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Characterization of Hyaluronan-Decorated Flexible Liposome Loaded with Curcumin

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ABSTRACT

Psoriasis is a widely known chronic skin disease with no cure. Psoriasis causes a rash with itchy, scaly patches. The most common topical treatment of psoriasis is the use of corticosteroids as anti-inflammatory agents, which can result in side effects such as skin thinning, acne, and swelling. Alternatively, a natural ingredient, curcumin, can be employed as an anti-inflammatory agent. However, curcumin has weak water solubility, limited bioavailability, quick metabolism, and quick systemic elimination; hence modification is required to increase its effectiveness. To increase the effectiveness of curcumin, it can be encapsulated in an active delivery system. Curcumin can be directly delivered to the disease cells by decorating the delivery system with a functional ligand such as hyaluronan (HA). Cell adhesion protein CD44, which is expressed in several inflammatory diseases, serves as a cell surface receptor for hyaluronan. In this study, hyaluronan was conjugated to a flexible liposome. Thin-film hydration method was used to prepare the flexible liposome. The delivery system was characterized using Dynamic Light Scattering, Transmission Electron Microscopy, Fourier Transform Infrared Spectroscopy, and High-Performance Liquid Chromatography. HA-decorated liposomes obtained had an average size of 95.48 nm (with 0.247 polydispersity index), had a zeta potential of -49.3 mV, was spherical, and had an average encapsulation efficiency of 34.01%. Additionally, absorbance bands from FTIR spectra showed that HA and curcumin functional groups were observed in the decorated liposome. The results from the characterization study showed that HA-decorated flexible liposomes loaded with curcumin with acceptable characteristics had been obtained.

1. Introduction

Psoriasis is a lifelong immune-mediated inflammatory skin disease with a strong genetic component with nearly 90% heritability [1]. Symptoms include rashes and itchiness commonly found on the scalp, palms, and vaginal area. In psoriasis, the cell adhesion protein CD44 protein is significantly elevated compared to normal skin, indicating inflammation [2]. Targeted delivery systems using hyaluronan (HA) as a ligand have been developed to target the inflammatory agents. Current techniques for treating psoriasis include topical applications of corticosteroids, systemic

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therapy, and biologics. Unfortunately, the disadvantage of the most common treatment of psoriasis i.e., corticosteroids, is long-term side effects [3]. Curcumin, a plant extract, is a potential natural treatment for psoriasis due to its anti-inflammatory properties and ability to treat wounds [4]. Curcumin's reported pharmacological activities include antiviral, antifungal, antioxidant, and anti-inflammatory [5].

The stratum corneum (SC), the outermost layer of human skin, restricts the amount of medication absorbed via the skin, leading to low clinical efficacy and drug waste after topical administration [6]. Moreover, to be more effective, the drug should be targeted directly at the affected cells. One potential target is CD44, which is over-expressed when psoriasis breaks out. In this study, HA was used as the targeting ligand [7].

In this research work, a flexible liposome was employed to encapsulate curcumin. To improve the delivery system, the flexible liposome was proposed to be decorated with HA. To realize this, HA was to be conjugated to dipalmitoylphosphatidylethanolamine (DPPE) before preparing the curcumin loaded flexible liposome [8]. FTIR was employed to analyze whether HA was successfully conjugated to DPPE. To determine the suitability of the transdermal carrier, the hyaluronan-decorated flexible liposome loaded with curcumin was characterized in terms of size, shape, zeta potential, and encapsulation efficiency.

2. Methodology

To prepare the HA-DPPE conjugation, 150 mg HA was dissolved in 10 ml of methanol and dimethyl sulfoxide (1:1 v/v) and was stirred at 60℃. After 30 min, DPPE was added to the HA solution previously dissolved at 19 mg in 1 ml methanol and chloroform (1:1 v/v). The pH was then adjusted to 4.5. Then, the mixture was stirred for 2 hours at 60℃. The stirred mixture solution was left for 24 hours in an incubator shaker at 37℃.

Next was the preparation of hyaluronan-decorated flexible loaded with curcumin. Flexible liposomes were prepared using a standard thin-film hydration method with modification [9]. The ratio of 1:1 solvent of chloroform and ethanol was used. In phase A, 29.94 mg of β-sitosterol and 2.994 mg of curcumin were dissolved in 15 ml of ethanol. Next, in phase B, 150 mg of L-α-phosphatidylcholine, 0.00238 ml of TWEEN 80, and 0.00284 ml of SPAN 80 were dissolved in 15 ml chloroform before mixing and stirring both phase A and phase B accordingly. The HA-DPPE conjugation was added drop-wise into the flexible liposome mixture whilst stirring with a magnetic stirrer at 60℃. The mixture was then placed in a pear-shaped flask and a rotary evaporator was employed to remove the solvents and obtain a thin film layer. Parameters of the rotary evaporator were set to constant throughout the process where the pressure was set at 175 mbar, 5℃ of the cooling chambers, vapor temperature at 28℃, heating bath at 65℃, and 150 rpm of flask rotation. The process took about 40 minutes. Next, 50 mL of phosphate buffer solution (PBS), 0.1mM at pH 6.8, was added to the dried thin film. The hydration of the thin film was allowed to proceed for about an hour at 65℃ and 120 rpm rotation. The hydrated thin film was then removed from the flask and allowed to swell at room temperature for around another hour. After that, the solution was briefly sonicated using a probe sonicator for an allocated time at 30% amplitude. After that, the sample was kept at 4°C.

