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RESEARCH ARTICLE

Characterisation and *in vitro* antimicrobial potential of liposome encapsulated silver ions against *Candida albicans*

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ABSTRACT

Liposomes are biocompatible, biodegradable, controlled delivery systems with the ability to encapsulate both lipophilic and hydrophilic compounds, including metal ions. Liposome encapsulated Ag^+ (lipo-Ag^+), prepared by reverse-phase evaporation, was used as a controlled delivery system against *Candida albicans*. Characterisation of the lipo-Ag^+ indicated that the multilamellar vesicles with diameters ranging between $\approx$0.5 and 5.0 μm showed potential as a controlled delivery system to consistently deliver Ag^+ to *C. albicans*. Results from inductively coupled plasma (ICP) analysis showed higher association of cell bound Ag^+ at 15 mins post exposure when compared to unencapsulated Ag^+. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) indicate detrimental effects of Ag^+ on *C. albicans* cell structure. These effects along with the ICP results also correlate with previously reported time kill experiment observations.

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Candida albicans; controlled release; liposomes; silver; wound management

Introduction

Although conventional antibiotics are regarded as effective antimicrobial agents, there is concern about their side effects and the increasing incidence of antibiotic resistant microorganisms (Karpanen et al., 2008). This has led to re-examination of the use of alternative antimicrobial agents, such as metal ions and/or plant extracts, which often attack multiple target sites, thereby reducing the risk of resistant strain development (Kim et al., 2008; Chang et al., 2010). Liposomes are spherical, lipid bilayer vesicles with a large aqueous inner-core for encapsulation and delivery of active agents. Encapsulation of antimicrobial agents in liposomes could provide protection from enzymatic and immunological inactivation (Drulis-Kawa and Dorotkiewicz-Jach, 2010). Additionally, the liposome's capacity to bind water may aid moisture retention which is conducive for tissue repair (Reimer et al., 2000). The capacity to transport both hydrophilic and hydrophobic materials, allows a wide range of antimicrobial agents to be incorporated into liposome vesicles.

Chronic and slow/non-healing wounds require extensive management to speed up the recovery process. Wound dressings and devices are designed to strategically manage the complexity of different wounds; these developments have been extensively discussed in a recent review by Martin et al. (2013). Generally, the main challenges in managing a chronic wound environment include (Martin et al., 2013):

(i) the ability to deliver sufficient drug or antimicrobial agent whilst maintaining bioavailability at microbicidal concentrations;
(ii) maintain control on the amount of wound exudate;
(iii) reduce the risk of uneven antimicrobial deposition at the wound site, and
(iv) the ease of wound dressing application.

The uneven distribution of drug or exposure to sub-lethal antimicrobial concentrations can lead to development of resistance among wound infecting pathogens. In addition, uneven drug deposition may also induce toxic side effects to healthy cells, thus leading to tissue necrosis. Wound dressings act as a barrier to reduce external microbial infection, absorb or donate fluid and can deliver antimicrobial agent(s) (Martin et al., 2013).

The use of metals as disinfecting agents has been practised since ancient times. Silver, copper, zinc, iron, cobalt and magnesium have all been used to disinfect water, treat diseases and wound infections. In modern society, antimicrobial metals are often incorporated into surfaces for food preparation, home appliances, surgical tools, indwelling medical devices, dental fillings, paint ingredients and water treatment systems (Rusin et al., 2003; Kim et al., 2008; Robinson et al., 2010). Despite their antimicrobial properties, treatment with many metals is limited in humans due to their toxicity at high concentration. However amongst metals with potentially therapeutic applications, silver has a long history of use as an effective antimicrobial agent (Warriner and Burrell, 2005; Yamanaka et al., 2005; Mooney et al., 2006) due to its comparatively low toxicity to human cells (Lansdown and Williams, 2004; Drake and Hazelwood, 2005). In its non-ionised form, silver is an inert metal that does not react with human cells (Leak and Johnson, 2007). The extensive antimicrobial activity of silver is derived from the ability of its ions to bind to thiol groups which leads to disruption of membrane integrity, inhibition of vital enzyme function, impairment of solute and electron transport system; all

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of which results in abnormal cell function and eventually cell death (Castellano et al., 2007; Low et al., 2013). In addition to its anti-infective role, silver also enhances wound healing, which could be especially useful in the management of severe wounds such as burns and slow/non-healing ulcers. Recently, in response to issues surrounding antibiotic resistance, topical application of silver compounds has increased in popularity due to their multi-target action in microbial cells (Castellano et al., 2007; Ansari et al., 2011). However, in common with many drug treatments, over exposure to the agents causes unwelcome side effects. Long-term topical exposure to high concentrations of Ag^+ leads to argyria, a build up of metallic silver (Ag^0) in the dermis causing an irreversible blue-grey discolouration of the skin. Some patients treated with silver containing-dressings reported the occurrence of skin rashes, stinging and burning sensations when treated with silver impregnated-dressings (Lansdown and Williams, 2004).

