**Enhancing the loading capacity of kojic acid dipalmitate in liposomes**

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**Abstract**

Kojic acid (KA) is a natural product, highly sensitive to light and heat, commonly used as a skin-whitening agent in its dipalmitic ester form as kojic acid dipalmitate (KDP). The bioavailability of KA is poor due to its high hydrophilicity, therefore KA was converted to a hydrophobic compound by esterification with two palmitic acids. The loading of KDP to liposomes is extremely low because it was loaded to the lipid bilayer compartment of the liposomes, which constitutes only small percentage of the liposomes. The aim of the following research was to enhance the loading capacity of KDP to liposomes. Liposomes were prepared by the thin lipid film hydration method. The particle size, polydispersity index (PDI) and zeta-potential (ζ-potential) of the liposomes were analysed by measuring dynamic light scattering (DLS) using photon correlation spectroscopy. KDP was loaded to the liposomes by active loading method. The stability study of the prepared liposomes loaded with KDP was studied over 2 months. KDP was quantified using an optimized HPLC method. Atomic force microscopy (AFM) was used to study the morphology of the liposomes.The particle sizes of the formulated liposomes were in the range of 80 - 100 nm. The liposomes were homogeneous with PDI less than 0.2 and average ζ-potential of -0.5 to -0.6 mV. The liposomes particle size, PDI and ζ-potential did not reveal a significant changes over two months, reflecting a stable liposomes. The loading capacity (LC%) of KDP was increased from 0.61% to 28.12 % i.e. 46 times increase. AFM study revealed spherical liposomes in the, homogeneously distributed and within 100 nm in diameter. The liposomes were successfully prepared and loaded with KDP. Liposomes loaded with KDP were stable over the predetermined time scale. The LC% was significantly increased from 0.61 % to 28.12 %, allowing a promising formulation of KDP to enhance its bioavailability and therapeutic capacity.

**Keywords:** Kojic acid dipalmitate; loading capacity; active loading; liposomes

**1. Introduction**

 Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone) is a natural product widely used as a skin-whitening agent because it inhibits tyrosinase enzyme and has antibacterial action. Kojic acid is produced by food fermentation and mushroom by various fungi and bacteria such as *Aspergillus* and *Penicillium* (Kang *et al.*, 2009, Lim *et al.*, 2009, Liu *et al.*, 2009, Oresajo and Yatskayer, 2009). In cosmetics, the inhibitory effect and storage properties of kojic acid are inadequate due to its labile oxidative properties which can be accelerated in the presence of light and heat (Balaguer *et al.*, 2008). Therefore, many semisynthetic kojic acid derivatives have been synthesized (Kang *et al.*, 2009). The C-7 hydroxyl group is usually modified to form esters or hydroxyphenyl ether or is used to form glycosides or peptides derivatives (Kang *et al.*, 2009). Kojic acid is added to cosmetics by means of its dipalmitic ester, that is, as kojic acid dipalmitate (KDP), which is hydrolyzed by means of esterases located in skin cells producing an *in situ* liberation of kojic acid (Balaguer *et al.*, 2008).

Liposomes are widely used as encapsulating agent to overcome obstacles to cellular uptake to target sites in vivo and improve delivery efficacy of compounds (Tonggu and Wang, 2019). Liposomal formulations have been proposed as a means of improving the therapeutic efficacy of poorly bioavailable drugs (Torchilin, 2005). Liposomes has been proven to be used as carrier system for neurological disease (Vieira and Gamarra, 2016), in pharmaceutics (for instance cancer therapy) (Borresen *et al.*, 2018, Yuba, 2018), cosmetics (for instance carrier for vitamins) (Mozafari, 2005) and food industries (for instance encapsulate bioactive food compounds to protect food from degradation and spoilage or to improve flavouring and nutritional properties) (Shukla *et al.*, 2017).

