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Antimicrobial activity of ZnO-doxycycline hyclate thermosensitive gel

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ABSTRACT: Zinc oxide (ZnO) is a prototype of micro- and nanomaterials widely studied in different fields due to many promising new applications. Various zinc oxides (typical, micronized, powder, tetrapod I, and tetrapod II ZnO) have been characterized using SEM. The particle size of zinc oxides was analysed using laser light diffraction spectroscopy. Effects of types and amounts of ZnO on the antimicrobial activity were evaluated using *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* as standard microbes. Lutrol F127 systems comprising ZnO and doxycycline hyclate were developed. Cytotoxicity of test samples and gel was investigated on human gingival fibroblast and macrophage cell line U937. Particle size analysis revealed that micronized ZnO was smaller than ZnO powder, ZnO BP, tetrapod I, or II zinc oxides. The particle size of tetrapod zinc oxides was larger than the others since they exhibited an arm structure configuration. The antibacterial activity depended on the particle size of ZnO, whereas the antifungal activity was less affected by the particle size. Increasing ZnO in the doxycycline hyclate-Lutrol F127 systems decreased the inhibition zone diameters against all test microbes investigated with agar diffusion method because it retarded drug diffusion. This effect could prolong the doxycycline hyclate release. *N*-methyl-2-pyrrolidone could enhance the antifungal activity of doxycycline hyclate thermosensitive gel. The developed system could inhibit bacteria in the oral cavity. All 10 mg/ml test samples were not toxic to human gingival fibroblast or macrophage cell line, however Lutrol F127 and doxycycline hyclate showed a slight effect but no significant difference from that of the control group. Therefore, ZnO combined with doxycycline hyclate could prolong the inhibition of microbes in the form of thermosensitive gel to use in localized periodontitis therapy.

KEYWORDS: N-methyl-2-pyrrolidone, periodontitis therapy

INTRODUCTION

Metal oxides such as MgO, CaO, and ZnO possess antibacterial activity against some bacteria strains such as *S. aureus* and *E. coli*[1]. Topical ZnO is an efficacious, painless, and safe therapeutic option for wart treatment[2]. Nowadays with nanotechnological advances, ZnO can be fabricated and formed in many different shapes as nanorods, nanohelixes, nanobows, nanowires, nanocombs, nanosheets, nanobelts, and more exotic branched structures such as tetrapods and multipods, the dimensions of which are often closely related to specific properties and uses. ZnO nanostructures have been prepared using templates, physical vapour deposition, electrodeposition, thermal evaporation or hydro-thermal, and solvo-thermal methods[3]. There are many reports exhibiting the antimicrobial activity of ZnO. ZnO had bactericidal action against *Staphylococcus aureus* and *Pseudomonas aeruginosa*[4]. ZnO nanoparticle exhibited significant antifungal properties against important plant pathogenic fungal such as *Botrytis cinerea* and *Penicillium expansum*[5]. ZnO nanorod arrays diminished the growth of *Candida albicans* with stable action for two months[6]. Many factors related to the efficiency for antibacterial activities of the metal oxide powders such as concentration, particle size, and specific surface area[7]. Nevertheless, the mechanisms of the antimicrobial activity of ZnO particles are not well understood. The generation of hydrogen peroxide by this substance after activation might be a main factor for the antimicrobial activity[1]. While the binding of the particles on the bacteria surface due to the electrostatic forces could be one of the

mechanisms of action[8]. Yamamoto et al[7] reported that the antibacterial activity depended on the surface area and concentration, while the crystalline structure and particle shape showed the little effect. Typically, the larger the concentration and surface area the larger the antibacterial activity[7].

Lutrol F127 or Poloxamer 407 have been used as thermosensitive polymer exhibiting a solubilizing property[9]. Lutrol F127 gel strength increased with temperature and concentration. This property might be altered by drugs or additives. Diclofenac, ethanol and propylene glycol weaken Poloxamer 407 gel, whereas sodium chloride, sodium monohydrogen phosphate, and glycerine strengthen it[10,11]. Bioadhesive force generally increased with gel strength and its value is modified by the same parameters (i.e., temperature and polymer concentration). The solvents or ionic agents might alter the adhesion characteristics. NaCl has been included in this gel to prolong the residence-time in the site of administration[10]. Lutrol F127 system showed the Newtonian flow at low temperature (4 °C), and becomes non-Newtonian with sharply increase in viscosity when the temperature is increased[12,13]. Our studies also reported a decrease of gelation temperature of Lutrol F127 as the ZnO BP amount is increased[10]. The power law kinetic implied that the release rate of doxycycline hyclate decreases as the amount of ZnO BP increases. System containing ZnO BP could prolong the drug release from Lutrol F127 systems[12]. Doxycycline hyclate is a well known broad-spectrum bacteriostatic agent which has been recommended for using in periodontitis treatment. This antibiotic inhibits the bacteria protein synthesis due to the disruption of transfer RNA and messenger RNA at the ribosomal sites systems[13].

N-methyl-2-pyrrolidone (NMP) is the lactam of 4-methylaminobutyric acid which is colourless with high-boiling point, low viscosity, low toxicity, and good biocompatibility[14]. It is a water-miscible organic solvent used as a good solvent for drugs with pour water solubility. The solubility parameter of NMP is similar to that of ethanol and dimethyl sulphoxide (DMSO)[15]. NMP increased transdermal absorption of some drugs such as phenolsulfonaphthalein, ibuprofen, and flurbiprofen[16,17] and also estradiol[18]. Atrigel is the commercial injectable system based on poly (DL-lactide) in NMP used for the periodontal therapy[19,20].

