

# Proteinase K/Retinoic Acid-Loaded Cationic Liposomes as Multifunctional Anti-Acne Therapy to Disorganize Biofilm and Regulate Keratinocyte Proliferation

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**Background:** Simultaneous anti-*Cutibacterium acnes* and anti-inflammatory actions are highly beneficial in treating acne vulgaris. In this study, we present novel anti-acne nanovesicles based on liposomes loaded with proteinase K (PK), retinoic acid (RA), and soyaethyl morpholinium ethosulfate (SME) to achieve an effective and safe treatment.

**Materials and Methods:** This study examined in vitro planktonic and biofilm *C. acnes* elimination, as well as the keratinocyte proliferation suppression by liposomes. The multifunctional liposomes for treating *C. acnes* in mice were also evaluated.

**Results:** We acquired multifunctional liposomes with a size of 71 nm and zeta potential of 31 mV. The antimicrobial activity of SME was enhanced after liposomal encapsulation according to the reduction of minimum bactericidal concentration (MBC) by 6-fold. The multifunctional liposomes exhibited a synergistically inhibitory effect on biofilm *C. acnes* colonization compared with the liposomes containing PK or those containing SME individually. The adhesive bacterial colony in the microplate was lessened by 62% after multifunctional liposome intervention. All liposomal formulations tested here demonstrated no cytotoxicity against the normal keratinocytes but inhibited *C. acnes*-stimulated cell hyperproliferation. The in vitro scratch assay indicated that the liposomal RA—but not free RA—restrained keratinocyte migration. The animal study showed that free RA combined with SME and multifunctional nanovesicles had a similar effect on diminishing *C. acnes* colonies in the skin. On the other hand, liposomes exhibited superior performance in recovering the impaired skin barrier function than the free control. We also found that RA-loaded nanovesicles had greater skin tolerability than free RA.

**Conclusion:** The cationic liposomes containing dual PK and RA represented a potential treatment to arrest bacterial infection and associated inflammation in acne.

**Keywords:** liposome, acne, *Cutibacterium acnes*, proteinase K, retinoic acid, skin delivery

## Introduction

Acne vulgaris is a chronic inflammatory and recurrent skin disorder involved in appendages. The major symptoms of acne include comedones, pustules, papules, and erythema. The sebaceous gland hyperactivity leads to increased sebum secretion, *Cutibacterium acnes* proliferation, and hyperkeratosis.<sup>1</sup> Acne affects about 10% of the global population and >80% of young adolescents, ranking as the 8th most prevalent disease.<sup>2</sup> Although not life-threatening, acne produces significant psychological

effects of depression, anxiety, and insomnia.<sup>3</sup> The pathogenesis of acne demonstrates that *C. acnes* colonization activates keratinocytes and sebocytes to induce inflammation, resulting in follicular occlusion and hyperkeratinization.<sup>4</sup> *C. acnes*, a Gram-positive anaerobic bacterium, is reported to present >30% of the skin microbiota in acne patients.<sup>5</sup> Acne vulgaris therapy focuses on the intervention of antibiotics and anti-inflammatory agents. However, the prolonged use of antibiotics causes skin toxicity and drug-resistant *C. acnes* development. The resistance of *C. acnes* to antibiotics such as clindamycin, erythromycin, and tetracycline has gradually emerged.<sup>6</sup> The American Academy of Dermatology thus recommends the shortest possible times for the use of antibiotics on acne treatment.

*C. acnes* generates biofilm in the sebaceous glands to upgrade antibiotic resistance, comedones, and inflammation. This *C. acnes* biofilm incidence is greater in acne patients compared to healthy subjects.<sup>7</sup> Biofilm is a community of bacteria encased in an extracellular polymeric substance (EPS) attached to a surface. Sessile *C. acnes* in the EPS matrix can withstand the immune response and become resistant to antibiotics that fail to deliver into the biomass.<sup>8</sup> Thus, the novel acne treatment targeting *C. acnes* biofilm and the prevention of antibiotic resistance is urgent. The nanoparticles provide insight into improving the outcome of antibiofilm efficacy. Among these, liposomes have received significant attention in eradicating biofilm bacteria due to their capability to penetrate EPS and their versatility as antimicrobial agents.<sup>9</sup> Liposomes are made of phospholipid bilayers surrounding an aqueous core. They are widely employed in industrial and clinical applications because of their possible scale-up, biocompatibility, high drug encapsulation, and controlled drug delivery. These lipid-based nanovesicles can facilitate fuse with bacterial membranes and biofilm and are attributed to the affinity of phospholipid bilayers with biofilm.<sup>10</sup> The extracellular proteins are vital for the early adhesion of *C. acnes* biofilm to the surface.<sup>11</sup> Proteinase K (PK) is reported to be effective for digesting proteinaceous adhesins during the attachment of EPS.<sup>12</sup> Therefore, we aimed to design antibiofilm liposomes by encapsulating PK into the nanovesicles. The cationic liposomes loaded with quaternary ammonium cationic surfactants developed as broad-spectrum antibacterial agents have been studied previously.<sup>13,14</sup> Recently, soyaethyl morpholinium ethosulfate (SME) as a cationic surface active compound has broadened the perspective in nanomedicine due to its antibacterial activity and good biocompatibility.<sup>15,16</sup> Hence, this study also utilized SME as the anti-*C. acnes* molecule in liposomes.

In addition to antimicrobial drugs, topical retinoic acid (RA) is the first-line treatment option for acne therapy. During acne therapy, RA inhibits keratinocyte proliferation, inflammation, and microcomedone production.<sup>17</sup> The topical RA delivery poses challenges, including instability, poor absorption, and skin irritation. Most of the RA-treated acne patients meet cutaneous irritation, such as edema and peeling.<sup>18</sup> The delivery systems based on nanocarriers can overcome the limitation of RA intervention.<sup>19</sup> The available management methods for acne are still limited and remain unchanged for decades. Nevertheless, efficient and safe approaches with fewer complications can remarkably improve patient compliance.

This work aimed to formulate multifunctional liposomes containing PK, RA, and cationic surfactant as a combined acne therapy. We began this investigation by evaluating the effects of anti-*C. acnes* on the liposomes in both planktonic and biofilm forms. Then, we explored the proliferation and migration inhibition of versatile nanovesicles against keratinocytes. Liposomes have been used to deliver drugs into the skin by facilitating skin permeation and protecting drugs from degradation.<sup>20</sup> Finally, we conducted the topical treatment of acne by liposomes in a mouse model to appraise the possible application.

## Materials and Methods

### Materials and Reagents

Soybean phosphatidylcholine (SPC, Phospholipon 80H) was purchased from American Lecithin Company. RA, cholesterol, and crystal violet were provided by Sigma-Aldrich. PK and cell counting kit-8 (CCK-8) were obtained from BioTools. SME was from Croda. Reinforced clostridial medium (RCM) was obtained from Merck. The PrestoBlue cell viability kit was provided by Invitrogen. The transwell plate for keratinocyte migration assay was purchased from Millipore. The IVISense Bacterial 250 fluorescent probe was bought from Perkin Elmer.

## Preparation of Multifunctional Liposomes

The lipid-based nanovesicles were fabricated using the thin-film technique. SPC (230 mg), cholesterol (70 mg), and RA (5 mg) were dissolved in chloroform/methanol (2:1, 10 mL). This dispersion was deposited as a thin film in a round bottom flask using a rotary evaporator at 50°C under a vacuum. After 24 h, the thin film was hydrated by double-distilled water (ddH<sub>2</sub>O) under a probe-type sonication (VCX 600, Sonics and Materials) at 37 W for 30 min to produce the final nanovesicles (NV). Subsequently, PK (100 mg) and SME (30 mg) were added into ddH<sub>2</sub>O and chloroform/methanol, respectively, if necessary. The code name for the nanovesicles containing PK or SME alone was NV/PK and NV/SME, respectively. Meanwhile, the combined PK and SME in the nanovesicles had been given a code name as NV/PK/SME.

## Physicochemical Features of the Liposomes

The average size, polydispersity index (PDI), and zeta potential of the prepared nanovesicles were determined using a laser scattering technique (Nano ZS90, Malvern). The nanodispersion was diluted 100-fold by ddH<sub>2</sub>O before the detection. The encapsulation percentage of RA in the liposomes was estimated by the ultracentrifugation method to separate the entrapped RA from the free RA. The nanovesicles were centrifuged at 48,000 xg and 4°C for 40 min. Further, the encapsulated drug in the precipitate and free drug in the supernatant were analyzed by high-performance liquid chromatography (HPLC, Hitachi Series-2). Then, the precipitate was dissolved by acetonitrile before analysis. The HPLC condition was described in a previous study.<sup>21</sup>

## Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

*C. acnes* (ATCC6919) was cultivated in RCM at 37°C under anaerobic status for 3 days. The MIC of liposomes was described as the lowest SME concentration necessary to inhibit *C. acnes* growth by serial dilution. The bacteria were diluted in RCM to obtain the optical density at 600 nm (OD<sub>600</sub>) of 0.01. The *C. acnes* solution was exposed to several dilutions of liposomal dispersions and then incubated at 37°C for 48 h. We used a microplate reader (Epoch2, BioTek) to determine the MIC at 600 nm. The colony-forming unit (CFU) of *C. acnes* was counted after liposome treatment for 48 h. The highest dilution of liposomes, resulting in a 99.9% eradication of *C. acnes* number, was acknowledged as MBC value.