 The active loading (also called remote loading) is defined as the ability to load sufficient amount of drug to liposomes to exert a therapeutic efficacy (Zucker *et al.*, 2009). Active loading depends, mainly, either on an ion gradient as reported by Bally *et. al.*, 1988 (Bally *et al.*, 1988) where they actively loaded dopamine into large unilamellar vesicles system or depends on pH gradients as reported by Mayer *et. al.*, 1986 (Mayer *et al.*, 1986), thus they actively loaded adriamycin into large unilamellar vesicles system. Both groups realised this action when they changed the intraliposomal solution from citrate buffer (pH 4) to HEPES buffer (pH 7.4) where the interliposomal composition and pH remain unchanged. This citrate method is still widely used for loading various anthracycline compounds (Dos Santos *et al.*, 2005).

Commonly, in passive loading, drug moves from point of high concentration to the point of low concentration. The driven forces of passive loading to the interliposomal compartment is the concentration gradient, thus Mao and his colleagues, 2019, has reported that vincristine was loaded passively to liposomes using concentration gradient method, however, low entrapment efficacy was achieved (Mao *et al.*, 2019). Therefore, they enhanced the entrapment efficacy *via* pH gradient method, whereas the pH of the intraliposomes were changed to generate the driven forces, increasing the entrapment efficacy to 90 %. It was, also, reported that the entrapment efficacy of doxorubicin was increased to 80 % by pH-gradient remote loading method (Cheung and Al-Jamal, 2019).

The aim of the following research is to increase the loading capacity of kojic acid dipalmitate in the liposomes using active loading technique using concentration gradient method.

**2. Material and methods**

**2.1. Materials**

Kojic acid dipalmitate (KDP) was purchased from Beijing Brilliance Biochemical Co., Ltd. (Hou Modern, China). Milli-Q water (HPLC grade), syringe filters (diameter 13 mm PTFE and pore size 0.22 μm NSTR) and centrifuge tube filters (Ultracel-30 with 30 kDa pore size) were purchased from Fischer Scientific, UK. Cholesterol, Tetrahydrofuran (THF), the polycarbonate filter papers (Whatman® Nuclepore™ Track-Etched membranes with diameter of 25 mm) have pore sizes of 200 nm, 100 nm and 50 nm, iron chloride hexahydrate, ammonium thiocyanate and Dulbecco’s phosphate buffered saline (PBS), were purchased from Sigma-Aldrich (UK). The 1,2-Distearoyl - sn - glycero - 3 - phosphocholine (DSPC) was purchased from Avanti Polar Lipids, Inc. (USA). Dialysis sacks (Float A-lyzer G2) has a pore size of 20 kD and a capacity of 5 ml was purchased from VWR international (UK). A PLGel Mixed-D (polystyrene/divinylbenzene co-polymer) (300 × 7.5 mm i.d., 5 µm particle size) analytical column and a PLGel Guard (50 mm × 7.5 mm i.d., 5 µm particle size) guard column made up of the same stationary phase were purchased from Polymer Laboratories (Church Stretton, Shropshier, UK). Nylon filter membrane was purchased from Sterlitech (Kent, USA). All other chemicals and reagents not quoted specifically were purchased from commercial sources at the highest quality available.