This study investigate the antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* of various zinc oxides. The thermosensitive ZnO gel was prepared using Lutrol F127 as gelling agent and its antimicrobial activity was tested with above microbes and other oral cavity bacteria. The effect of NMP on the microbial activity of this system was also investigated.

MATERIALS AND METHODS

Materials

Lutrol F127 (lot No. WPTX 607B) was purchased from Vita Co., Ltd., Bangkok. ZnO BP (lot no. 000150, Vidhyasom, Thailand), ZnO micronized (Namchiang, Bangkok), ZnO powder (Sigma-Aldrich Inc., Germany) was used as received. ZnO tetrapod I and II were synthesized by an oxidation reaction technique by heating the mixture of ZnO and hydrogen peroxide solution at 1000 °C as described[21], and were provided by Dr Supab Choopun, Department of Physics, Faculty of Science, Chiangmai University, Thailand. Doxycycline hyclate (Batch No. 20071121, Huashu pharmaceutical corporation, Shijiazhuang, China) was supplied by T. Man Pharma Ltd., Bangkok. *N*-methyl-2-pyrrolidone (NMP) (Fluka, New Jersey, USA) was used as received. The standard microbes used in this study were *Staphylococcus aureus* (ATCC 6538P), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 17110). Various oral cavity bacteria obtained from Faculty of Dentistry, Srinakharinwirot University, Bangkok, were also used for antimicrobial activity test. Sabouraud dextrose agar (SDA), Sabouraud dextrose broth, tryptic soy agar (TSA), and tryptic soy broth (Difco, USA) were used as medium for antimicrobial test. Dimethylsulphoxide (DMSO) (Merck, Germany), DMEM (Gibco, Auckland, New Zealand), MHB (Becton, Dickinson and Company, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, MANN Research Laboratories, New York) and foetal bovine serum (Gibco, Auckland, New Zealand) were used as received.

Characterization of various zinc oxides

Surface morphology of all materials was analysed using scanning electron microscopy (SEM) (Maxim 200 Camscan, Cambridge, England). The samples were sputter-coated with gold before examination. The particle size of various zinc oxides was measured using a particle size distribution analyser (Partica LA-950, HORIBA, Japan) with the widest measurement range (0.01–3000 μm). Distilled water was used as dispersing medium. Mean diameters of samples were determined (n=3).

Preparation of Lutrol F127 gel loaded with ZnO and doxycycline hyclate

Twenty percent w/w Lutrol F127 aqueous solutions were prepared by the cold method[22]. Briefly, Lutrol F127 was slowly added to cold water (4 °C). Each dispersion left in a refrigerator for 24 h at 4 °C then a clear solution was formed. To manipulate the various formulation properties, ZnO BP and doxycycline hyclate were added into the formula. Systems containing 20% w/w Lutrol F127 and various amount of ZnO BP such as 0.5, 1, 1.5, 2, 5, and 10% w/w were prepared. The 20% w/w Lutrol F127 systems containing 0% and 5% w/w doxycycline hyclate, with and without *N*-methyl-2-pyrrolidone (20% w/w) and various amount of ZnO BP were also prepared.

EVALUATION OF GEL PROPERTIES

Antimicrobial studies

Antimicrobial activity of the samples was tested using the direct contact method and the agar diffusion method. Standard microbes used in this study were: *Staphylococcus aureus* (ATCC 6538P), *Escherichia coli* (ATCC 10536), and *Candida albicans* (ATCC 17110). The culture media for antibacterial and antifungal assay were TSA and SDA, respectively. The numbers of the microbes with and without the presence in different ZnO concentrations were investigated to estimate the antibacterial activities of ZnO. For the antibacterial tests, different amounts of ZnO were added to 10 ml medium to get different concentrations in range of 5–200 mg/10 ml. The number of the bacteria was estimated by the direct plate counting method. Microbes with concentration of 10^4 colony forming units per millilitre (CFU/ml) were inoculated in a 10 ml broth medium as a blank control and in a solution mixture containing broth medium and different amounts of ZnO. The cultures were grown at 37 °C under an agitation condition (200 rpm) for 4 h. After dilution, 100 µl of the proper dilution was transferred directly onto the agar plate. The solution was then spread over the surface of the agar. Three agar plates were used for each dilution. After incubating at 37 °C for 24 h, the plates having an ideal number of colonies between 30 and 300 were counted. To be comparable, the inhibition ratio of the bacteria was evaluated as[23] $(A - B)/A$, where A is the number of bacterial colonies from the untreated bacteria suspension (without ZnO) and B is the number of bacterial colonies from the bacteria culture treated by ZnO. The same antibacterial tests were done on all types of ZnO.

Antimicrobial activities of prepared ZnO-doxycycline hyclate gels were determined using agar-cup diffusion method. The actively growing broth culture of microbes was prepared and the turbidity was adjusted to obtain approximately 10^8 cells/ml. Then, a swab was spread onto an agar plate in three directions to ensure complete the plate area and the spread culture were dried at ambient condition. The sterilized cylinder cups were placed carefully on the surface of the swabbed agar. The prepared gels were filled into the cylinder cup and incubated at 37 °C for 24 h. The antimicrobial activities were measured as the diameter (cm) of clear zone for growth inhibition. The tests were carried in triplicate and the mean inhibition zone ± S.D. were calculated.