## *C. acnes* Growth Curve

The growth inhibition of *C. acnes* by the lipid nanovesicles over 108 h was detected in 96-well microplates. The liposomes containing SPC at 0.72 µg/mL were inoculated with bacteria (OD<sub>600</sub>=0.01) at 37°C. The liposomes were diluted with RCM if necessary. The microplate reader was used to analyze each well's absorbance (600 nm) and reveal the bacterial growth in real-time.

## Disk Diffusion Assay

We placed *C. acnes* (OD<sub>600</sub>=0.7) on the agar plate. The prepared liposomes with different dilutions (1/32, 1/16, 1/8, 1/4, 1/2, and 1) and ddH<sub>2</sub>O were loaded onto the agar. After incubating for 48 h, the clear zone diameter without bacteria was measured.

## *C. acnes* Viability Assay by SYTO 9

The viability of *C. acnes* after treatment of nanovesicles for 24 h was visualized using SYTO 9 green fluorescent nucleic acid staining. *C. acnes* was grown to OD<sub>600</sub>=0.01 and then treated with liposomes at 37°C for 24 h. The microbes were incubated with SYTO 9. After a 15-min reaction, the strain was observed under confocal microscopy (TCS SP2, Leica).

## *C. acnes* Survival Outside and Inside the Biofilm

The bacteria were cultivated anaerobically using RCM at 37°C for 3 days. The culture was diluted to OD<sub>600</sub>=0.1 by RCM supplemented with 1% glucose. The bacterial suspension (1 mL) was pipetted into the 24-well microplate and incubated at 37°C for 3 days. The biofilm was developed on the well bottom after supernatant removal. The nanovesicles with

different dilutions (1/32, 1/16, 1/8, and 1/4) were treated to the biofilm for 24 h. Then, the recovered *C. acnes* outside and inside the biofilm were loaded on the agar plate to measure CFU.

## Biofilm Structure Observation by Scanning Electron Microscopy (SEM)

The SEM analysis was performed to visualize the effect of liposomes on biofilm formation. The *C. acnes* biofilm was treated with liposomes in the 24-well plate at 37°C for 24 h under an anaerobic condition. The well was washed twice with PBS and fixed in 3% glutaraldehyde and 2% paraformaldehyde for 2 h. After washing, the sample was dehydrated in a graded ethanol series (30%–95%), then dried and coated with gold. The sample was visualized under SEM (SU-8220, Hitachi).

## Biofilm Metabolic Activity

The *C. acnes* biofilm metabolism was determined using the PrestoBlue cell viability kit. PrestoBlue is a resazurin-based metabolism assay that detects metabolically active bacteria or cells. The *C. acnes* biofilm in the microtiter plate was treated with liposomes at 37°C for 10 h. After adding the PrestoBlue reagent (10%) to the biofilm, the plate was incubated for 1 h, and the fluorescence intensity was read at 570 nm (excitation) and 600 nm (emission) using a microplate reader.

## Biofilm Adhesion Assay

*C. acnes* biofilm adhesion was assessed in a static condition in the microplate. The *C. acnes* ( $OD_{600}=0.1$ ) allowed adhering at 37°C for 1 h. Subsequently, the nanovesicles were treated to the biofilm for 10 h. After rinsing with PBS and staining with 0.1% crystal violet, the CFU of the adherent microbes was measured.<sup>22</sup>

## Microfluidic Chamber Analysis

The *C. acnes* biofilm was developed in a flow cell chamber (Ibidi  $\mu$ -Slide 1<sup>0.2</sup> Luer) for 2 days under an anaerobic condition at 37°C. The nanovesicles and SYTO 9 were injected into the biofilm-loaded flow chamber by a syringe pump with a flow rate of 5  $\mu$ L/min. The liposome-treated biofilm attached to the chamber surface was visualized by confocal microscopy (TCS SP8X, Leica) from 45 to 225 min.

## Keratinocyte Viability Assay

The human-derived keratinocytes (HaCaT, AddexBio) were maintained in DMEM with 10% heat-inactivated FBS and 100 U/mL antibiotic/antimycotic. The keratinocyte viability after the nanovesicle intervention was explored by employing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The HaCaT ( $1 \times 10^5$  cells/mL) was exposed with liposomes containing RA (0–2  $\mu$ g/mL) at 37°C for 24 h. Meanwhile, the MTT reagent (0.5 mg/mL) in the culture medium (100  $\mu$ L) was incorporated into the cells and incubated for 3 h. The keratinocyte viability was detected using a microplate reader at 595 nm.

## Keratinocyte Proliferation Assay

HaCaT cells ( $5 \times 10^4$  cells/mL) were plated in a 96-well plate for 24 h. Then, the heat-killed *C. acnes* (h-*C. acnes*, the multiplicity of infection (MOI)=75) and liposomes were added into the wells for 24 and 48 h, respectively. The h-*C. acnes* was prepared by incubation at 100°C for 30 min.<sup>23</sup> The dead bacteria were stored at –20°C before use. The h-*C. acnes*-induced keratinocyte proliferation inhibited by RA-loaded nanovesicles was measured with CCK-8.

## Keratinocyte Migration Assay

The keratinocyte migration inhibition by RA-loaded liposomes was studied by in vitro scratch assay. HaCaT cells ( $4 \times 10^5$  cells/mL) were cultured in culture-insert with 2 wells (Ibidi) at 37°C for 24 h. The insert was then transferred by sterilized tweezers, followed by cell washing with DMEM to remove the detached cells.<sup>24</sup> Then, the h-*C. acnes* (MOI=75) and liposomes were added to the cell suspension. After a 20-h treatment, the image was taken with optical microscopy (Axiovert 200, Zeiss). The cell-free area was quantified using the ImageJ software.

## Keratinocyte Transwell Invasion Assay

The transwell invasion is another platform that rates the keratinocyte migration inhibition by the RA-loaded nanovesicles. This experiment was conducted in a transwell plate with a pore size of 8  $\mu\text{m}$ . In addition, the HaCaT suspension ( $5 \times 10^5$  cells/mL) was treated with h-*C. acnes* (MOI=75) or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , 20 ng/mL) for 24 h. The liposomes were also added into the cell suspension simultaneously. Then, the keratinocytes were diluted to  $1 \times 10^5$  cells/mL and placed on the upper chamber of the Transwell. The culture medium was subsequently filled into the lower chamber. After being incubated for 24 h, the crystal violet (0.1%) was used to stain the migrated HaCaT in the lower well. The image was taken by optical microscopy. The cell number in the lower well was also quantified using a microplate reader at 595 nm.

## Animals

The Balb/c mice and nude mice (6 weeks old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The Institutional Animal Care and Use Committee of Chang Gung University approved all animal experiments. The experimental processes complied with the European Community legislation (Directive 86/609/EEC).

## In vivo *C. acnes* Infection Treated by Topical Liposomes

The mouse was anesthetized to shave the dorsal skin. *C. acnes* suspension ( $1 \times 10^7$  CFU, 150  $\mu\text{L}$ ) was subcutaneously injected into the dorsal area. The liposomes (200  $\mu\text{L}$ ) were topically administered on the infected skin region every 24 h for 3 days, and the transepidermal water loss (TEWL) was recorded daily using Tewameter TM300 (Courage and Khazaka). After a 3-day treatment, the appearance of the skin was observed using a digital magnifier (Dino-Lite). The IVISense Bacterial 250 fluorescent probe was subcutaneously injected into the infection site to observe the *C. acnes* residence by the in vivo imaging system (IVIS, Lumina LT, PerkinElmer).

## In vivo Skin Tolerance of Topical Liposomes

The liposomal or free RA (0.2 mL) was added on a gauze pad ( $1 \times 1 \text{ cm}^2$ )—which was applied to the nude mice's back—and then fixed with Tegaderm dressing. The pad was removed daily; new ones were applied for 5 consecutive days. The TEWL was monitored every day. At the end of the experiment (Day 5), the skin appearance was recorded using a digital magnifier. After excising the treated skin area, the skin was sectioned for hematoxylin and eosin (H&E) staining to examine the histological change after formulation administration.

## Statistical Analysis

The Kruskal–Wallis test was employed to analyze the statistical difference in the data from different treatment groups. Meanwhile, a posthoc test to check the individual difference was carried out using Dunn's test. A 0.001, 0.01, or 0.05 level of probability was considered statistically significant.

