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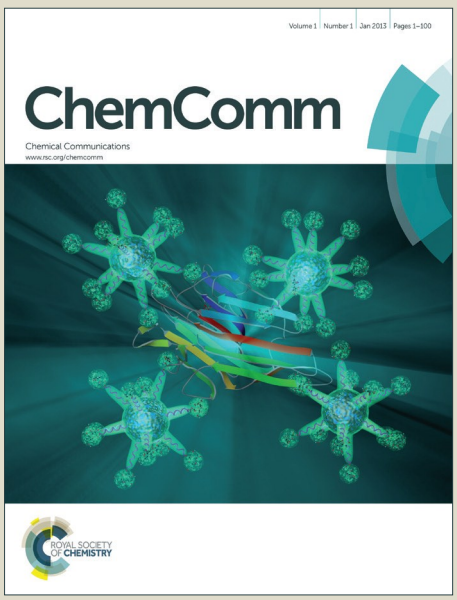

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Nanoliposomes Containing Eucalyptus Citriodora Antibiotics for Specific Antimicrobial Activity

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Bacterial infections are a serious issue for the public health and represent one of the major challenges of modern medicine. In this work, a selective antimicrobial strategy based on pore-forming toxin triggering, which is secreted by infective bacteria, was designed to fight *Staphylococcus aureus*. The antimicrobial activity is realized by employing *Eucalyptus citriodora oil* as antibiotic which in this study is encapsulated in nanoliposomes.

*Staphylococcus aureus*is a gram positive bacterium that is common cause of skin infections, respiratory disease and food poisoning.[1] The possibility of selectively killing the infectious bacteria such as *Staphylococcus aureus* is a significant issue in medical health and food science. Delivering therapeutic drugs to specific sites of action is a pursuing goal and a great challenge.[2] Generally, the nanoparticles and nanoliposomes encapsulating some agents can be prepared with surface modification, in order to specifically target and bind the receptors on the cell.[3, 4] However, the complexity and the off-targeting effect is the main hurdle in this common approach, and the drugs are not delivered to the target sites, which will result in a low delivery efficiency. An alternative to the active approach is the passive selective strategy, in which the agents are encapsulated within nanoparticles or liposomes and in such a way, antimicrobial agents are protected inside the nanocarriers without being released before meeting the infective target bacteria[5], which could secrete the toxin protein with the capability of triggering the release of therapeutic agents from the nanocarriers by disrupting the membrane of carriers.[6-8] It was proved that the α-haemolysin one kind of toxin protein secreted from *Staphylococcus aureus* can interrupt the liposome and trigger the release of dye molecule contained in the liposome[5].In this way, the side effect due to the nonspecific targeting and drug release could be eliminated. In addition, it is also a significant issue to increase the long term availability and efficiency of antimicrobial agents avoiding the loss of agents before utilization.

Herein, we reported an efficient approach towards passively selecting the infectious bacteria instead of actively targeting. The selective strategy we proposed is depicted in Figure 1.The antibiotic *Eucalyptus citriodora oil (E.c.oil)*[9] – extracted from *Eucalyptus* in which the dominant component is Citronellal – was encapsulated in nanoliposomes that could be triggered to release the citriodora oil to kill bacteria by the pore forming toxin protein secreted from infected bacteria such as *Staphylococcus aureus.* However, the non-infective bacterium such as *Escherichia coli (E. coli.),* some of which was common found in lower intestine of warm-blooded organisms, were not attacked and survive in this strategy in view that no pore forming toxin secreted from *E. coli*.

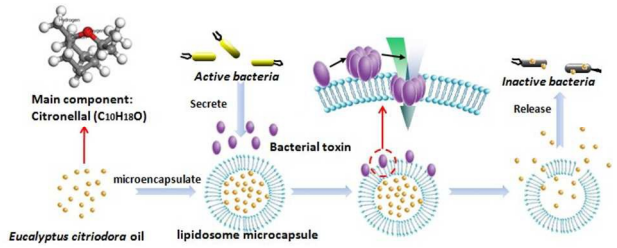

Figure1. Schematic of pore-forming toxin triggered *Eucalyptus citriodora oil* (*E.c. oil*) release from liposome.