Antimicrobial studies against oral cavity bacteria

The bacterial strains tested for anti-microbial activity were: *Streptococcus sobrinus*, *Streptococcus milleri* group, *Streptococcus salivarius* N01, *Streptococcus salivarius* N02, *Streptococcus mitis* whereas the pathogen strain were *Streptococcus mutans* ATCC 27175, *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans* using disk a diffusion susceptibility testing method as described previously[24]. When a suspension of an actively growing microbe was obtained, the turbidity was adjusted to fix the turbidity with UV-vis spectrophotometer (Perkin-Elmer, Germany) at wavelength of 625 nm. The test bacterial cell suspension was spread on Mueller Hinton agar for 10 min before disk placing. The 10 µg/ml test samples were prepared in DMSO. The disk was loaded with 10 µl of the test sample solutions and dried. The blank disk loaded with 10 µl DMSO and the 30 µg vancomycin disk were used as negative and positive controls, respectively.

The minimum inhibition concentration (MIC) of all ZnO samples, Lutrol F127, NMP and prepared gels were measured using the broth microdilution method according to the CLSI protocol[24,25]. The assay was performed in a 96-well microtitre plate with a 100 µl final volume per well. Each well contained 20 µl of the serially diluted test sample under test, 20 µl of the test bacteria suspension in MHB at 10^6 CFU/ml and 60 µl fresh MHB culture broth. Methicillin and vancomycin (10 µg/ml) were employed as the positive control while the MHB-diluted DMSO solvent (same concentrations as the test sample) without the test sample was used as the negative control. The assay plate was incubated at 37 °C for 24 h, whereupon the growth of the test bacteria was examined in terms of the turbidity of the culture. The MICs were determined as the lowest concentration of the test compound at which

no turbidity from microbial growth could be observed with a microplate spectrophotometer (Packard Fusion) at a wavelength of 600 nm. The turbidity of this concentration was lower than that of the positive control $> 90\%$.

Cytotoxicity

The preparation and culture of human gingival fibroblasts followed a described method[26]. Cells with the passage of 5 were cultured in DMEM containing 10% fetal bovine serum and 100 µg/ml penicillin and 100 µg/ml streptomycin and incubated at 37 °C in the 5% CO_2 atmosphere. The percentage of cell viability of human gingival fibroblasts and also macrophage cell line U937 was determined after incubating the cell lines with 10 mg/ml test samples for 72 h with MTT method as described[27].

RESULTS AND DISCUSSION

Morphology of all materials was characterized using SEM. SEM images of various zinc oxides are shown in Fig. 1. ZnO BP appeared as a group of hexagonal crystals with diameter in the range of 0.20–0.70 µm, and length in the range of 0.30–1.20 µm. The spherical ZnO agglomerated of various sizes (0.10–2.60 µm) of micronized ZnO is shown in Fig. 1. The shape of powder ZnO and micronized ZnO were spherical but the size of powder ZnO (< 0.20 µm) was smaller than that of micronized forms (0.05—0.70 µm). ZnO tetrapods comprised four needle-like arms with pyramidal tips with various particle sizes.

Particle size of all samples were determined using laser light diffraction spectroscopy using distilled water as a dispersing medium. Micronized ZnO exhibited the smallest particle size (4.436 µm). The particle size of powder ZnO (13.585 µm) was smaller than that of ZnO BP (18.427 µm), tetrapod I ZnO (33.072 µm), and tetrapod II ZnO (37.41 µm), respectively. Due to their structural arm characteristic, ZnO tetrapod I and II were larger than ZnO BP, micronized, and powder ZnO. According to the morphological study from the SEM, the structures of both tetrapod ZnO were four needle-like arms with pyramidal tips and various particles sizes. The particle size measured by the Malvern Nano-Sizer, which is the hydrodynamic diameter, was expected to be larger than the actual size. Thus the average particle sizes of all samples measured by this method was bigger than the one found in the SEM image (Fig. 1), which might be the aggregates of particles[28].

The growth inhibition of microbes (*S. aureus*, *E. coli*, and *C. albicans*) was studied by the direct-exposure method with colony count. The amount of

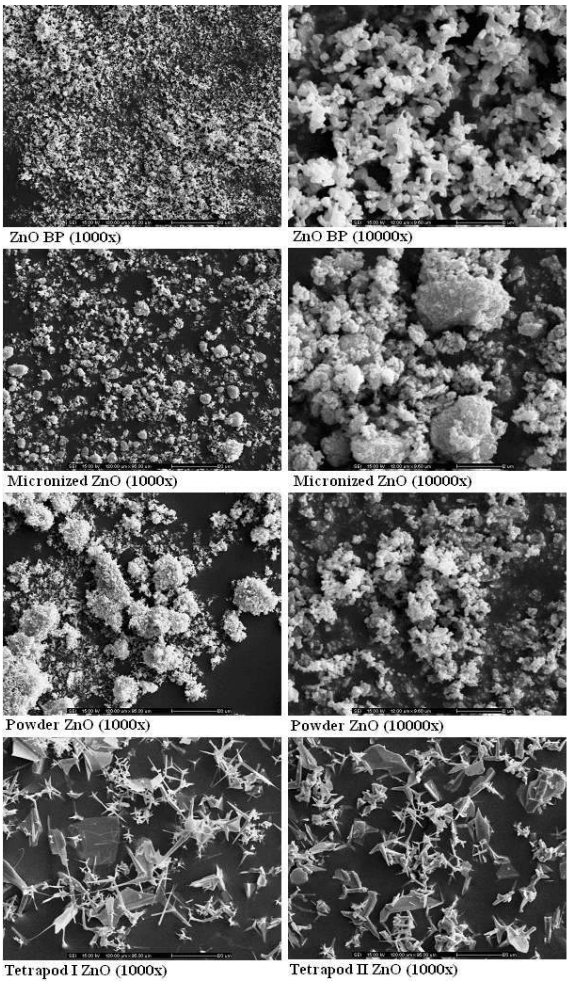

Fig. 1 SEM micrographs of different forms ZnO: ZnO BP, Micronized ZnO, Powder ZnO, Tetrapod I ZnO, and Tetrapod II ZnO.