## Results

### Physicochemical Features of the Liposomes

All liposomes were prepared using the thin-film method. The size of blank liposomes (NV) was 82 nm, as determined by dynamic light scattering (Table 1). On the other hand, PK (NV/PK) incorporation increased the hydrodynamic diameter to 112 nm, whereas SME incorporation (NV/SME) reduced the vesicle size to  $66 \pm 0.4$  nm. The combined PK and SME in liposomes (NV/PK/SME) exhibited an average size of  $71 \pm 0.6$  nm. PDI was below 0.32 for all liposomes tested, verifying a narrow distribution and homogeneity in size. NV and NV/PK were negatively charged at  $-35 \pm 0.8$  and  $-47 \pm 0.9$  mV, respectively, because of SPC's anionic feature. The nanovesicles became positively charged after adding cationic surfactant SME (NV/SME and NV/PK/SME). The RA loading did not significantly change the liposomal diameter and surface charge because of the very low percentage (0.05%) of RA in the liposomes. NV and NV/PK showed more than 80% encapsulation percentage for RA loading. SME incorporation (NV/SME and NV/PK/SME) reduced RA encapsulation to about 63%.

**Table 1** The Characterization of Liposomes by Vesicle Size, Polydispersity Index (PDI), Zeta Potential, and RA Encapsulation Percentage

Formulation	Size (nm)	PDI	Zeta Potential (mV)	RA Encapsulation (%)
NV	81.5±0.3	0.32±0.01	-34.5±0.8	84.3±0.01
NV/PK	111.8±0.9	0.17±0.01	-46.7±0.9	80.2±0.02
NV/SME	65.7±0.4	0.32±0.01	32.3±4.9	64.9±0.03
NV/PK/SME	71.2±0.6	0.26±0.01	31.3±1.5	62.7±0.04

**Note:** Each value represents the mean±SEM (n=3).

**Abbreviations:** NV, nanovesicles; PK, proteinase K; SME, soyaethyl morpholinium ethosulfate.

**Table 2** The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Free PK, Free SME, and Liposomes Based on SME Concentration (µg/ml)

Formulation	MIC (µg/mL)	MBC (µg/mL)
Free PK	>125	>125
Free SME	0.98–1.96	46.88–93.75
NV	>125	>125
NV/PK	>125	>125
NV/SME	0.49	15.63–31.25
NV/PK/SME	0.49	7.81–15.63

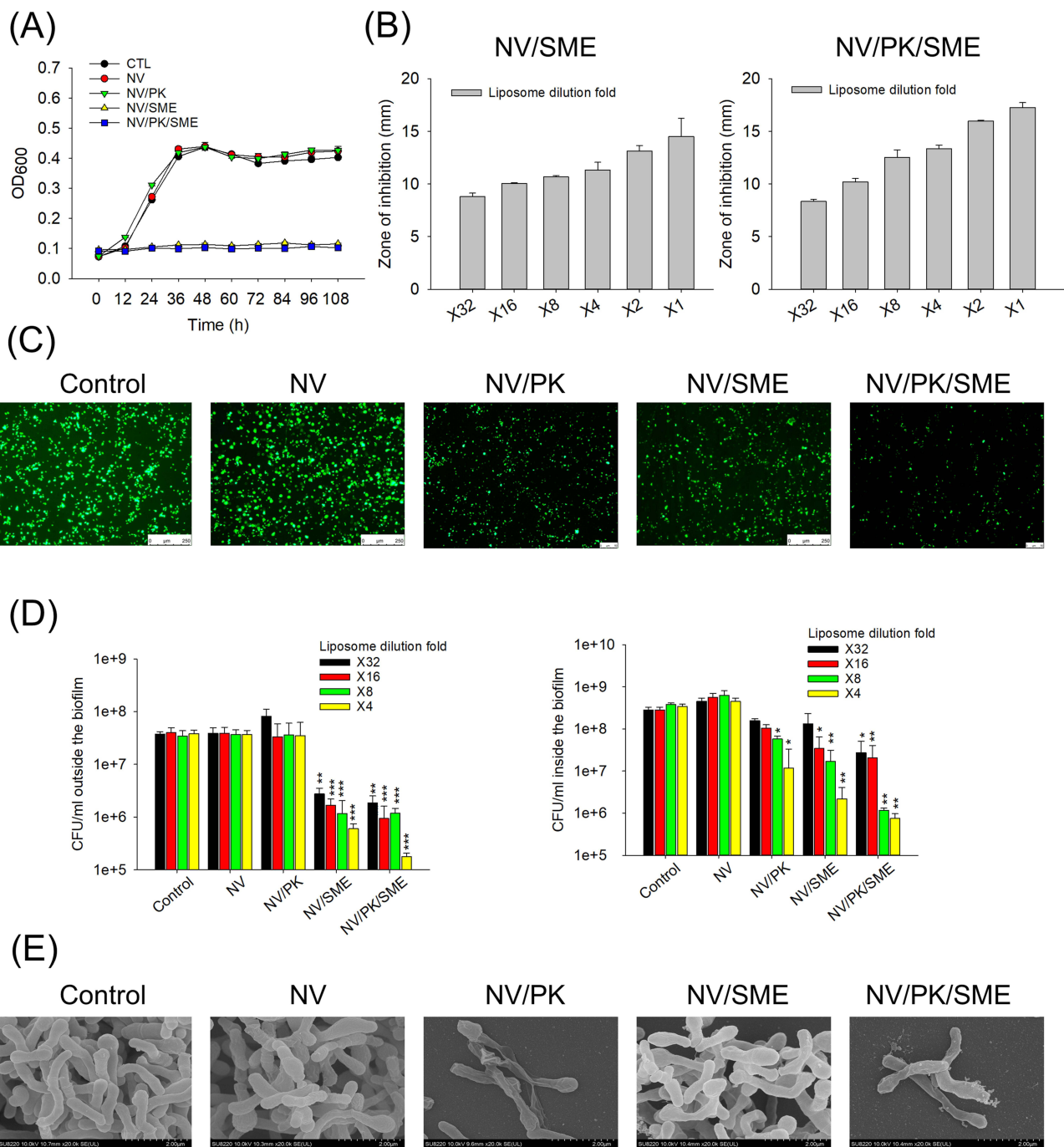
**Note:** All data are presented as the range of three experiments.

## Anti-*C. acnes* Activity of the Liposomes

We first measured MIC and MBC values of free and liposomal PK or SME. MIC is recognized as the lowest concentration to inhibit bacterial growth by serial dilution. Noteworthy, the formulations without SME (free PK, NV, and NV/PK) showed no effect on *C. acnes* growth suppression. The MIC levels of free SME were determined as 0.73–1.47 µg/mL for planktonic *C. acnes* (Table 2). The SME inclusion in liposomes (NV/SME and NV/PK/SME) decreased MIC to 0.49 µg/mL. This 2–4-fold reduction indicated that the nanovesicles offered dose attenuation in terms of SME concentration. MBC is the lowest bactericidal concentration that kills 99.9% of microbes. The MBC against *C. acnes* was 46.88–93.75 µg/mL for free SME (Table 2). The SME loading in liposomes (NV/SME) led to greater bactericidal activity with MBC of 15.63–31.25 µg/mL. Meanwhile, the PK inclusion in SME-loaded nanovesicles (NV/PK/SME) further enhanced the bactericidal effect (MBC=7.81–15.63 µg/mL). The effect of the nanovesicles on the growth curve of *C. acnes* was assessed at an SME concentration of 15.63 µg/mL (MBC of the NV/SME). The time-kill curves for liposomal SME (NV/SME and NV/PK/SME) indicated that MBC was sufficient to inhibit almost all *C. acnes* (Figure 1A). The bacterial population incubated with the nanovesicles without SME (NV and NV/PK) demonstrated no *C. acnes* growth inhibition.

The prepared liposomes were diluted to different folds by ddH<sub>2</sub>O to measure the *C. acnes* inhibition zone in the disk diffusion test. There is no clear inhibition zone in the agar for the liposomes without SME (NV and NV/PK) (Figure 1B). NV/SME and NV/PK/SME exhibited a large inhibition zone on the agar media in a concentration-dependent manner. The inhibition zone against *C. acnes* ranged between 8.3 and 14.5 mm for NV/SME. Adding PK in the liposomes (NV/PK/SME) further enlarged the inhibition zone to 8.8–17.3 mm. The *C. acnes* viability was visualized using SYTO 9 as a fluorescent dye. In the confocal image, the blank liposomes (NV) could not diminish live bacteria compared to the untreated control (Figure 1C). An inferior green intensity due to SYTO 9 was detected after NV/PK and NV/SME treatments, suggesting the decrease of live *C. acnes*. Inserting the combined PK and SME in liposomes (NV/PK/SME) further decreased the green fluorescence, confirming the synergistic effect on *C. acnes* killing.