The antibiotic used in this work (*E.c. oil)* was extracted from the *Eucalyptus* that is a large genus of the *Myrtaceae* family that comprises about 900 species and sub-species. More than 300 species of this genus contain volatile oil in their leaves, and the antimicrobial activity of *Eucalyptus* species essential oils against a wide range of microorganisms has been demonstrated in previous studies[9, 10]. The *E.c. oil* contains Citronellal that was proved to be the main component and contributes mostly to the antimicrobial activity[10, 11]. The antimicrobial activity was of Citronellaland *E.c. oil*was tested, which presented the similar effect of killing bacteria (Figure. S1). Recently, *E.c. oil* has attracted the attention of scientists due to its biocompatibility, large availability and easy extraction. However, the *E.c. oil* is volatile and chemically unstable to some extent in the presence of air, light, moisture and high temperatures. Hence, it is beneficial to microencapsulate *E.c. oil* before treating bacterial infections to limit active ingredients loss during processing and storage[12]. The antimicrobial activity of *E.c. oil* utilized in this work was explored (Figure 2a) – *E.c. oil* was proved to be a good antimicrobial agent with broad spectra antimicrobial property supported by the similar MIC and MBC value against 7 different pathogens displayed (Table S1 in Supporting Information). The

antimicrobial activity of *E.c. oil* against the *E. coli* and *S. aureus* was further explored. Generally, the population of *E. coli* and *S. aureus* reduced significantly when *E.c. oil* (200 ppm) was added after 2 hours incubation with the bacteria. Subsequently, both *E. coli* and *S. aureus* were killed entirely after 4 hours (Figure S2 in Supporting Information). The cell lysis rate of *E.c.oil* against the *E.coli* and *S. aureus* further proved the antimicrobial ability of this extracts (Figure $2a_1$). In addition, the antimicrobial mechanism of *E.c. oil* was discovered to inhibit the DNA and ATP metabolism of bacteria such as *E.coli* and *S. aureus* (Figure $2a_2$ and $2a_3$), which is observed from the quantification of DNA by using the DAPI staining method and ATP bioluminescence measurement, respectively (Supporting methods). Based on the antimicrobial agent, we developed a stimuli-response system for killing the specific bacterium with high efficiency and long term availability by entrapping the *E.c.oil* into the nanoliposome we synthesized.

The nanoliposomesprepared[13]by optimal protocol were obtained after several trials with different surfactants or different ratios of components with fixed surfactant (Table S2 - S4 in Supporting Information).It was composed of egg L-a-phosphatidylcholine (PC) (20 mg/ml, 1000 mg) and cholesterol (C) (4 mg/ml, 200 mg), chloroform (50 ml), phosphate buffered saline (PBS) (50 ml)polyvinylpyrrolidone(PVP) (50 mg) were necessary as well in the nanoliposome formation. The size of nanoliposome in this case is optimized (~ 63.9 nm) compared to other conditions characterized by DLS (Figure $2b_1$, larger diameter with lowest PDI factor). The morphology of nanoliposome was observed as well by atomic force microscopy (AFM) (Figure $2b_2$). *E.c. oil* was utilized (in the following concentrations: 2.00 mg/ml (I), 4.00 mg/ml (II) and 6.00 mg/ml (III), respectively) as antimicrobial agent mixed with PC and Cholesterol – the final nanoliposomes containing *E.c. oil* were obtained by vesicle extrusion. The diameter of nanoliposomes containing *E.c. oil* is displayed[14] in Figure $2c_1$. The entrapment efficiency of nanoliposome for antimicrobial agents was investigated (Table 1). Compared to the other nanoliposomes entrapping the *E.c. oil*, the concentration with 4 mg/mL was optimized for encapsulation not only for the high entrapment efficiency but also for the homogeneity (Figure $2c_1$ and $2c_2$). It is observed that the diameter of nanoliposome increased after entrapping the *E.c. oil*. Furthermore, the *E.c. oil* entrapped in the nanoliposome was also explored by Fourier transform infrared spectroscopy (FT-IR).The represented peaks (1722.78 cm^{-1}, 2715.66 cm^{-1} for –CHO and 1450.53 cm^{-1},1376.87 cm^{-1} for –CH3) of *E.c. oil* almost disappeared after that was entrapped into nanoliposome, compared to the naked one. Therefore, we succeeded in encapsulating the *E.c. oil* into the nanoliposomes. The surface potential was -25 mV (Figure S3), suggesting that the negative charge on the surface of nanoliposome could avoid non-specific binding to the membrane of bacterium.