ZnO BP in the range of 5–200 mg/10 ml was tested for antimicrobial activity. The inhibition profiles of ZnO are shown in Fig. 2. At lower concentration of 50 mg/10 ml, ZnO BP inhibited *S. aureus* growth stronger than *C. albicans* or *E. coli*. At 5 mg/10 ml, ZnO BP inhibited *S. aureus* growth about 90%. Approximately 50% growth inhibition was found for both *C. albicans* and *E. coli* at all concentrations. At lower concentration such as 50 mg/10 ml, ZnO BP inhibited *C. albicans* better than *E. coli*. These results indicated that the ZnO could inhibit all microbes (*S. aureus*, *E. coli*, and *C. albicans*). ZnO inhibited about 90% of *S. aureus* growth and about 50% of *C. albicans* growth at concentration of 5 mg/10 ml, whereas ZnO inhibited *E. coli* in a dose dependent fashion from 0–100 mg/10 ml ZnO and the highest

inhibition was 50% of *E. coli* growth. Many reports showed that ZnO could be used as antimicrobial agent[29–32], as it exhibited antimicrobial activity against *S. aureus*[29] and *E. coli*[7,30]. The antibacterial activity of ZnO increased with the concentration[29] in agreement with the present investigation of anti-*E. coli*. Tam et al[33] reported that ZnO is more effective for gram-positive than gram-negative bacteria because the formers have simpler cell membrane structure. The antifungal activity was evident in the carbon samples containing ZnO[32]. The ZnO-eugenol cement was more effective in reducing *Candida* spp. colony than the glass ionomer cement, with zerocounts after 48 h[31,34], whereas the present study implies that ZnO could inhibit about 50% of the *C. albicans* after 4 h. All these results support the antifungal activity of ZnO. Many studies suggest that the release of highly active free radicals after ZnO activated with light radiation may be a mechanism responsible for antibacterial activity[35]. Zn ions released from the particle might bind to proteins to deactivate them, interact with microbial membrane to cause structural change and permeability, or interact with microbial nucleic acids to prevent microbial replication. Other proposed mechanism involves an accumulation of ZnO particles in the microbial membrane resulting in membrane disorganization and microbial cellular internalization[36]. However, it is still not clear yet whether the antibacterial activity of ZnO could be attributed to the damage of cell membranes, which lead to leakage of cell contents and cell death[33]. The difference in activity against these two types of bacteria can be attributed to different organization of the cell wall. Gram-positive bacteria typically have one cytoplasmic membrane and thick wall composed of multilayers of peptidoglycan[37,38]. Gram-negative bacteria have more complex cell wall structure, with a layer of peptidoglycan between outer membrane and cytoplasmic membrane[30,37,38]. Therefore, the cell wall membrane of gram-positive bacteria may be damaged more easily. Membrane damage attributed to photocatalytic processes is a mechanism proposed to explain the antibacterial activity of some metal oxides[37,38].

The inhibition profiles against *S. aureus*, *E. coli*, and *C. albicans* of various ZnO are shown in Fig. 3. The control shows initial amount of cell without addition of the test samples. ZnO at concentration of 5 mg/10 ml was used for *S. aureus* and *C. albicans* whilst a concentration of 150 mg/10 ml was used for *E. coli*. Antibacterial activity of various zinc oxides against *S. aureus* and *E. coli* was not different, significantly ($p > 0.05$). Micronized ZnO exhibited an antibacterial activity against *S. aureus* higher than that of ZnO powder, ZnO BP, ZnO tetrapod II, or I, whilst antibacterial activity of powder ZnO against the *E. coli* growth was higher than that of micronized, BP, tetrapod I and II forms. Antifungal activity for different type of ZnO against *C. albicans* showed no significant difference ($p > 0.05$). All types of ZnO could inhibit *C. albicans* growth as the same level. Thus the particle size of ZnO affected the antibacterial activity but not antifungal activity. Particle size and shape of ZnO have been reported previously to affect its antibacterial activity[39]. The antibacterial activity of ZnO increased with the smaller particle size. The enhanced antimicrobial activity of smaller particles was attributed to the higher surface area to volume ratio. More particles of smaller size could cover a bacterial colony better resulting in the generation of a larger number of active oxygen species released from ZnO surface to destroy bacteria more effectively[40].

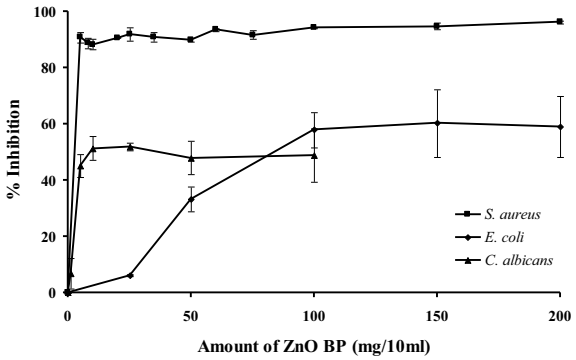

Fig. 2 Inhibition profile towards *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* of ZnO BP (n=3).