Candida albicans (*C. albicans*) is a yeast-like fungus in the polymicrobial community of commensals colonising the skin and mucosal surfaces of healthy individuals (Moyes and Naglik, 2011). Whilst rarely causing infection, trauma associated with wounds may result in pathogenic invasion by these opportunistic microorganisms (Dryden, 2009). Superficial (mucosal) candidal infections are usually not life threatening and commonly observed in otherwise healthy individuals (Rogers et al., 2013). However, *Candida* spp. colonisation poses a major risk factor in the development of systemic candidal infection (candidaemia), especially in immunocompromised individuals (Giglio et al., 2012; Gow and Hube, 2012).

In previous studies, we have reported the feasibility of using liposome encapsulated silver ions against common representatives of skin pathogens (*Pseudomonas aeruginosa*, *Staphylococcus aureus* and *C. albicans*) (Low et al., 2013). The aim of this study is to demonstrate the *in vitro* delivery of liposome encapsulated silver ions and their effect on *C. albicans* cells.

Materials and methods

Sterile malt extract broth (MEB) and malt extract agar (MEA) (Lab M, Heywood, UK) were prepared according to manufacturer's recommendations. Silver solutions were prepared by dissolving sufficient silver nitrate ($AgNO_3$ – 99.85% purity: Acros Organics, Geel, Belgium) in sterile distilled water (SDW). Contamination checks were performed by plating 100 μL samples on MEA. No microbial colonies were observed following 24 h incubation at 37 °C.

Cacodylate buffer (0.2 M) was prepared by dissolving 42.8 g sodium cacodylate (BDH Lab Supplies, Poole, UK) in 800 mL distilled water, followed by 6.9 mL 1 M hydrochloric acid (HCl) (Fisher Scientific, Loughborough, UK). The solution was adjusted to pH 7.3 with HCl and made up to a final volume of 1 L with SDW. The 2.5% v/v glutaraldehyde was prepared by adding 10 mL glutaraldehyde (25% v/v, BDH Lab Supplies, Poole, UK) to a mixture of 50 mL cacodylate buffer and 40 mL SDW. The 1.0% w/v osmium tetroxide was prepared by dissolving 0.1 g osmium tetroxide (BDH Lab Supplies, Poole, UK) in 5 mL SDW followed by subsequent addition of 5 mL cacodylate buffer. The alcohol solutions at 30%, 50%, 70% and 90% v/v were prepared by adding sufficient absolute alcohol (Fisher Scientific, Loughborough, UK) to SDW.

Preparation and characterisation of liposome encapsulated Ag^+ (lipo-Ag^+)

Liposome suspensions (10 mg/mL) were prepared by the Reverse-phase Evaporation Vesicles (REV) method adapted from Szoka and Papahadjopoulos (1978). Phosphatidylcholine (PC) and cholesterol (CH) (Sigma grade ≥99%, Sigma Aldrich, Gillingham, UK) 2:1 molar ratio were dissolved in chloroform (≥99%, Sigma Aldrich, Gillingham, UK). This organic lipid solution was added slowly into the aqueous phase, which consists of 2.5% w/v $AgNO_3$ solution (one part organic lipid into 10 parts aqueous phase) with constant magnetic stirring in a sterile beaker. The beaker was loosely covered with aluminium foil to reduce light oxidation whilst still allowing solvent (chloroform) evaporation. Lipid emulsions were left to stir for 8 h to form a homogenous liposome suspension before centrifugation (Mistral 1000 centrifuge, MSE, London, UK) at 4700 r.p.m. (4000 *g*) for 15 min. The supernatant was discarded and the pellet (lipo-Ag^+) re-suspended in SDW up to the initial volume. Control liposomes (lipo-H_2O) were prepared in a similar manner using SDW to replace the 2.5% w/v $AgNO_3$ solution. The prepared stock of liposomes were stained using 0.01% w/v acridine orange (BDH Lab Supplies, Poole, UK) and viewed under the fluorescence microscopy (Nikon "Eclipse" ME600, Nikon Corp., Tokyo, Japan; Camera: Spot RT Colour, Diagnostic Instruments Inc., Sterling Heights, MI) using the Nikon G2A filter at 1000 × magnification. The size (diameter) distribution of the prepared liposomes was measured using Image-Pro software. Re-suspended lipo-Ag^+ liposome pellets were analysed to determine the amount of encapsulated Ag^+ (ICP analysis). Timed samples were also taken from the lipo-Ag^+ suspensions and the Ag^+ content of the sample supernatants used to show the release of Ag^+ from the liposomes. The kinetics of Ag^+ release from the liposomes was analysed by fitting the data to zero order, first order, Higuichi and Korsmeyer-Peppas equations (Costa and Lobo, 2001).

Inoculum preparation

Freeze dried cultures of *C. albicans* (NCYC 854) from the University of Wolverhampton stock culture collection were resuscitated in sterile MEB and incubated at 37 °C for 48 h. Prior to experimental use, overnight broth cultures were diluted with sterile broth to give a starter inoculum culture of approximately 1×10^7 colony forming units (CFU)/mL.

