZnO-NPs with LP (a triple combination) led to a saturation state of albumin permeation which not only did not possessed the additive enhancing effect caused by the two kinds of the NPs, but also was not significantly more effective than the combination of ZnO-NPs and LP.

The huge enhancing effect of the combination of the inorganic NPs and LP observed in this study can be attributed to the totally different mechanisms of two major types of enhancers used in this study (NPs and organic solvents). Apparently the two mechanisms could complete the enhancing effects of each other leading to a discriminating skin permeation of albumin.

According to Figure 4, the skin distribution of the CaP-NPs proved their spreading into all skin layers. In contrast, the ZnO-NPs mainly deposited in the SC and epidermis.It means that the concentration of ZnO-NPs in the SC (the main impermeable skin layer) was much more than CaP-NPs. Therefore, ZnO-NPs could alter the SC more than CaP-NPs and thus enhance the albumin permeation more than CaP-NPs.

CONCLUSION

In the absence of enhancers, albumin did not considerably penetrate the skin. LP was the most effective enhancer for permeation of albumin among the organic solvents used in this study. CaP-NPs and ZnO-NPs enhanced the permeation of albumin but their strong enhancing effect was observed in the presence of LP.The enhancing effect of ZnO-NPs was more than CaP-NPs.The obtained data proved a considerable synergistic enhancing effect caused by the inorganic NPs and the organic solvent, LP.Using a triple combination of both kinds of NPs and LP revealed a saturation state of the albumin skin permeation and did not lead to an increase in albumin permeation compared to using one kind of the NPs and LP. In this study, we suggest using a combination of ZnO-NPs and LP to effectively enhance the skin permeation of albumin or other proteins in dermal delivery of proteins.

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CONFLICT OF INTEREST

The author reports that there are no conflicts of interests.

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