The phase transition, rheological behaviour, syringeability, and drug release of the aqueous ZnO-Lutrol F127 systems were previously reported by our group[12]. NMP influences both the sol-gel and gel-sol transition of systems. Lutrol F127 system with NMP could shift the sol-gel transition to a lower temperature, but the gel-sol transition to a higher temperature. The Lutrol F127 gel containing NMP showed a Newtonian flow characteristic at 4 °C, while non-Newtonian flow characteristic appeared when the temperature was increased to 28 °C and 37 °C. The flow curves of the Lutrol F127 systems containing different doxycycline hyclate with 20% w/w NMP shifted to a higher shear rate when the 20% w/w NMP was added and the flow curve of all gel systems were shifted to high shear stress as the temperature was increased. The addition of NMP into systems containing ZnO decreased the viscosity of the sys-

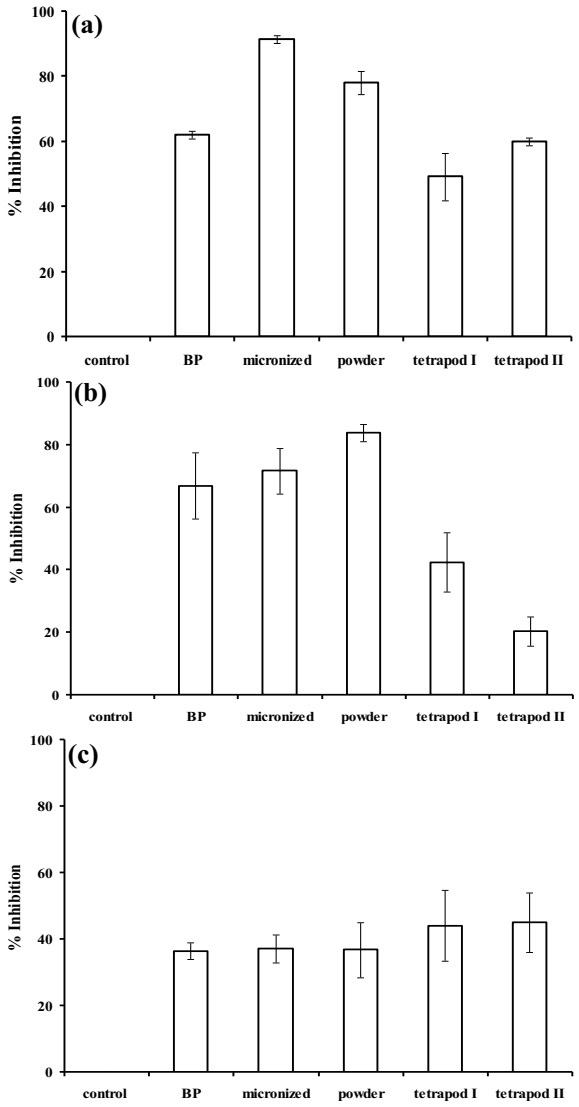

Fig. 3 Inhibition profiles against (a) *S. aureus*, (b) *E. coli*, and (c) *C. albicans* of various ZnO at concentration of 5 mg/10 ml (n=3).

tem. However, the flow curve also shifted from low shear stress to higher shear stress as the temperature was increased. The decreased gelation temperature resulted from the interaction between the hydrophobic portion of the polymer molecules, which could disrupt the micellar structure and increase the entanglement of micelles[41]. All systems could be delivered from a syringe through a needle and could change from solution at low temperature into viscous gel at higher temperature. Doxycycline hyclate released from gel without ZnO as a biphasic characteristic, with a relatively fast release at the initial hours, then the drug release was almost completed and the amount of drug released was about 90% after 10 h. The apparent prolongation of doxycycline hyclate release was found as amount of ZnO was increased[12]. Because the antimicrobial activity of ZnO BP did not apparently differ from that of the other zinc oxides and its derivative typically used in pharmaceuticals, the prepared gels containing different amount of ZnO BP were tested for the antimicrobial activity against *S. aureus*, *E. coli*, and *C. albicans* using the agar diffusion method. There was no inhibition zone for the Lutrol F127 gel without ZnO BP indicating that Lutrol F127 had no antimicrobial activity. All gels containing different concentrations of ZnO BP showed no inhibition zone, since ZnO BP could not diffuse pass the gels into the agar. Thus the antimicrobial activity of prepared gel containing ZnO BP was not detected in this agar diffusion method. Therefore, the direct contact method was used to assay the antimicrobial activity of gel preparation. The percentage of growth inhibition against *S. aureus*, *E. coli*, and *C. albicans* are shown in Fig. 4. The percentage of growth inhibition against *S. aureus* of the gel base containing 20% w/w NMP was $31.8 \pm 4.9\%$ which was less than that without NMP which was $54.5 \pm 3.5\%$. This result suggested that the 20% w/w NMP did not enhance the antibacterial activity of the system. The growth inhibition against *S. aureus* of the prepared gel containing ZnO alone was increased as the ZnO amount was increased. Whereas the percentage of growth inhibition against *S. aureus* by the prepared gel containing 20% w/w NMP and ZnO (1%, 5% or 10% inhibition against *E. coli* by the prepared gels without NMP containing 1, 5, and 10% w/w ZnO were not different. The addition of 20% w/w NMP into the systems containing ZnO (1, 5, and 10%w/w) could inhibit *E. coli* growth as 27.5 ± 7.0, 51.4 ± 3.9 and $63.3 \pm 4.8\%$, respectively, (Fig. 4b). The percentage of growth inhibition against *C. albicans* by the prepared gel containing 5 and 10% w/w ZnO were not different but higher than by that of 1%w/w ZnO. The percentage of the growth inhibition against *C. albicans* of the prepared gels loaded with 20%NMP was less than that without NMP (Fig. 4c). However, the pure NMP (20%w/w) were tested antimicrobial activity using direct contact method. The percent inhibition against *S. aureus*, *E. coli*, and *C. albicans* were $26.3 \pm 8.0\%$, $49.6 \pm 10.9\%$, and $1.6 \pm 0.8\%$, respectively. These results show that adding of 20%w/w NMP into the Lutrol F127 systems containing ZnO does not enhance the antimicrobial activity of these systems, but increasing the amount ZnO does. The antibacterial behaviours of ZnO nanofluids against