**2.2. Methods**

**2.2.1. Preparation of liposomes**

Liposomes were prepared by the thin lipid film hydration method adapted from Isailović *et. al*., 2013 with major modifications (Isailović *et al.*, 2013). DSPC (20 mg) was mixed with cholesterol (5 mg) and KDP (0.5 mg) in a 250 ml round bottom flask. Solvent (methanol: chloroform, 1:1), 1 ml, was added and the mixture gently shaken by hand until a clear solution was obtained. The solvent was completely evaporated using a rotary evaporator (Buchi Rotavapor® R-100, Switzerland) set at 200 mbar pressure, 60 ºC and 100 rpm. The resultant thin lipid film was further dried by freeze-drying (Martin Christ alpha 1-4 LD plus, Germany) at -55 ºC for 30 min. Then, the thin lipid film was rehydrated with 4 ml of PBS and warmed to 60 ºC using a rotary evaporator at 100 rpm for 5 - 10 min. The suspension was inspected visually to ensure that all the lipid thin film had detached from the wall of flask. The resultant suspension, containing multi-layer vesicles (MLVs), was passed through 5 cycles of freezing (using liquid nitrogen) and thawing (using a water bath at 60 ºC). The suspension was extruded 10 times through a 0.2 μm polycarbonate membrane using a Lipex extruder (Lipex Biomembranes, Inc., Canada). The polycarbonate membrane was equilibrated with 5 ml PBS after installation in the extruder. The extruding pressure was 20 bar and the temperature was 60 ºC. The extrusion was subsequently repeated with smaller pore size membranes (i.e. 0.1 μm and 0.05 μm). The final suspension of liposomes was clear, transparent and bluish in appearance. The same procedure was repeated without KDP to produce the KDP-free liposomes. The resultant liposomes suspension was dialysed using a Pur-A-Lyzer™ Maxi Dialysis Kit (MWCO 12 - 14 kDa) (Sigma, UK) to remove free KDP. The KDP loaded into liposomes was then quantified using HPLC.

**2.2.2. Measurement of size, polydispersity index and zeta-potential**

The particle size, polydispersity index (PDI) and zeta-potential (ζ-potential) of the liposomes were analysed by measuring dynamic light scattering (DLS) using photon correlation spectroscopy (Malvern Nano-Zs, Malvern Instruments, UK) at 25 °C and 173o angle. The instrument was fully automated, hence 50 μl of the liposomes suspension was placed in a disposable capillary cell (DTS1070) for analysis of size and ζ-potential. Optimisation of the measurement and the noise were carried out automatically.

**2.2.3. HPLC for quantification of KDP**

 HPLC analysis was based on the method described by Balaguer *et. al.* 2008 (Balaguer *et al.*, 2008). A shimadzu LC system, equipped with a shimadzu FCV-20AH2/20AH6 high-pressure pump, DGU-20A on-line degasser and SPD-20A UV-VIS detector (Tokyo, Japan), was employed. The injection was carried out by means of SIL-20A auto-sampler. A computer equipped with a LC system manager software connected to the LC system was used to process all chromatographic data. Chromatography was performed on PLGel mixed-D (polystyrene /divinylbenzene co-polymer) (300 × 7.5 mm i.d., 5 µm particle size) analytical column and PLGel column Guard (50 × 7.5 mm i.d., 5µm particle size) made up of the same stationary phase. The mobile phase was THF (100 %). Each sample was filtered prior to injection by using a nylon filter **(Sterlitech, Kent, USA)** and the aliquot was filled into the vials and then injected (30 µl) into the HPLC apparatus using multiple auto-sampler system. The flow rate was set at 1.5 ml. min-1.

**2.2.4. Active loading of KDP**

The process of active loading was adapted from Hayes *et. al.*, 2014 with major modifications (Hayes *et al.*, 2014). The liposomes were prepare as described earlier in section 2.2.1. No KDP was added to the liposomes during the preparation process. Following the preparation process of liposomes (i.e. clear, transparent and bluish solution in appearance was obtained), the process of active loading was started. KDP stock solution, 10 mg.ml-1, was prepared in DMSO. Then, 100 μl of KDP stock solution (containing 1000 μg of KDP) was mixed with 980 μl of liposomes suspension. A cloudy suspension was formed and agitated in a shaking water bath (Boekel Grant ORS 200 orbital, USA) at 60 ºC at 75 shake per min for 3 min followed by 21 shake per min for 27 min. The resultant clear liposomes suspension was kept on ice for 15 min and became cloudy again. The cloudy liposomes suspension was centrifuged (Minispin, Thermo Scientific, UK) at 8000 x g for 5 min and the supernatant was removed and kept at 4 ºC until use. The resultant liposomes suspension was dialysed using a Pur-A-Lyzer™ Maxi Dialysis Kit (MWCO 12 - 14 kDa) (Sigma, UK) to remove free KDP (Fig. 1). The KDP loaded into liposomes was then quantified using HPLC.