Furthermore, we examined the capability of liposomes to eradicate the *C. acnes* biofilm. The *C. acnes* CFU outside and inside the biofilm was counted with a log transformation. NV and NV/PK were devoid of the activity to lessen the



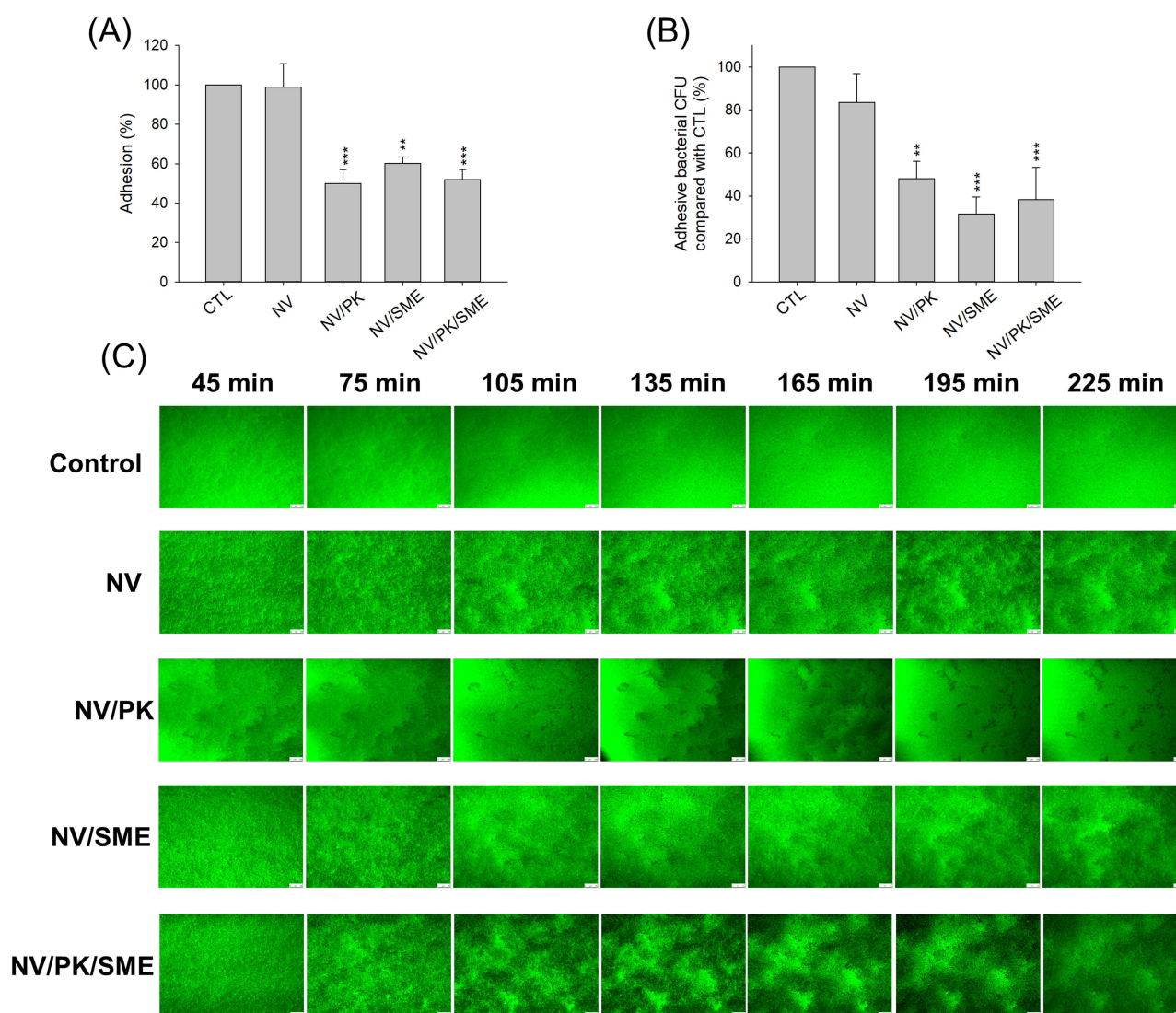
**Figure 1** Determination of anti-*C. acnes* activity in planktonic or biofilm forms by liposomes: **(A)** the time-killing curves of liposomes during 108 h; **(B)** the diameter of inhibition zone (mm) after liposome treatment in the agar plate; **(C)** confocal image of live *C. acnes* stained with SYTO 9 after liposome treatment; **(D)** *C. acnes* CFU outside and inside the biofilm after liposome treatment; and **(E)** the biofilm structure observed by SEM after liposome treatment. All data are presented as the mean of three experiments  $\pm$  S.E.M. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , or \* $p < 0.05$  as compared to the untreated control group.

viable bacteria outside the biofilm (the left panel of Figure 1D). The bacteria outside the biofilm showed a significant decrease in CFU upon treatment with SME-loaded liposomes (NV/SME and NV/PK/SME). The combination of PK and SME demonstrated a 2-log reduction of the *C. acnes* population outside the biofilm. The blank nanovesicles (NV) lacked the capacity to eradicate *C. acnes* inside the biofilm (the right panel of Figure 1D). A dose-dependent inhibition of biofilm *C. acnes* was found for NV/PK and NV/SME. The combined treatment using PK and SME appeared to be synergistic when *C. acnes* CFU was decreased inside the biofilm. SEM observation allows the understanding of biofilm

structure change after liposome interaction. In the untreated control, *C. acnes* formed a dense biofilm composed of microbe aggregate with a pleomorphic rod shape (Figure 1E). The three-dimensional and well-structured network was still observed after the treatment of blank liposomes (NV). Treatment with NV/PK resulted in disaggregated and elongated bacteria with wrinkled and punctured membranes. NV/SME-treated biofilm was associated with a disintegrated cluster, along with the destabilization of the bacterial membrane. NV/PK/SME abolished the biofilm matrix together with the elongated and ruptured *C. acnes* appearance.

## Biofilm Metabolic Activity and Adhesion

An initial surface contact followed by irreversible adhesion is required for biofilm formation. Here we examined whether the liposomes could prevent the adherence of biofilm in the microtiter plate. The biofilm adhesion percentage estimated by PrestoBlue determined the adhesion level by the metabolic activity of *C. acnes*. The biofilm treated with NV revealed an adhesion percentage similar to the untreated control (Figure 2A). We found that when biofilm was incubated in the presence of NV/PK, the PK-loaded liposomes suppressed the adhesion to  $50 \pm 7.2\%$ . Compared to the control, the adhesion treated with



**Figure 2** Determination of anti-adhesion activity of biofilm *C. acnes* by liposomes: **(A)** the adhesion percentage of the biofilm detected by PrestoBlue after liposome treatment; **(B)** the adhesive *C. acnes* CFU percentage of the biofilm detected after liposome treatment; and **(C)** the biofilm mass attached on the surface of microfluidic chamber observed by confocal microscopy. All data are presented as the mean of three experiments  $\pm$  S.E.M. \*\*\* $p < 0.001$  or \*\* $p < 0.01$  as compared to the untreated control group.