After the encapsulation, the *E.c. oil* can only be specific triggered to release for killing the bacterium, but cannot display any antimicrobial activity in the nanoliposome. In case of *S. aureus*, the antimicrobial activity was displayed mainly due to the pore forming toxin secreted from *S. aureus* making the release of *E.c. oil* from the nanoliposomes. The test of the pore forming toxin protein (the α-haemolysin) secreted from Staphylococcus aureus was made by means of immunoblotting (Figure S4). The results showed that the protein extracted from bacterial cultures was consistent with pore forming toxin protein (the α-haemolysin) standard substance. The population of *S. aureus* declined after incubating the nanoliposomes entrapped *E.c. oil* with the bacterium for 24 hours. The almost entirely killing of the bacteria

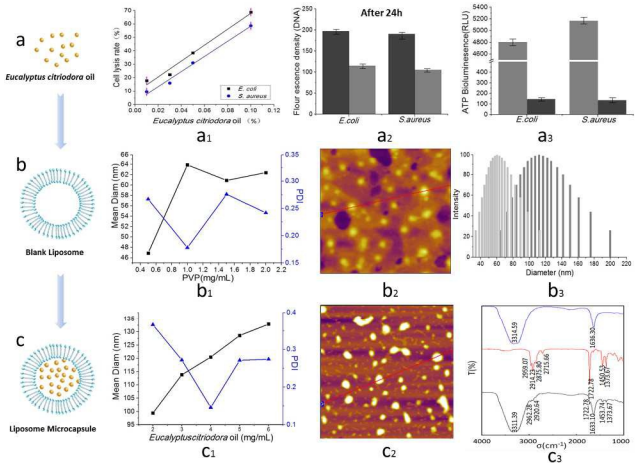

Figure 2. (a). Antimicrobial activity of the *E.c. oil*. (a_1) The cell lysis rate of *E.c. oil* against the *E. coli* and *S. aureus*. (a_2) DNA metabolism of bacterium *E. coli* and *S. aureus* before and after the *E.c. oil* treatment. (a_3) ATP metabolism of bacterium *E. coli* and *S. aureus* before and after the *E.c. oil* treatment. (a_3) (b) Preparation of nanoliposome. (b_1) Diameters of the nanoliposomes characterized by DLS. (b_2) Morphology of nanoliposomes tested by AFM. (b_3) Histogram of the diameter of nanoliposome before and after entrapping *E.c. oil*. (c). Encapsulation of *E.c. oil* into nanoliposomes. (c_1)Diameters of nanoliposomes entrapping the *E.c. oil*. (c_2) Morphology of nanoliposomes entrapping the *E.c. oil* characterized by AFM. (c_3) FT-IR spectra of *E.c. oil* before and after encapsulation.

was observed after 48 hours of incubation (Figure 3a). However, in the case of *E. coli*, there is no antimicrobial activity displayed for the *E.c. oil* entrapped in the nanoliposome (Figure 3a). The morphological change before and after administration of *E.c. oil* were investigated by transmission electron microscopy (TEM) (Figure 3b and 3c). *S. aureus* are collapsing and shrinking after the addition of *E.c. oil*. The DNA metabolism of bacterium was also affected and DNA synthesis was inhibited as observed by fluorescence microscopy due to the addition of *E.c. oil* (Figure 3e and 3f). The population of *E. coli* rarely changed during 96 hours monitoring. The morphology of *E. coli* and DNA metabolism of bacterium were not affected (Figure 3d and 3g) although in the presence of *E.c. oil,* which suggested that *E.c. oil* entrapped in the nanoliposome did not present any antimicrobial activity against the *E. coli*.

Gas chromatography (GC) was also utilized to analyse the chemical composition of *E.c. oil* before and after entrapping compared to the standard sample of citronellal in GC analysis, as well as both in the incubation with *E.coli* and *S. aureus*(Figure 3h). The characteristic peak (Figure 3h) is hydroxycitronellal

($C_{10}H_{18}O$) that is the main component of *E.c. oil*. It is observed that before the *oil* was entrapped, however, it cannot be detected in the GC analysis after the encapsulation of *E.c. oil* (the curve in the middle of Figure 3h).It is indicated that *E.c. oil* has been completely encased in the nanoliposome and almost no leakage occurred. The same phenomenon was detected and no hydroxycitronellal peak was observed when the nanoliposome was added in the culture medium with *E. coli*, which means that side effect due to premature drug leakage and nonspecific drug release were avoided. The peak was redetected when the liposomes were added into the culture medium with *S. aureus* (curve on the bottom of Figure 3h). The *E.c. oil* was released under the pore-forming toxin.