**Figure 1.** Schematic diagram of the liposomes active loading of KDP.

#### 2.2.5. Estimation of the loading capacity of the KDP

To estimate the concentration of the KDP loaded into the liposomes, a sample of 100 μl was taken from the liposomes suspension before and after dialysis and mixed with 900 μl methanol to dissolve the liposome and release the loaded KDP for further quantification using HPLC. The loading capacity were calculated according to equation (Isailović *et al.*, 2013):

$ (LC\%) = \frac{Encapsulated KDP}{Lipid content } ×100$ ……………………………………….……….....… [1]

Quantification of DSPC was by the method of Stewart *et. al.*, 1980 with minor modifications (Stewart, 1980). Ammonium ferrothiocyanate solution, 0.1 M, was prepared by dissolving iron chloride hexahydrate and ammonium thiocyanate to 0.1 M in 100 ml Milli-Q water. A standard solution of DSPC (1 mg.ml-1) was prepared in chloroform. To identify the optimal wavelength to detect the reaction product, preliminary studies were carried out. A solution composed of 20 μl DSPC (20 μg.ml-1), 980 μl chloroform and 1000 μl ammonium ferrothiocyanate, 0.1 M, was placed in a 2 ml Eppendorf tube and vigorously mixed for 20 min using a shaker (Edmund Buhler, 7400 Tubingen, Germany) at 420 shake per min. Then, the mixture was centrifuged at 800 x g for 5 min (Minispin, Thermo Scientific, UK). The lower layer was carefully removed and the absorption profile measured over the wavelength range of 350 - 650 nm using a spectrophotometer (Perkin Elmer Lambda 12). Chloroform was used as a blank. In routine studies, the DSPC content of liposomes was quantified as described above and the absorption measured at 420 nm using a spectrophotometer (Perkin Elmer Lambda 12).

**2.2.6. Atomic force microscopy**

Atomic force microscopy (AFM) was used to study the morphology of the liposomes as described previously (Plochberger *et al.*, 2017, Duan *et al.*, 2018). Freshly cleaved mica was mounted on a metal disc and 20 μl samples were deposited on the mica for 20 min. Samples were washed five times with filter sterilised Milli-Q water, air dried at room temperature and analysed using a Picoforce Nanoscope V Multimode atomic force microscope (Bruker) using tapping mode with scan rates between 0.5 and 1.5 Hz and a resonant frequency range of 270 - 460 kHz. Image capturing was conducted after specifying representative areas. The resolution of the images was 512 x 512 points and the height and amplitude error mode images (5 μm x 5 μm) were used after flattening using Nanoscope software v7.2.

**2.2.7. Statistical analysis**

 Statistical analysis was carried out using IBM SPSS software version 23 and Excel 2016. Results were considered significantly different when student-p value was less and equal to 0.05. All tests and analysis were replicated a minimum of three times and the presented data were mean ± standard deviations.

**3. Results and discussions**

**3.1. Liposome's particle size, PDI and** ζ**-potential**

The particle sizes of the formulated liposomes were in the range of 80 - 100 nm as shown in Fig. 2 (A). The presence of DMSO during the preparation of liposomes (i.e. active loading process) affect the particle size as there was an increase of about 10 – 15 nm more than the other formulations. Stability of the liposomes was monitored by measuring the particle size during 60 days. The size of the liposomes, PDI, and ζ-potential were measured on the day 1, 7, 30 and 60 after preparation. The measurements were carried out for the liposomes formulation prepared with passive and active loading of KDP. The liposomes were homogeneous with PDI less than 0.2 as shown in Fig. 2 (B). In particular, the average ζ-potential was in the range of -0.5 to -0.6 mV as shown in Fig. 2 (C).

**Figure 2.** (A) the particle size, (B) the PDI and (C) ζ-potential of the liposomes loaded with KDP pasively and actively over 60 days. The results represented the mean of the three independent replicates ± standard deviation.