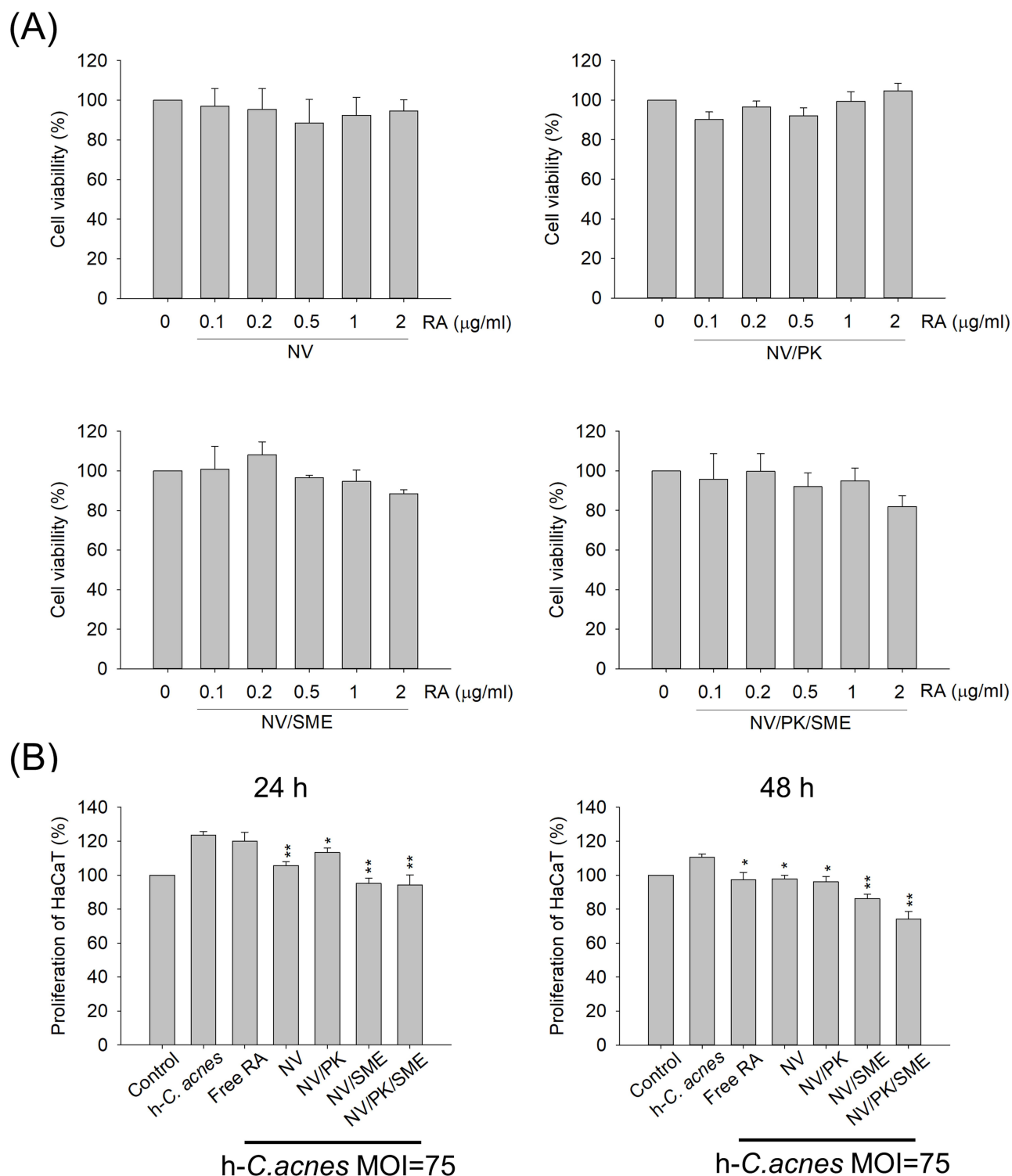


NV/SME and NV/PK/SME displayed a metabolic activity of  $60\pm 3.2\%$  and  $52\pm 5.1\%$ , respectively. We also determined the *C. acnes* CFU adhered in the microplate after a 10-h liposome treatment, while the blank liposomes (NV) were devoid of *C. acnes* adherence (Figure 2B). PK incorporation (NV/PK) significantly decreased the adherence to  $48\pm 8.1\%$ . The bacterial adhesion was inhibited by SME-loaded liposomes (NV/SME and NV/PK/SME) to a similar level to NV/PK. The flow cell chamber assay was established to monitor the effect of liposomes on surface attachment and biofilm formation. The biofilm patch was formed by the preferential adhesion of *C. acnes* to the microfluidic channel. The continuous flow of ddH<sub>2</sub>O (control) did not eliminate the biofilm attached to the channel surface (Figure 2C). A similar phenomenon was observed in the confocal image of NV-treated biofilm. A partial dispersal of the adhered biofilm occurred after the continuous injection of NV/PK and NV/SME into the flow cell. Biofilm observation by confocal microscopy displayed a significant matrix degradation and structural integrity loss after combined treatment with PK and SME (NV/PK/SME). This phenomenon suggested a synergistic effect on biofilm destruction.

## Keratinocyte Proliferation Regulation by the Liposomes

The multifunctional liposomes were loaded with RA to treat acne-associated keratinocyte proliferation and migration. The biocompatibility of the nanovesicles on the human keratinocyte cell line was tested using an MTT assay. Five different concentrations (0.1–2  $\mu\text{g/mL}$ ) of RA loaded in liposomes were incubated with keratinocytes for 24 h. The result showed that the liposomes were not cytotoxic at concentrations up to 2  $\mu\text{g/mL}$ , as the cell viability was kept close to the untreated control (Figure 3A). The keratinocyte viability was found to be  $>80\%$  for all RA liposomes tested. This finding suggests that the liposomes can be used for inhibiting keratinocyte activation at  $\leq 2 \mu\text{g/mL}$  without causing damage to mammalian cells. The *C. acnes*-induced proliferation and migration of keratinocytes are crucial factors in acne development. We conducted a CCK-8 assay to investigate the effect of RA-loaded nanovesicles on keratinocyte cell proliferation regulation. HaCaT cell proliferation was increased by 1.3-fold after a 24-h stimulation with h-*C. acnes* (the left panel of Figure 3B). Meanwhile, free RA at 2  $\mu\text{g/mL}$  did not reduce the h-*C. acnes*-induced proliferation. Treatment with different types of RA-loaded liposomes significantly suppressed proliferation. The liposomes containing SME (NV/SME and NV/PK/SME) showed greater proliferation repression than those without SME (NV and NV/PK). The free RA exhibited the ability to suppress proliferation as the incubation time was prolonged to 48 h (the right panel of Figure 3B). The inhibition level of the liposomes without SME (NV and NV/PK) was comparable to that of free RA. Similar to the 24-h treatment, the inhibition was pronounced with 48 h of incubation by SME-containing nanovesicles (NV/SME and NV/PK/SME).

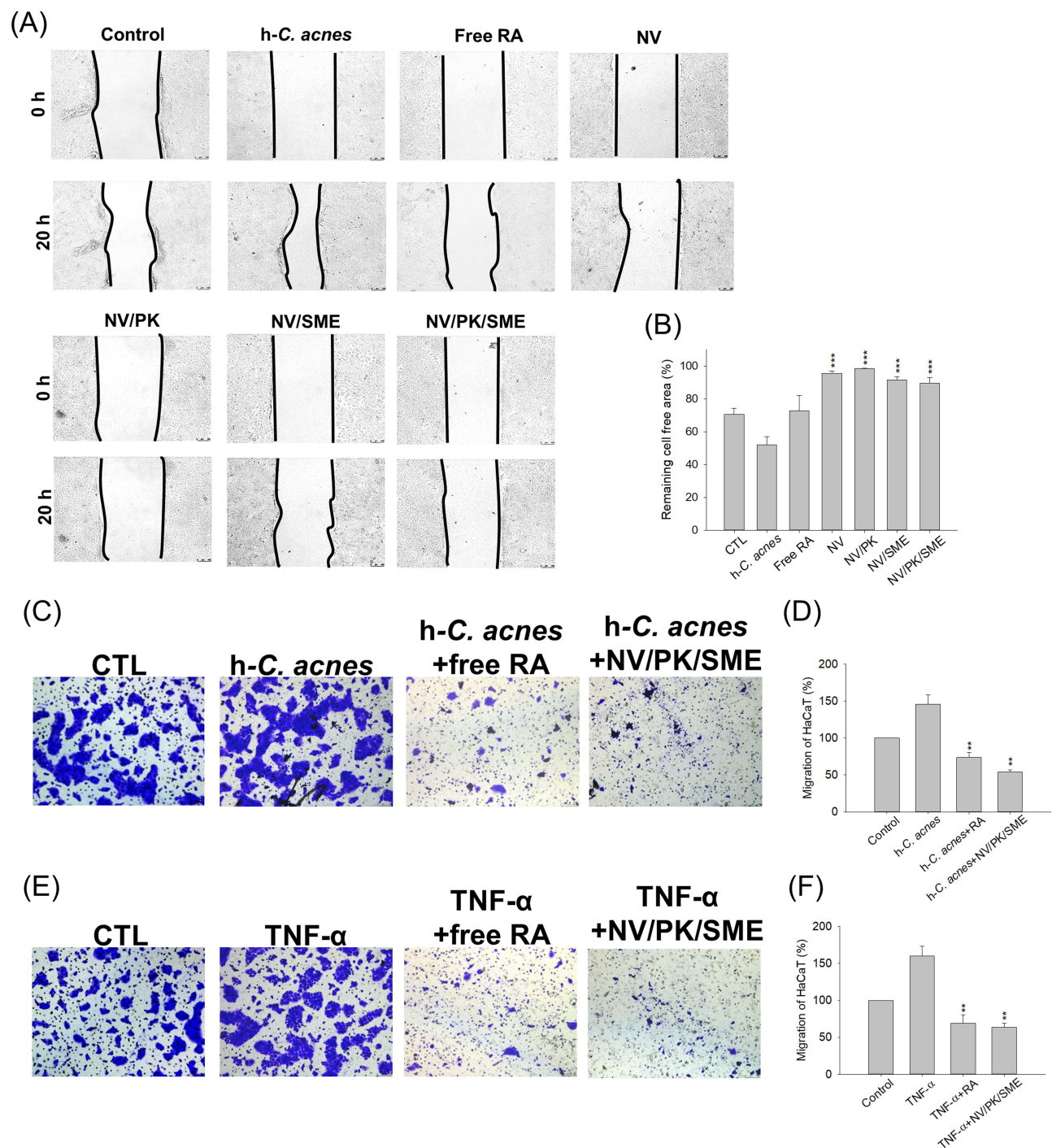
The cell-based scratch analysis was utilized to examine cell proliferation and migration. This evaluation can unveil whether a specific compound or liposomal formulation slows down the rate of proliferation or migration. The HaCaT cells that underwent no treatment (control) displayed wound closure after a 20-h incubation (Figure 4A). A further wound closure due to the proliferation and migration of keratinocytes was visualized 20 h later using h-*C. acnes*. No significant suppression in scratch closure was detected in the free RA (2  $\mu\text{g/mL}$ ) treatment. Migratory keratinocytes were inhibited by all nanoformulations tested. We calculated the remaining cell-free area in the culture insert (Figure 4B). The areas for the four liposomal formulations were approximately 90%–98%. The transwell invasion test is another evaluation platform that detects cell migration. Keratinocytes were subjected to the upper layer of the transwell; the number of keratinocytes migrating to the bottom layer was appraised. The crystal violet colorimetric analysis indicated a significant cell migration after a 24-h incubation (Figure 4C). This migration was exceptionally elevated with h-*C. acnes* stimulation by 1.5-fold compared with the basal control. A substantial suppression was noticed after free RA and equivalent NV/PK/SME liposomes were treated. The violet crystal quantification result showed that free and liposomal RA reduced the migration by 49% and 63%, respectively (Figure 4D). The migration of HaCaT cells was increased by activating another stimulator TNF- $\alpha$  (Figure 4E). Free RA and NV/PK/SME provided considerably significant inhibition on the migration. The migration was decreased by 57% and 60% with the treatment of free and liposomal RA, respectively (Figure 4F).