For size, PDI and zeta potential characterization, a Litesizer 500 (Anton Paar, Malaysia) was used. The measurements of the flexible liposomes were determined based on dynamic light scattering (DLS). Measurements were performed at an angle of 175◦, 1.33 material refraction index, and constant temperature of 25℃. Three measurements of each sample were taken using a clear disposable cell. For each sample, the number and duration of runs were optimized to produce results

that met the standards for measurement quality. The samples were dissolved in distilled water in a ratio of (1:9 dilution) before the measurement.

To examine the shape, transmission electron microscopy (TEM) was used. The sample was dropped on a special copper mesh of the electron microscope. The excess liquid from both samples was removed using filter paper before 2% of the phosphotungstic acid solution was added for negative staining for 20 s. The sample was let dry at room temperature before being examined using TEM [10].

FTIR analysis was performed to identify functional groups and characterize bonding information. All samples were freeze-dried prior to testing. An FT-IR spectrometer, Spectrum 1000, was used to obtain FT-IR spectra. The IR spectra were captured between 4000 and 400 cm^{-1} [11].

Curcumin was analyzed using High-Performance Liquid Chromatography (HPLC) from Perkin Almer (St. Louis, MO, USA). The system has a photodiode array detector and the Empower™ 3 computer integrating device. Chromatographic separations of samples were accomplished using the C18 column (150 mm x 4.6 mm, 5µm). The mobile phase was an aqueduct mixture of acetonitrile and acetic acid (55:45, v/v), flowing at a rate of 1 mL/min, and the column oven temperature was fixed at 30℃. The injection volume was 5µL, and the wavelength of the DAD detector was set to 425 nm.

A freshly synthesized flexible liposome was isolated from the unentrapped extracts using the mini-column centrifuge method [12]. A column prefilled with G-50 Sephadex gel was used. The small columns were centrifuged at 1000 rpm for three minutes to remove the excess buffer. The center of the column was filled with the samples, which later was centrifuged. The recovered aliquots of flexible liposomes were disrupted in ethanol at a ratio of 1:1. A solution containing 45% deionized water with 1 % acetic acid and 55% acetonitrile was used to suspend the samples. The resulting dispersion was thoroughly mixed for 10 minutes while continuously stirred and then filtered using a 0.45 m membrane filter [13]. The HPLC system was used to determine the drug loading. With an injection volume of around 5µL and a flow rate of 1.0 mL/min, HPLC was run continuously. Three measurements of each sample were taken and strong peaks of curcumin were detected at 4.19 min retention time. The following equation was used to determine the encapsulation efficiency.

$$\mathrm{EE}(\%) = \frac{\text{Extract in liposome}}{\text{Extract in liposome+Free extract}} \quad (1)$$