Particle size is considered an important parameter because it is directly related to the stability, release and biodistribution of the liposomes (Mozafari *et al.*, 2008). Particle size and PDI were always taken into consideration due to the distribution measure of the liposomes as PDI of 0 reflects an entirely monodisperse and 1 for completely heterodisperse liposomes.

In contrast to the report by Lopez-pinto *et. al*., 2005 (Lopez-Pinto *et al.*, 2005) who stated that adding co-surfactant such as ethanol would decrease the particle size of the liposomes due to the charge modification of the system, we have noticed that DMSO has increased the particle size which could be due to an interaction with DSPC. The finding that DMSO has increased the particle size of the formulated liposomes was in consistent with the findings of Bonora *et. al*., 2005 (Bonora *et al.*, 2005) as they suggested that the presence small sulfoxide amount could cause partial dehydration of the lipid surface, modification on the water structure and eventually affect biomembrane.

 Liposomes produced by thin-film re-hydration method were reported to be homogeneous (Budai *et al.*, 2004). The physical stability of the liposomes can be greatly influenced by ζ-potential by determining the electrostatic repulsion between the particles. In addition, the dominant component on the particle surface could be identified with assist of ζ-potential. In our experiment, a negative charge of liposomes was obtained for good electrostatic stabilisation as vesicle aggregation and fusion were prevented. This was confirmed by no changing in the particle size for more than 10 % over 60 days. The surface charge did not be affected by the presence of KDP, reflecting a deep entrapment inside the membrane for passive loading formulations and this was consistent with the finding of Bojana *et. al*., 2013 (Isailović *et al.*, 2013).

**3.2 Estimation of the LC% of the liposomes**

The passive loading of the KDP into liposomes depended mainly on hydrophobic interaction and association with DSPC bilayer structure. Accordingly, liposomes were able to incorporate KDP but in low percentages for the passive loading methods of 0.61 % as the lipid bilayer constitute only small fractions of the liposomes (Table 1 and Fig. 3). This percentages corresponding to the amount of the hydrophobic moieties in the lipid of the liposomes as KDP is hydrophobic. Moreover, a significant amount of KDP could be lost during extrusion process because of the shear forces on the MLV during preparation, favouring leakage and loss from liposomes (Isailović *et al.*, 2013). Also, the unilamellar liposomes were reported to offer less space for incorporation of lipophilic drug or instance KDP (Caddeo *et al.*, 2008). Furthermore, it was reported that the presence of cholesterol in the formulation increases the cohesiveness of the lipid bilayer membrane, decreasing the hosting capability of resveratrol (Kutchai *et al.*, 1983).

Active loading revealing significantly higher LC% as compared to the passive loading of KDP. The concentration gradient technique forced KDP to be incorporated into the core of the liposomes, thus LC% of 28.12 % was achieved. When the temperature of liposomes was increase up to the transmission phase temperature, the holes opened in the lipid bilayer. KDP permeated from intraliposomal to interliposomal medium own to the concentration gradients. This gradient is a driving force for permeation of KDP, leading in their equilibration of the concentration on both sides of the liposomes bilayer, and this was in agreement with the report of Lasic and his colleagues (Lasic *et al.*, 1995). The result of active loading based on concentration gradient was, also, consistent with the finding of Tardi and his colleagues, who achieved 80 % loading capacity of irinotecan, based on a modified transition metal encapsulation procedure, without a pH gradient method (Tardi *et al.*, 2007).

**Table 1.** The LC% of the liposomes for the passive and active loading of KDP. The results represented the mean of the three independent replicates ± standard deviation.

|  |  |
| --- | --- |
| **Liposomes** | **LC%** |
| Passive loading of KDP | 0.61 ± 0.08 |
| Active loading of KDP | 28.12 ± 2.19 |

\*

**Figure 2.** LC% of the liposomes loaded passively and actively with KDP. Data are the mean ± standard deviation of three independent experiments. \* p ≤ 0.01

KAD added to the external medium during the active loading process was able to cross the lipid bilayer and the permeability depends completely on the permeability coefficient (Clerc and Barenholz, 1995). The concentration of KDP was higher in the intraliposomal medium. The concentration gradient between the intraliposomal and interliposomal medium generated driven force which enforce KDP to enter the interliposomal medium of the liposomes. This driven force increased the LC% of the liposomes (Mayer *et al.*, 1986, Bally *et al.*, 1988).