**Figure 3** The effect of liposomes on the cytotoxicity and proliferation regulation of human keratinocytes: **(A)** the cell viability of keratinocytes determined by MTT assay; and **(B)** the proliferation of *h-C. acnes*-stimulated keratinocytes after liposome treatment. All data are presented as the mean of three experiments  $\pm$  S.E.M.  $^{**}p < 0.01$  or  $^{*}p < 0.05$  as compared to the *h-C. acnes* treatment group without liposome intervention.

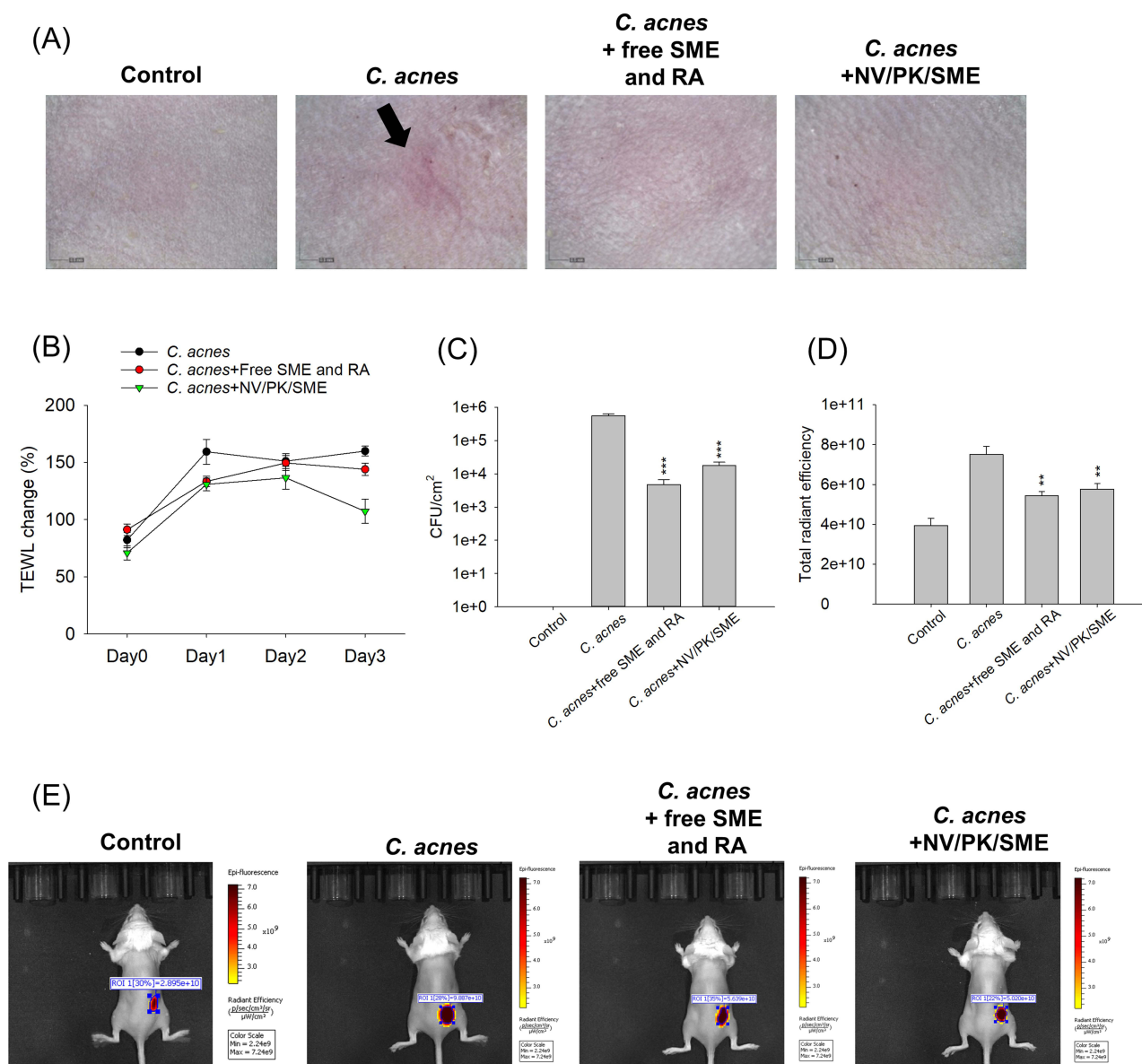
## In vivo *C. acnes* Infection Treated by Topical Liposomes

The in vivo anti-*C. acnes* effect of the multifunctional nanovesicles (NV/PK/SME) loaded with RA was evaluated in mice. Free RA combined with SME was also tested at a dose equivalent to that in liposomes. The animals were subcutaneously infected with *C. acnes*, followed by topical application of free drugs or liposomes. In the microscopic



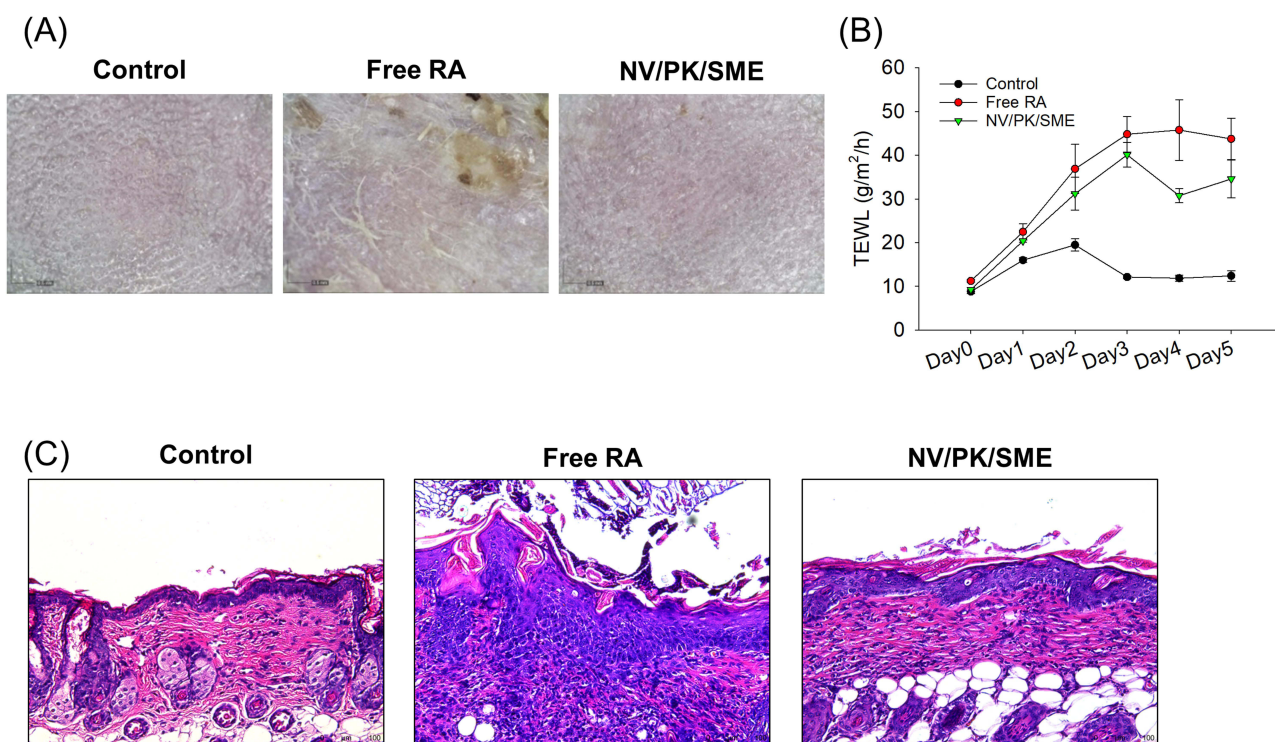
**Figure 4** The effect of liposomes on the proliferation and migration regulation of human keratinocytes: **(A)** the migration of h-C. *acnes*-stimulated keratinocytes after liposome treatment as determined by in vitro scratch assay; **(B)** the measurement of remaining cell free area based on the in vitro scratch assay; and **(C)** the migration of h-C. *acnes*-stimulated keratinocytes after liposome treatment as determined by in vitro transwell assay; **(D)** the measurement of crystal violet-stained h-C. *acnes*-stimulated keratinocytes in the bottom layer based on the in vitro transwell assay; **(E)** the migration of TNF- $\alpha$ -stimulated keratinocytes after liposome treatment as determined by in vitro transwell assay; and **(F)** the measurement of crystal violet-stained TNF- $\alpha$ -stimulated keratinocytes in the bottom layer based on the in vitro transwell assay. All data are presented as the mean of three experiments  $\pm$  S.E.M. \*\*\* $p < 0.001$  or \*\* $p < 0.01$  as compared to the stimulator treatment group without liposome intervention.