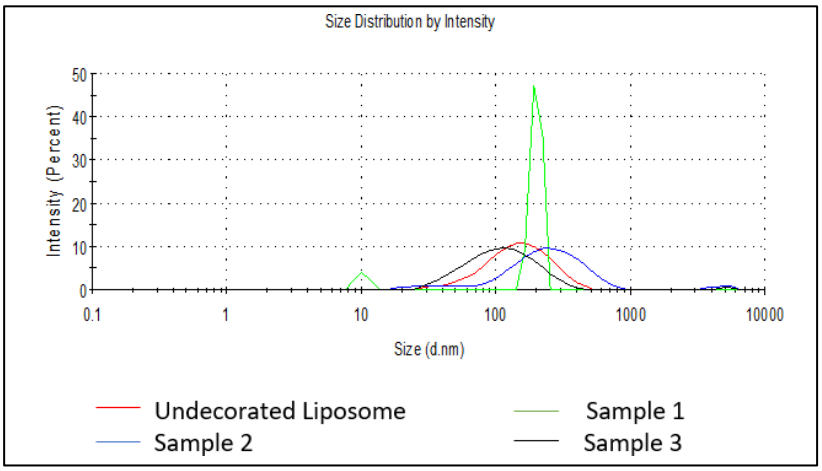
3. Results

In the first sample, only a small sample of HA-decorated flexible liposomes loaded with curcumin (HA-L1) was prepared and thus the sample was sonicated using a water bath ultrasound for 1 minute. HA-L1 exhibited an unusually large size of 3247 nm, indicating a poor-quality result (Table 1, Figure 1). The zeta potential analysis was not conducted. This large size could be attributed to a water bath ultrasound and a small suspension. Next, a bigger sample of HA-decorated flexible liposomes loaded with curcumin (HA-L2) was prepared and the sample was sonicated using a probe sonicator for 1 minute. The second sample showed improved size (179.2 nm), but the zeta potential was not ideal, and the polydispersity index (PDI) indicated a comparatively widely spread size distribution, potentially leading to liposome aggregation. The decision was then made to change the sonication time for the third sample (1 minute plus another 2 minutes after 24 hours). This resulted in the preparation of smaller size and stable HA-decorated flexible liposomes loaded with curcumin (HA-L2) with a size of 95.48 nm, a lower PDI of 0.247, and a stable zeta potential of -49.3 mV. Undecorated and unloaded flexible liposomes were prepared as a reference. However, for the undecorated and unloaded liposomes, the sonication time was 3 minutes in a go. The size and zeta potential obtained for the unloaded loaded undecorated flexible liposomes were acceptable for a transdermal carrier.

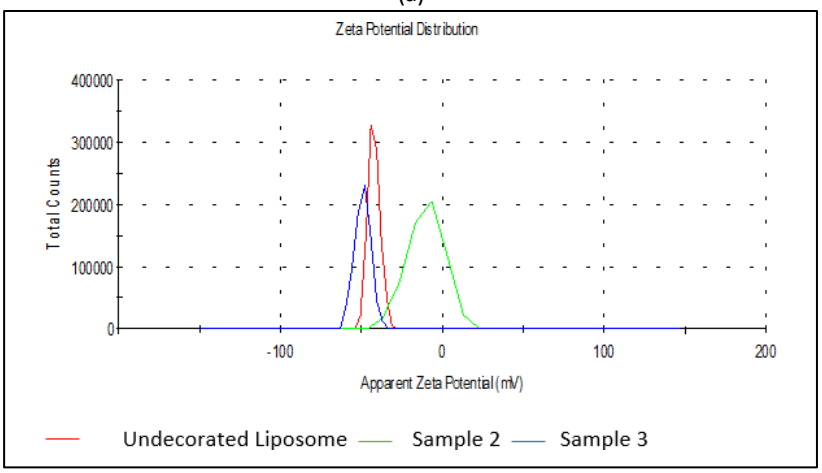
However, the size of the unloaded undecorated liposomes was smaller than the loaded decorated liposomes, possibly due to differences in sonication methods. Overall, both samples demonstrated good uniformity and stability based on the PDI and zeta potential values. Zeta potential determination is an important characterization technique to assess nanoparticle surface charge and physical stability. A zeta potential value outside the range of +30 mV to -30 mV provides sufficient repulsive forces for colloidal stability [14].

Table 1
Size, PDI and zeta potential analyses of the formulations

Sample	Average size (nm)	PDI	Zeta potential (mV)
HA-L1 (Sample 1)	3247	1.000	-
HA-L2 (Sample 2)	179.2	0.414	-10.3
HA-L3 (Sample 3)	95.48	0.247	-49.3
Undecorated flexible liposome	127.0	0.243	-42.4



(a)



(b)

Fig. 1. (a) Size dispersion and (b) zeta potential of different samples of flexible liposomes

FTIR analyses were carried out to determine the existence of functional groups and their interactions in the formulation. Figure 2 shows the spectra obtained for dipalmitoylphosphatidylethanolamine (DPPE), hyaluronan (HA), lecithin, HA conjugated to DPPE (HA-DPPE), curcumin (CUR), undecorated unloaded liposome (EL), curcumin loaded undecorated liposome (CUR-L) and curcumin loaded HA decorated liposome (HA-L). The spectra show prominent

peaks. The characteristic peaks for curcumin 1280 cm^{-1} pertained to the olefinic C-H bending vibration with a small shift on the spectrum of CUR-L and are associated with the amide linkages (-NH_2- and -NH-). The N-O symmetric stretching and C-O bond vibrations of curcumin are seen in the CUR-L system. The HA spectrum shows the amide bond of the NH_2. The chemical overlay in the mixture's spectrum proves the existence of the single compound's linkage or existence.