The long term efficiency of the antimicrobial activity of *E.c. oil* entrapped in nanoliposome was explored compared to the naked *E.c. oil* (Figure 3i). Initially, the naked *E.c. oil* displayed the fast antimicrobial activity in the first 4 days, while the one entrapped in the nanoliposome showed the slow activity. However, the naked *E.c. oil* rarely showed the antimicrobial activity 4 days later when more bacterium appeared, and the population of *S. aureus* did not decrease. The *E.c. oil* entrapped in the nanoliposome could maintain the antimicrobial activity 4 days later, and still worked after 8 days. It is proved that the *E.c. oil* entrapped in nanoliposome possessed the longer term efficiency of the antimicrobial activity than the naked one.

The stability of the nanoliposome including *E.c. oil* was also an important factor to evaluate. The samples are emulsions composed of nanoliposome containing *E.c. oil*. They could rarely change in the storage on the shelf from 1 day to 90 days. (Figure S5). The availability of the nanoliposome containing *E.c. oil* to *S. aureus* bacterium was further examined with the agents for 7 days storage, 30 days storage and 90 days storage, respectively (Figure S6). The antimicrobial activities of three samples with different storage time are all efficient as freshly prepared samples (Figure 3b). Nanoliposomes including *E.c. oil* we prepared possessed good stability and the high efficient antimicrobial activity to *S. aureus* though it is stored for a long time. Finally, for the negative control experiment, the pure nanoliposome was proved to be no effect on antimicrobial activity (Figure S7).

In summary, the nanoliposome containing the *E.c. oil* (a novel antibiotic extracted from plant) presented excellent antimicrobial activity. After the encapsulation, the agents can only be specifically released from the nanoliposome to kill *S. aureus* mainly due to the pore forming toxin secreted from which the specific triggering and long term availability of this agent entrapped in nanoliposomes were proved as well. After long time storage, the efficiency of liposomes entrapping the *E.c. oil* were also satisfying. This work may engage the attention of the diversity of researchers in biomaterial science, nanomedicine, and microbiology and chemistry.

The authors acknowledge the financial support from National Natural Science Foundation of China (grant no.31301573), National Science Foundation for Post-doctoral Scientists of China (grant no.12M511223), Natural Science Foundation of Jiangsu Province (grant no.BK20130493), Jiangsu University research foundation (grant no. 11JDG050, 14JDG021) and Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Notes and references

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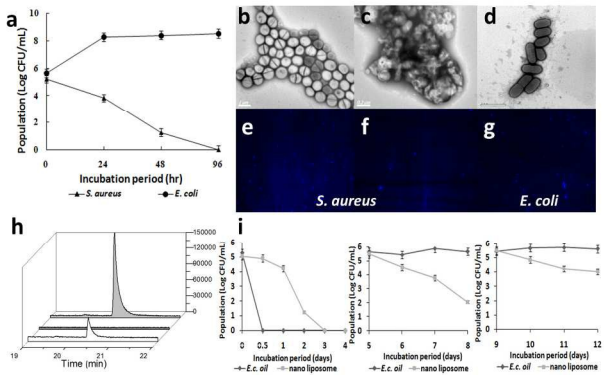

Figure 3. The selective antimicrobial activity of the *E.c. oil* entrapped in nanoliposomes (a) The bacterium viability of *S. aureus* and *E. coli* after the treatment of *E.c. oil* entrapped in nanoliposomes. (b and c) TEM images of morphology of *S. aureus* bacteria before and after incubation with *E.c. oil* entrapped in nanoliposomes. (d) TEM images of morphology of *E. coli*.(e and f) DNA metabolism of *S. aureus* before and after the treatment of *E.c. oil* entrapped in nanoliposomes tested by fluorescence microscopy. (g) DNA metabolism of *E. coli* tested by fluorescence microscopy. (h) GC detection of *E.c. oil* before entrapment, after entrapment, and nanoliposome containing *E.c. oil* incubating in the culture medium with *S. aureus*.(i) The cell viability of *S. aureus* monitored from 1 day to 12 day treated by naked *E.c. oil* and the one entrapped in nanoliposomes. More *S. aureus* were added at 5 day and 9 day. There is no effect on killing *S. aureus* in the sample treated by naked *E.c. oil* but efficiency in the sample treated by *E.c. oil* entrapped in nanoliposome.

† Electronic Supplementary Information (ESI) available: Experimental details, material preparation, characterization, and the surface potential result and antimicrobial activity result of nanoliposome with Eucalyptus citriodora oil for long time storage. See DOI: 10.1039/b000000x/

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