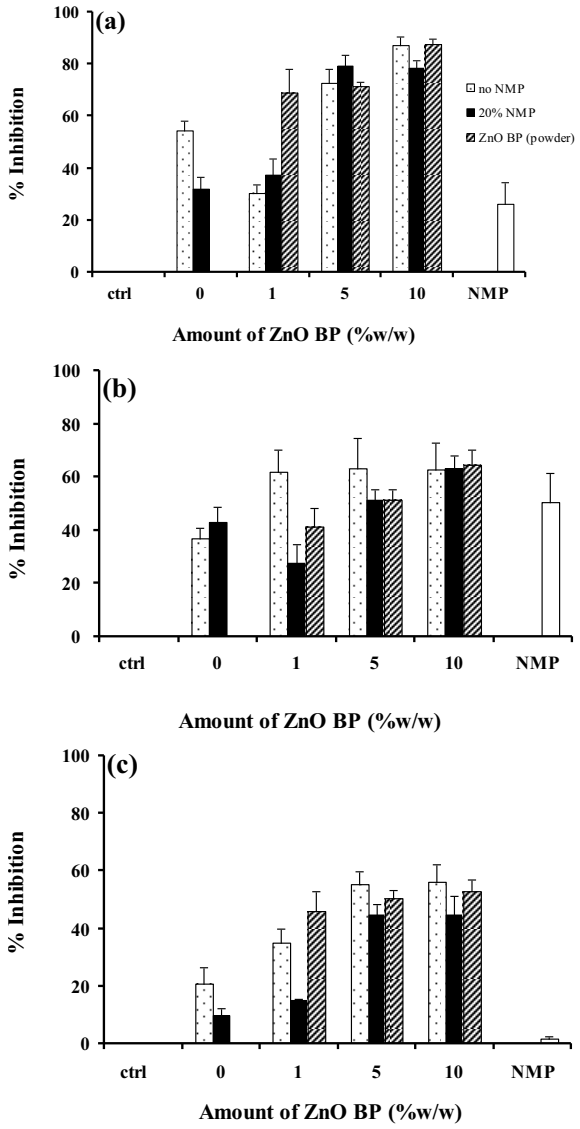

Fig. 4 Percentage of (a) *S. aureus*, (b) *E. coli*, and (c) *C. albicans* growth inhibition of Lutrol F127 gel containing different amount of ZnO and *N*-methyl-2-pyrrolidone (n=3).

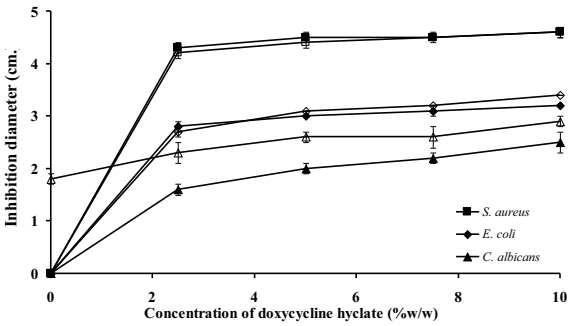

Fig. 5 Inhibition zone diameter of 20%w/w LutrolF 127 gel containing different concentrations of doxycycline hyclate obtained from the agar diffusion method. Filled symbols represent the prepared gel without NMP, and open symbols represent the prepared gels with 20%w/w NMP (n=3).

E. coli bacteria shows that the number of the bacterial colonies dropped by increasing ZnO particles[39].

The 20%w/w Lutrol F127 gels containing various amount doxycycline hyclate with or without 20%w/w NMP were tested their antimicrobial activity against *S. aureus*, *E. coli*, and *C. albicans* using the agar diffusion method (Fig. 5). Because the doxycycline hyclate has a potent antibacterial activity, it was difficult to observe when the direct contact method was employed since the complete eradication of microbe was evident. From agar diffusion test, the inhibition zone diameter against *C. albicans* of the gel base containing 20% w/w NMP was 1.8 ± 0.1 cm, whereas the gel base containing and without 20% w/w NMP did not exhibit the inhibition zone against *S. aureus* and *E. coli*, indicating that the addition of 20% w/w NMP could inhibit *C. albicans*. The inhibition zone diameter were increased as the doxycycline hyclate concentration was increased, indicating the antimicrobial activity of doxycycline hyclate was dose dependent. The inhibition zone diameter against *S. aureus* was greater than that against *E. coli* and *C. albicans*, indicated that the doxycycline hyclate influenced on the gram-positive bacteria greater than gram-negative bacteria and fungal, respectively. Generally, doxycycline is bacteriostatic against a wide variety of organisms, both gram-positive and gram-negative. The addition of NMP into the gel system affected the activity against *C. albicans* since the inhibition zone of the prepared gel with 20%w/w NMP were notably higher than that without NMP. Therefore, NMP enhanced the antifungal activity of doxycycline hyclate. NMP is the lactam of 4-methylaminobutyric acid. It is colourless with high boiling point, low viscosity, low toxicity, and good biocompatibility[42]. Inhalation exposure of pregnant rats to NMP during the entire post-implantation phase of gestation was neither teratogenic nor embryo-lethal[43]. Oral administration of NMP produced developmental toxicity below maternally toxic levels[44]. The solubility parameter of NMP is similar to that of ethanol and dimethyl sulphoxide[45]. Therefore NMP could solubilized the lipid in fungal cell membrane and enhance the drug penetration and action.