appearance of the mouse skin, the subcutaneous invasion of *C. acnes* resulted in the skin abscess with erythema signs (the arrow in Figure 5A). Both free SME/RA and liposomes were protected against the cutaneous damage caused by infection. The TEWL was significantly increased by *C. acnes* injection (Figure 5B), suggesting a skin barrier dysfunction after the infection. The drug formulations partially recovered the increased TEWL, with liposomes showing superior



**Figure 5** In vivo anti-acne effect of liposomes in the *C. acnes*-infected mouse model: **(A)** the appearance of mouse skin surface; **(B)** the TEWL level of the mouse skin; **(C)** *C. acnes* CFU in skin; **(D)** the average radiance intensity of *C. acnes* burden (labeled with Xenolight Bacterial Detection Probe) in skin of the mouse quantified by in vivo imaging system; and **(E)** the *C. acnes* burden in skin of the mouse observed by in vivo imaging system. All data are presented as the mean of six experiments  $\pm$  S.E.M. \*\*\* $p < 0.001$  or \*\* $p < 0.01$  as compared to the *C. acnes* infection group without RA treatment.

recovery than free SME/RA. The *C. acnes* colonization in the infected skin area was  $5.5 \times 10^5$  CFU/cm<sup>2</sup> after bacterial invasion for 3 days (Figure 5C). A 2-log reduction of CFU was found after topical delivery of the free drug group. The number of CFU in the skin treated with the multifunctional liposomes was significantly lower than that of the control with a 1.5-log reduction. The Xenolight Bacterial kit was injected into the *C. acnes*-infected region to visualize the bacterial aggregate by IVIS. There were some bacteria-associated signals in the healthy animals (Figure 5D and E), which could be derived from the resident bacterial flora other than *C. acnes*. The bacterial administration increased the fluorescence signal in the skin, suggesting a successful *C. acnes* colonization. The topical delivery of free SME/RA and nanovesicles decreased *C. acnes* accumulation with a comparable level. Both the fluorescence zone and intensity were lowered by drug treatment.



**Figure 6** The in vivo skin tolerance of the liposomes in the healthy mouse: **(A)** the appearance of mouse skin surface; **(B)** the TEWL level of the mouse skin; and **(C)** the H&E-stained histology of mouse skin treated by liposomes. All data are presented as the mean of five experiments  $\pm$  S.E.M.

## In vivo Skin Tolerance of Topical Liposomes

The possible skin irritation elicited by topical RA was analyzed in vivo. The photograph of the mouse dorsal skin treated with ddH<sub>2</sub>O for 5 days (control) exhibited an unharmed appearance with a smooth surface (Figure 6A). Some skin lesions were detectable in the free RA-treated group that revealed erythema, crust, and edema in the superficial layer. We observed fewer skin lesions in the liposome-treated group than in the free RA. The ddH<sub>2</sub>O treatment (control) slightly increased TEWL on Day 1 and Day 2; then, the value recovered to baseline control after 3 days (Figure 6B). Free RA greatly disrupted the skin barrier function as evidenced by a large increase of TEWL. This increase could be partially relieved after encapsulation of RA into nanovesicles. The ddH<sub>2</sub>O application exhibited no histopathological change based on H&E staining (Figure 6C). Free RA induced scaling, epidermal hyperplasia, and immune cell infiltration after a consecutive 5-day challenge. In the case of topical liposome application, the skin section manifested persistence against epidermal thickening and inflammatory cell diffusion. The signs of these damages showed less severity than those of free RA treatments.

## Discussion

The dual therapy of antibacterial and anti-inflammatory agents is commonly used for acne treatment. However, the currently available treatments are often unsatisfactory for complete response because of antibiotic resistance and skin irritation.<sup>25</sup> The failure of antibiotic acne treatment can be associated with biofilm-mediated resistance. While most antimicrobial agents are designed to eradicate planktonic microbes, they are usually devoid of treating biofilm infection.<sup>10</sup> One of the attempts to overcome the resistance threat is designing antibacterial nanoparticles. In this study, we developed cationic liposomes incorporated with PK as antibiofilm formulations to efficiently eliminate *C. acnes*. The combined PK and SME revealed a synergistic effect in diminishing the *C. acnes* burden in the biofilm. This combination also synergistically disintegrated the biofilm architecture. The liposomes were also loaded with RA to prevent keratinocyte proliferation and migration induced by *C. acnes*. Topical RA treatment is ideal for providing increased local drug bioavailability. Skin irritation during topical RA therapy can lower compliance with long-term

treatment.<sup>26</sup> An efficient topical anti-acne therapy should deliver the drugs to the target skin site with minimal irritation. Notably, our liposomes effectively lessened *C. acnes* accumulation and skin toxicity in the mouse model.

SME was the main active in the liposomes for eradicating *C. acnes*. The cationic surfactants can be utilized as capping agents to change the physicochemical characteristics of the nanoparticles. The nanovesicle size was decreased by SME incorporation because of the emulsification effect of the surfactant to reduce surface-free energy. All liposomal formulations developed in this study exhibited a size of <120 nm, confirming the nano-sized range of the prepared products. The surface charge is an index used to probe the stability of nanoparticles. It is generally regarded that the nanovesicles with zeta potential above +30 mV or below -30 mV are highly stable due to the electrostatic repulsion.<sup>27</sup> Meanwhile, the zeta potential data implied high stability of the as-prepared liposomes. Due to the extremely high lipophilicity, RA was expected to be accommodated in the phospholipid bilayers of liposomes. The RA encapsulation could reach >80% in liposomes; however, SME incorporation reduced the encapsulation percentage to 60%. Because free RA could be removed by ultracentrifugation, the complete encapsulated form of RA in liposomes was used throughout all experiments. The liposomes were diluted by ddH<sub>2</sub>O for drug dose calibration to assure the equivalent RA concentration in different formulations for testing.

Our data demonstrated that the cationic liposomes facily inhibited *C. acnes* growth in the planktonic form. The bacterial membrane behaves as a barrier to retain cellular homeostasis. Thus, the damage in the bacterial membrane can disrupt the physiological function, leading to cell death. It has been reported that liposomes are fusogenic to bacterial membranes due to the resemblance between the phospholipid bilayers and bacterial plasma membrane.<sup>28</sup> This fusion effect promotes the bactericidal activity of antibiotics against drug-resistant pathogens.<sup>9,29</sup> A similar result was found in our study, where the liposomal SME was superior to free SME in eradicating planktonic *C. acnes*. The anti-*C. acnes* activity of SME was attributed to the electrostatic interaction between the protonated amine moieties of SME and anionic teichoic acid in the bacterial membrane,<sup>16</sup> resulting in membrane disturbance and intracellular protein leakage. Teichoic acid in the Gram-positive bacteria membrane is a common binding site of the nanovesicles.<sup>30</sup> Gram-positive bacteria tend to possess looser cell walls than Gram-negative bacteria. The nanovesicles possibly penetrated across the loose membrane, permitting the entrance into the cytoplasm and consequently disorganizing the bacterial structure. The nanoparticle size affects the interaction with the microbial membrane.<sup>31</sup> The liposomes with SME showed a very small size of about 70 nm that could invade the internal components of bacteria.

The transition of planktonic bacteria to biofilm involves adhesion to the surface. The biofilm network of *C. acnes* is composed of proteins, extracellular DNA, and carbohydrates.<sup>32</sup> These components not only develop the mature biofilm but also play a vital role in early surface attachment and colonization. *C. acnes* biofilm acts as an adhesive in the sebum, generating microcomedone.<sup>11</sup> We found that the biofilm was disorganized and detached from the microplate well after the treatment of PK- and/or SME-loaded nanovesicles based on the PrestoBlue and CFU assays. Once delivered into the biofilm, the effect of antimicrobial agents depends on bacterial membrane damage or metabolic process interference.<sup>33</sup> The PrestoBlue assay indicated the reduction of metabolic activity in the adhered *C. acnes* biofilm treated by PK/SME-loaded liposomes. This phenomenon may have resulted from the death of biofilm *C. acnes* or merely the metabolism disturbance in live bacteria without death. The multifunctional liposomes significantly reduced microbial CFU in the adhered biofilm, suggesting that *C. acnes* death—but not metabolism disturbance—was the predominant mechanism of liposome-mediated detachment. Biofilm-associated protein (Bap) is a multi-domain and cell surface-anchored protein that plays a crucial capacity in the early adhesion of biofilm development. PK is reported to be effective for Bap degradation.<sup>34</sup> It also enzymatically degrades proteinaceous adhesin to hamper biofilm development.<sup>35</sup> In addition to PK, we found that SME in liposomes retarded the biofilm adhesion, possibly due to the *C. acnes* killing by the cationic surfactant, lessening the bacterial colonization to form biofilm.