**3.3. Morphological examinations of the liposomes using atomic force microscopy**

Morphological examination of the liposomes using atomic force microscopy (AFM) was carried out to analyse the dimensions, shape and substructure of the liposomes.

In the liposomes passively loaded with KDP and subjected to AFM, the AFM showed particles with diameters less than 200 nm (Fig 3, A). In contrast, for actively loaded liposomes, the diameters of the liposomes were slighly increased more than 200 nm (Fig 3, B). The particle analysis of the liposomes passively loaded with KDP revealed particle sizes of a mean z-height of 2.3 nm (range 0.663 - 10.978 nm) and a mean diameter of 167.3 nm (range 103.4 - 422.8 nm) (Fig. 3, C). On the other hand, the particle analysis. the liposomes actively loaded with KDP revealed particle sizes of a mean z-height of 5.252 nm (range 1.242 - 16.36 nm) and a mean diameter of 207.8 nm (range 110.2 - 438 nm) (Fig. 3, D).

|  |  |  |
| --- | --- | --- |
|  | **(I)****Passive loading of KDP** | **(II)****Active loading pf KDP** |
|  | **(A)** | **(B)** |
| **2D image** | C:\Users\SARMAD\Desktop\dpip\Papers for publication\To do work\Enhancing the loading capacity\passive 2d_2.png | C:\Users\SARMAD\Desktop\dpip\Papers for publication\To do work\Enhancing the loading capacity\active 2d_2.png |
|  | **(C)** | **(D)** |
| **3D image** | C:\Users\SARMAD\Desktop\dpip\Papers for publication\To do work\Enhancing the loading capacity\passive 3d.png | C:\Users\SARMAD\Desktop\dpip\Papers for publication\To do work\Enhancing the loading capacity\active 3d.png |

Figure 3. Representative AFM images: 2D (A and B) and 3D (C and D) of the liposomes incubated with the KDP passively (column I) and actively (column II). AFM was carried out using a Picoforce Nanoscope V Multimode atomic force microscope.

In AFM studies, the liposomes passively loaded with KDP possessed a mean z-height of 2.3 nm (range 0.663 - 10.978 nm) and a mean diameter of 167.3 nm (range 103.4 - 422.8 nm) (Fig. 3, C), consistent with literature findings of Parbhu and his colleagues (Parbhu *et al.*, 2002). The mean z-height of the particles in the liposomes was increased from 2.3 nm to 5.25 nm and also the mean diameter of the particles was increased from 167.3 nm to 207.8 nm, when active loading technique was used (Fig. 3). The reason of inreasing the size of the particles could be due to the effect of using a cosolvent DMSO. It has been reported by Bonora *et. al*., 2005 that using DMSO increased the perticle size of liposomes (Bonora *et al.*, 2005) as they suggested that the presence small sulfoxide amount could cause partial dehydration of the lipid surface, modification on the water structure and eventually affect biomembrane, which is consistent with our findings. However, Lopez-pinto *et. al*., 2005 reported that adding co-surfactant such as ethanol would decrease the particle size of the liposomes due to the charge modification of the system (Lopez-Pinto *et al.*, 2005), which was inconsistent with our findings.

**4. Conclusion**

 The liposomes were successfully prepared and loaded with KDP using active loading method. Loading of KDP using concentration gradient as a driving force resulted in significant increasing the loading capacity of KDP. The liposomes loaded with KDP were stable two months and there was no significant change in particle size, PDI and zeta-potential. The resulted liposomes were spherical in shape the distribution of the particles were uniform, allowing a promising formulation of KDP that could be used clinically.

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**Declaration of interest**

 The author declares no conflict of interest and has received no payment for the preparation of this manuscript.

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