The effect of ZnO and NMP on the antimicrobial activity of doxycycline hyclate gels is shown in Fig. 6. The agar diffusion method was tested for the antimi-

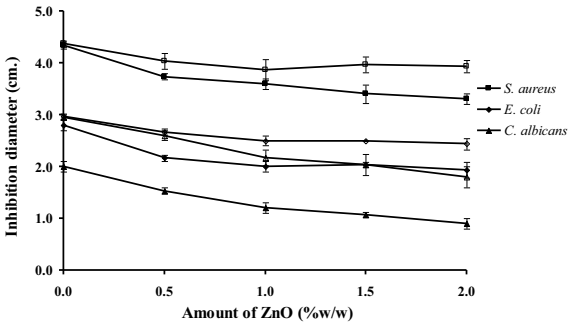

Fig. 6 Inhibition zone of 5%w/w doxycycline hyclate gels containing different ZnO concentrations with and without NMP against *S. aureus*, *E. coli*, and *C. albicans*. (Open symbols show the prepared gel with NMP. Filled symbols show the prepared gel without NMP) (n=3).

crobial activity against *S. aureus*, *E. coli*, and *C. albicans*. Inhibition zone diameters against *S. aureus* and *E. coli* of the doxycycline hyclate gels with or without NMP were not different. Whereas the inhibition zone diameters against *C. albicans* of the doxycycline hyclate gels with 20%w/w NMP were higher than that without NMP, suggesting that NMP enhances the antifungal activity. The inhibition zones of the prepared gel containing 0.5% w/w ZnO were lower than that without ZnO, indicating that the diffusion of doxycycline hyclate from gel decreases when ZnO is present. For *S. aureus*, the inhibition zone of the doxycycline hyclate-Lutrol F127 gel were decreased with the amount of ZnO, whereas the inhibition zone of the prepared gel with 20% NMP did not depend on the ZnO amount. For *E. coli* and *C. albicans*, the inhibition zone of the doxycycline hyclate-Lutrol F127 gel with or without 20% w/w NMP were decreased when the amount ZnO were increased. These results showed that the amount of ZnO affected the diffusion of drug from gels. The complex formation between doxycycline and metal ion such as Zn^{2+} has been reported. Since this specie could attach either to the tricarbonyl-methane or the phenol-diketone area of this drug molecule[46] therefore the drug release depended on the dissociation of this complex which could retard the drug diffusion into the agar in antimicrobial test and also the prolongation of drug release in dissolution study in the previous work[12]. This prolongation of drug release with ZnO and its antimicrobial activity exhibited its good properties for sustaining doxycycline hyclate delivery.

All zinc oxides could not inhibit all test microbes as presented in Table 1. The 100 µg/disk Lutrol F127 could inhibit *S. milleri* group whereas the 100 µg/disk

Table 1 Zone of inhibition from disk diffusion test of disk containing 100 µg/disk test samples.

Test Sample	Zone of inhibition (mm)							
	S. milleri group	*S. mitis*	*S. salivarius* N01	*S. salivarius* N02	*S. mutans* ATCC 27175	*S. sobrinus*	*P. gingivalis*	*A. actinomycetemcomitans*
ZnO BP	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
ZnO micronized	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
ZnO tetrapod I	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
ZnO powder	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
Lutrol F127	18	< 8	< 8	< 8	25	< 8	< 8	< 8
Doxycycline hyclate	32	17	13	13	36	19	< 8	< 8
NMP	< 8	< 8	< 8	< 8	10	< 8	< 8	< 8
Lutrol 20%	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
Lutrol 20% + NMP 20%	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
Lutrol 20% + NMP 20% + doxycycline hyclate 5%	22	< 8	< 8	< 8	32	8	< 8	< 8
Lutrol 20% + NMP 20% + doxycycline hyclate 5% + ZnO BP 1%	17	< 8	< 8	< 8	26	< 8	< 8	< 8
Vancomycin	> 30	> 30	> 30	> 30	> 30	> 30	23	21

* NMP 0.1% v/v was used.

doxycycline hyclate could inhibit all microbes except *P. gingivalis* and *A. actinomycetemcomitans*. However, doxycycline hyclate at 1000 µg/disk could inhibit these two bacteria. NMP, gel bases containing NMP or without NMP showed no detectable antibacterial activity against any of the test strains. Doxycycline hyclate gel inhibited *S. milleri* group and *S. mutans* ATCC27175 whereas this gel comprising ZnO reduced in the bactericidal activity however the increased amount of this system to 1000 µg/disk enhanced the inhibition since all microbes could be inhibited (Table 2).