Although some antibiotics can eradicate *C. acnes*, the clinical efficacy is usually limited because of the biofilm-associated recolonization within a few weeks.<sup>8</sup> Once the biofilm is established, the thick EPS prevents host defense and antibiotic penetration, resulting in antibiotic resistance. It appeared that not only the adhesion inhibition but the multifunctional liposomes were also successful in abolishing pre-existing biofilm. The multifunctional liposomes disrupted the biofilm structure and killed the biofilm *C. acnes*. There is a complex channel network inside the *C. acnes* biofilm network.<sup>9,16,36</sup> The nanoparticles with positive charge can interact with negatively charged EPS for facile biofilm entrance.<sup>37</sup> PK was expected to impair the biofilm structure after liposome delivery into the biofilm. PK is proven to cleave the C-terminal peptide bonds of amino acids for protein digestion.<sup>38</sup> Biofilm is produced by viable microbes. Thus, enzymatic treatment should be

combined with antimicrobial agents for successful biofilm dispersal. SME in liposomes played a major role as anti-*C. acnes* agents to eliminate biofilm. The biofilm CFU result verified the synergistic antibiofilm activity of the combined PK and SME.

Besides the enzymatic degradation of biofilm, the role of PK in the liposomes should be probed. We observed that NV/PK could decrease live *C. acnes* stained by SYTO 9. However, NV/PK demonstrated no bactericidal effect in the MIC/MBC and growth curve. SYTO 9 is a green dye binding to bacterial nucleic acids to determine cell viability. It is reported that serine proteases such as PK degrade proteins of the bacterial membrane to cause the disturbance of cell wall maintenance and membrane construction.<sup>39</sup> Our SEM image illustrated a damaged bacterial envelope in the *C. acnes* biofilm after a challenge by NV/PK. Proteinases may also degrade the synthase of DNA and RNA polymers.<sup>40</sup> This effect contributed to the less SYTO 9 fluorescence in *C. acnes* after NV/PK intervention. It was speculated that this degradation could mildly decrease nucleic acids but maintain bacterial viability, as detected in MIC/MBC and the time-killing curve. Nevertheless, we found a stronger bactericidal effect of cationic liposomes (NV/SME) after PK incorporation (NV/PK/SME) based on MBC. This finding indicated that PK assisted in the anti-*C. acnes* activity of SME.

Keratinocytes account for 95% of all cells in the cutaneous epidermis. They play a central role in the pathogenesis of acne. *C. acnes* activates Toll-like receptor (TLR)-2 and -4 in keratinocytes to evoke an inflammatory response and oxidative stress, consequently promoting keratinocyte proliferation and migration.<sup>41</sup> *C. acnes* can directly activate keratinocytes to prompt local inflammation by releasing cytokines interleukin (IL)-1 $\beta$ , IL-6, and TNF- $\alpha$ .<sup>42</sup> This inflammation precedes microcomedone generation to serve as a trigger for follicular hyperkeratinization. RA inhibits keratinocyte inflammation through the downregulation of TLR-2 expression, controlling the signs of acne vulgaris.<sup>43</sup> The regulation of keratinocyte proliferation by RA is mediated by the binding to specific nuclear receptors, eliciting the transcription of target genes. Our findings showed that free RA at 2  $\mu\text{g/mL}$  was defective in inhibiting keratinocyte proliferation in the CCK-8 assay. RA incorporation into nanovesicles was favorable for restraining the proliferation and migration of HaCaT cells, with the SME-loaded liposomes (NV/SME and NV/PK/SME) proving the highest inhibition. This incorporation into liposomes protected RA from degradation. The antiproliferative effect of the liposomes could possibly be related to the cellular uptake, enabling prompt internalization in the cytosol and RA release to interact with more targets. The positive charge of the nanoparticles facilitates cellular ingestion due to the enhanced affinity with negatively charged cytomembrane.<sup>44</sup> The PK presence in nanovesicles generally signified no effect on keratinocyte proliferation and migration.

RA incorporation into nanovesicles such as liposomes is proven to ameliorate the absorption into the skin.<sup>45</sup> Our in vivo study found that the topical application of multifunctional liposomes (NV/PK/SME) led to the keratinization normalization and disintegrated barrier recovery in the *C. acnes*-infected mice. The bacterial invasion in the skin was also reduced after topical liposome application. The liposomes can augment the drug contact time on the skin surface, after which the nanovesicles fuse with the intercellular lipids of superficial skin. This effect causes a looser and permeable skin barrier for improved drug delivery.<sup>46</sup> The intercellular space between the keratinocytes in the stratum corneum is reported to be 50–70 nm.<sup>47</sup> Thus, multifunctional nanovesicles of approximately the same size could pass through this space and into the deeper skin strata for treating acne. The positive charge of the multifunctional liposomes was also preferable in penetrating across the negatively charged lipid bilayers of stratum corneum.<sup>48</sup> On the other hand, the positive charge is beneficial for liposome transport into the appendages since the hair is also negatively charged. Another advantage of topical liposome delivery is its occlusive feature to form a dense film on the skin surface. This film retards TEWL and enriches skin hydration to enhance drug permeation through the skin.<sup>49</sup> Our data confirmed the reduced TEWL after liposome administration. *C. acnes* is largely found in the lipid-rich area of the skin, reaching a density of about  $10^6$  CFU/cm<sup>2</sup>.<sup>50</sup> Notably, the *C. acnes* colonization after subcutaneous bacterial injection in the present study approached this value. *C. acnes* impairs epidermal barrier property by stimulating keratinocyte proliferation.<sup>51</sup> It was inferred that topical liposomes could facilitate penetrate the skin to inhibit *C. acnes* infection, arresting inflammation and keratinocyte hyperproliferation to effectively treat the infected wound.

The first-line therapy for acne is topical RA medication, which usually makes retinoid dermatitis of irritation, erythema, and eschar.<sup>19</sup> Its combination with antibiotics or benzoyl peroxide causes more adverse effects, inflicting a higher incidence of withdrawal than monotherapy.<sup>52</sup> An effective topical acne therapy should deliver RA to the target site and prevent skin irritation with good tolerability. Topical RA exfoliates the superficial skin layer by loosening intercellular connections in the stratum corneum, leading to increased TEWL.<sup>43</sup> Although RA effectively inhibits keratinocyte hyperproliferation in acne skin, this drug enhances skin proliferation in healthy skin.<sup>53</sup> RA can regulate keratinocyte proliferation and differentiation

based on different conditions. The in vitro MTT assay showed that the liposomes were not cytotoxic to the HaCaT cell line. The in vivo tolerance displayed that the liposomes prevented RA-related skin irritation by reducing epidermal hyperplasia and immune cell infiltration. This irritation prevention could be ascribed to the RA encapsulation in the nanovesicles to avoid direct contact of the acidic moiety with the skin. A previous study<sup>26</sup> has also proven that the cream form with RA molecules encapsulated inside the oil droplets is better tolerated than the gel form.

The antibacterial effect of SME achieved the anti-acne potential of the multifunctional nanovesicles collaborated with the inflammation inhibition of RA. Moreover, the PK inclusion in the liposomes reduced the adhesion and development of *C. acnes* biofilm, ameliorating the susceptibility to antibacterial agents and the host immune system. *C. acnes* is also responsible for many infectious diseases, including folliculitis, periodontitis, and rhinosinusitis.<sup>25</sup> The developed liposomes are expected to have a wide application against different types of *C. acnes* invasion. However, further investigations are still needed to explore the possible application. Currently, available management methods for acne, such as RA, benzoyl peroxide, and azelaic acid, have led to skin irritation. If patients' skin demonstrates intolerance, it is recommended to terminate the topical therapy. The liposomes developed in the study were promising in improving tolerability and patient compliance because of their therapeutic efficacy and safety.

## Conclusion

This study designed multifunctional nanovesicles to suppress *C. acnes* infection and the associated keratinocyte proliferation. An important finding of this work is that the liposomes had a strong anti-*C. acnes* potential against planktonic and biofilm forms. The fusion and electrostatic interaction between the cationic liposomes and negatively charged bacterial membrane and biofilm were the major mechanisms revealing greater antimicrobial activity than the free control. In addition, PK incorporation in the nanovesicles increased the susceptibility of bacterial effect and biofilm eradication. Further, the liposomes efficiently arrested the proliferation and migration of the activated keratinocytes. Liposomal RA improved skin tolerability compared to the free form. Moreover, the experimental data of the liposomes suggests that the versatile liposomes can potentially be used as an anti-acne agent against *C. acnes*.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors have declared no competing interests.

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