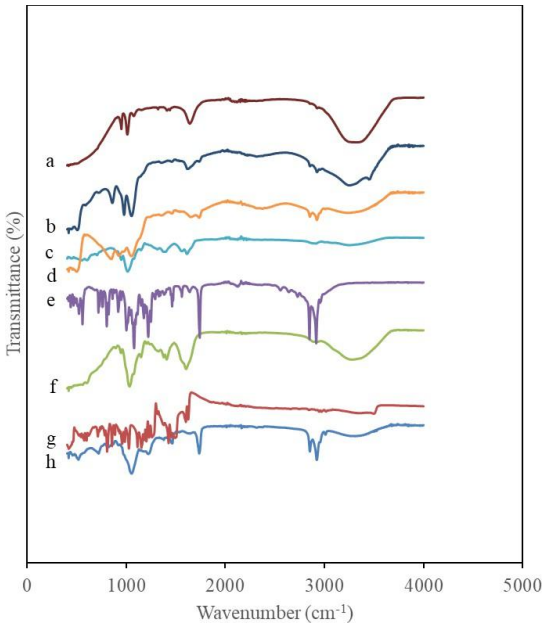


Fig. 2. FTIR Spectra of (a) HA-L (curcumin loaded HA-decorated liposome) (b) CUR-L (curcumin loaded undecorated liposome) (c) HA-DPPE (d) EL (unloaded undecorated liposome) (e) DPPE (f) HA (g) CUR and (h) Lecithin

Curcumin loaded and HA decorated flexible liposomes were then subjected to TEM analysis. The TEM image at 50000x magnification on a 50 nm scale length revealed a spherical vesicle at the appropriate size as determined using DLS analysis (Figure 3).

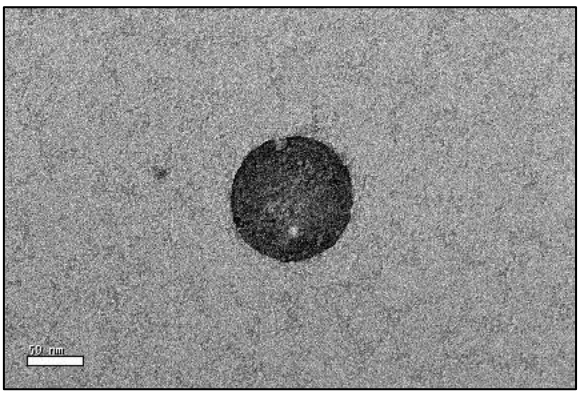


Fig. 3. TEM micrograph of curcumin loaded HA decorated flexible liposome with 50000x magnification at 50 nm scale length

Curcumin was analyzed using High-Performance Liquid Chromatography (HPLC) from Perkin Almer (St. Louis, MO, USA). Figure 4 shows the calibration curve obtained for the curcumin standard used in this study. The coefficient of correlation (R^2) value obtained was 0.9953. The observed retention time was around ±4.19 min.

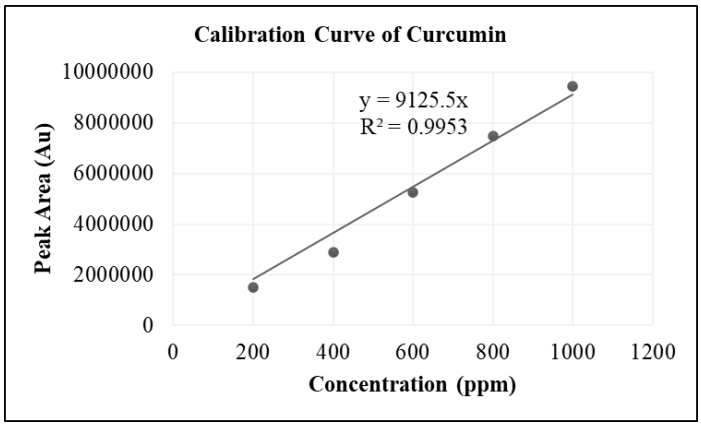


Fig. 4. Calibration curve of curcumin

The encapsulation efficiency calculated using Eq. (1) was quite low at 34.01%. It might be because some curcumin was not completely dissolved in the solvent, resulting in a low concentration of curcumin in the liposome. Liposome contamination was also possible when the metal probe tip was used during the sonication process. The phospholipids and encapsulated chemicals are both prone to degradation, leading to very low EE [15].

4. Conclusion

The conjugation of HA-DPPE was successfully obtained and characterized using FTIR. It was observed to be well-mixed in a gel texture. Curcumin loaded HA decorated flexible liposomes were successfully prepared using the HA-DPPE. The decorated flexible liposomes show the following characteristics: an average size of 95.48 nm (with 0.247 polydispersity index), a zeta potential of -49.3 mV and were spherical in shape. The encapsulation efficiency was quite low at 34.01% indicating further modifications in the formulation and/or process parameters should be made. To improve it, further research must be conducted to find the best solvents and the operating conditions for the solubility of HA and curcumin.

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