MIC of zinc oxides was higher than 100 µg/disk whilst that of NMP, Lutrol F127, Lutrol 20%, Lutrol 20% + NMP 20% was higher than 5 µg/ml. The MIC of system containing 20%NMP and 5% doxycycline hyclate is presented in Table 3. Therefore, this system exhibited an antimicrobial activity against the test bacteria. Because it also affected the normal flora the careful consideration should be performed for the long usage of this system. Cytotoxicity of test samples on human gingival fibroblasts and macrophage cell line are shown in Table 4. All 10 mg/ml test samples were not toxic to these two cells however Lutrol F127 and doxycycline hyclate showed the slight effect but not significant difference from that of control group. Lutrol F127 which is nonionic surfactant consisting

Table 2 Zone of inhibition from disk diffusion test of disk containing 1000 µg/disk test samples.

Test Sample	Zone of inhibition (mm)							
	S. milleri group	*S. mitis*	*S. salivarius* N01	*S. salivarius* N02	*S. mutans* ATCC 27175	*S. sobrinus*	*P. gingivalis*	*A. actinomycetemcomitans*
ZnO BP	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
ZnO micronized	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
ZnO tetrapod I	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
ZnO powder	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
Lutrol F127	18	< 8	< 8	< 8	25	< 8	< 8	< 8
Doxycycline hyclate	32	17	13	13	36	19	32	31
NMP	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
Lutrol 20%	< 8	< 8	8	< 8	< 8	< 8	< 8	< 8
Lutrol 20% + NMP 20%	< 8	< 8	< 8	8	< 8	< 8	< 8	< 8
Lutrol 20% + NMP 20% + doxycycline hyclate 5%	23	20	25	10	30	27	30	30
Lutrol 20% + NMP 20% + doxycycline hyclate 5% + ZnO BP 1%	20	12	29	< 8	26	17	18	20
Vancomycin	> 30	> 30	> 30	> 30	> 30	> 30	23	21

* NMP 0.1% v/v was used.

Table 3 MIC from microdilution method of test samples.

Test sample	MIC (µg/ml)					
	S. milleri group	*S. mitis*	*S. salivarius* N01	*S. salivarius* N02	*S. mutans* ATCC 27175	*S. sobrinus*
ZnO BP	> 100	> 100	> 100	> 100	> 100	> 100
ZnO micronized	> 100	> 100	> 100	> 100	> 100	> 100
ZnO tetrapod I	> 100	> 100	> 100	> 100	> 100	> 100
ZnO powder	> 100	> 100	> 100	> 100	> 100	> 100
Lutrol F127	> 100	> 100	> 100	> 100	100	> 100
NMP	> 5	> 5	> 5	> 5	> 5	> 5
Lutrol 20%	> 5	> 5	> 5	> 5	> 5	> 5
Lutrol 20% + NMP 20%	> 5	> 5	> 5	> 5	> 5	> 5
Lutrol 20% + NMP 20% + doxycycline hyclate 5%	0.0049	0.3125	> 5	> 5	0.0024	0.625
Lutrol 20% + NMP 20% + doxycycline hyclate 5% + ZnO BP 1%	0.0049	0.3125	> 5	> 5	0.0049	0.625
Methicillin	2	2	2	2	0.25	8
Vancomycin	2	2	2	2	2	4

polypropylene oxide and polyethylene oxide block copolymer therefore it could disturb the lipid in cell membrane but this effect should be less than that of sodium lauryl sulphate as previously reported tested with human gingival fibroblast[47]. Therefore the developed ZnO-doxycycline hyclate thermosensitive gel showed the ability to be used as local antimicrobial delivery for periodontitis treatment.

Table 4 Cytotoxicity of test samples on human gingival fibroblasts (HGF) and macrophage cell line U937 (MCL).

Test sample	ED_{50} (µg/ml)	Viability of HGF (%)	Viability of MCL (%)
Media Control	-	100.02 ± 0.01	100.01 ± 0.01
ZnO BP	> 10	100.46 ± 0.03	101.98 ± 0.02
ZnO micronized	> 10	100.39 ± 0.02	103.03 ± 0.12
ZnO tetrapod I	> 10	101.85 ± 0.01	98.52 ± 0.48
ZnO powder	> 10	100.07 ± 0.06	99.46 ± 0.09
Lutrol F127	> 10	97.47 ± 0.12	99.65 ± 0.13
Doxycycline hyclate	> 10	97.70 ± 0.12	98.18 ± 0.17
NMP*	> 10	99.77 ± 0.05	97.52 ± 0.19
Lutrol 20%	> 10	101.85 ± 0.04	100.85 ± 0.17
Lutrol 20% + NMP 20%	> 10	107.82 ± 0.08	101.09 ± 0.07
Lutrol 20% + NMP 20% + doxycycline hyclate 5%	> 10	102.07 ± 0.01	97.94 ± 0.04
Lutrol 20% + NMP 20% + doxycycline hyclate 5% + ZnO BP 1%	> 10	102.53 ± 0.01	101.36 ± 0.10

* 0.01% v/v NMP was used.

CONCLUSIONS

The particle size affected the antibacterial activity of ZnO, whereas the antifungal activity was less affected by the particle size. The increased ZnO amount into the doxycycline hyclate-Lutrol F127 systems decreased the inhibition zone diameters against all microbes since ZnO decreased the diffusion of drug from gels. This effect could prolong the doxycycline hyclate release, therefore ZnO and doxycycline hyclate thermosensitive gel could prolong the inhibition of microbes for using in localized periodontitis therapy as sustainable drug release system. However, this system affected the normal flora in oral cavity therefore the careful consideration for the long usage of this system should be conducted.

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