Inductively coupled plasma (ICP) analysis to determine the amount of Ag^+

Test flasks were set up to contain 90 mL of MEB containing sufficient agent to achieve the MLC (Low et al., 2013) upon the addition of inoculum culture (10 mL) to achieve a final cell concentration between 1×10^5 and 1×10^6 CFU/mL. The flasks were prepared in triplicate and incubated at 37 °C in a shaking water bath oscillating at 50 cycles per min (Mickle Laboratory Engineering Co. Ltd., Surrey, UK). Samples were taken at 15 min and at 2.5 h for ICP analysis to determine the amount of cell associated Ag^+. Prior to analysis on the ICP (Spectro Ciros Charged Coupled Device, CCD, Spectro, Kleve, Germany), 1 mL of sample was centrifuged at 13 000 r.p.m. (11 000 *g*) for 8 min (Sanyo, MSE Micro Centaur, London, UK). The resulting pellet was micro-digested by adding 1 mL of nitric acid (analytical grade inorganic acid, 69–72%, BDH, VWR International Ltd., Lutterworth, UK), mixed and allowed to react for 15 min. This was followed by addition of 0.5 mL hydrogen peroxide (100% volumes >30% w/v, Thermo Fisher Scientific Inc., Hemel Hempstead, UK). After 15 min, the micro-digested samples were transferred to glass test tubes and 8.4 mL of ultrapure water (Purelab Option-Q, Elga, High Wycombe, UK) was added.

Scanning electron microscopy (SEM)

Overnight cultures of *C. albicans* were treated with concentrations of Ag^+ at twice the MLC in MEB for 1.5 h in a 37 °C shaking water

bath. Following exposure, 1.5 mL samples were centrifuged using the microcentrifuge for 3 min. The supernatant was discarded and the pellet resuspended with 1.5 mL SDW. The content was centrifuged again, supernatant discarded and the pellet was post-fixed in gluteraldehyde and osmium tetroxide, before dehydration in a series of increasing ethanol concentrations. Samples were dried using a critical point dryer (Polaron, CPD7501, Quorum Technologies, Lewes, UK). The prepared samples were then kept in a desiccator until they were mounted on SEM stubs. The sample stubs were coated with gold particles prior to analysis using the SEM (Zeiss EVO®50, Carl Zeiss AG, Oberkochen, Germany). The cell dimensions (μm) were calculated using measurements made in mm from photomicrographs of the SEM images. Cell volumes were calculated from the equation for the volume of a prolate spheroid:

$$\text{Volume} = \frac{1}{6} \times \pi \times \text{width}^2 \times \text{length} \quad (1)$$

Transmission electron microscopy (TEM)

After 24 h exposure to lipo-Ag^+ (1.5 × MLC), a sample (1.5 mL) was centrifuged for 3 min at 3000 rpm (637 *g*) and washed with 2.5% v/v glutaraldehyde. Similar to pre-SEM preparation, the sample was post-fixed using glutaraldehyde, osmium tetroxide and dehydrated with increasing concentration of alcohol solution. The dehydrated sample was washed with propylene oxide/resin (ratio 1:1) and left for 45 min on a rotator at 4 r.p.m. for the embedding process to occur.

The sample was centrifuged and the pellet was re-suspended with resin, and the embedding process was allowed to continue in an oven at 60 °C under vacuum for 20 min. Following that, the resin was left to polymerise under atmospheric pressure in the oven for 16 h. The resin stub sample was then sectioned (Reichert-Jung Ultracut E ultramicrotome, Vienna, Austria) and mounted on formvar-coated gold 200 mesh grids (Agar Scientific, Essex, UK). This was stained using 30% w/v uranyl acetate in methanol and Reynolds lead citrate. Photomicrographs were captured using TEM (JEOL 1200EX, Tokyo, Japan).

Results

Liposomal preparation and characterisation

The prepared lipo-Ag^+ has a mean diameter of 2.24 μm whilst the empty liposomes (lipo-H_2O) have a mean diameter of 1.80 μm (Figure 1). Size distribution of lipo-H_2O appears to show a mono-disperse profile whereas the lipo-Ag^+ shows a poly-disperse profile of apparently two subpopulations of vesicles. ICP analysis indicated that the encapsulation efficiency achieved using this method to prepare lipo-Ag^+ was an average of 5.4% of Ag^+ available in the aqueous phase. The liposomes steadily released the encapsulated Ag^+ over time, with an apparently linear release over the first 2.5 h (Figure 2a). When Ag^+ release was analysed with zero order, first order, Higuchi and Korsmeyer–Peppas equations the results indicated that Ag^+ release from liposomes follows the Korsmeyer–Peppas equation (Figure 2b) with a correlation coefficients of >0.99 (Table 1), i.e. diffusional release over the first 2.5 h. The correlation coefficients for the other models (zero, first and Higuchi equations) were all ≤0.97. This demonstrates the feasibility of liposomes as controlled release delivery systems to provide constant release of Ag^+.

Liposomal Ag^+ release and cell uptake

The results from the ICP analysis of *C. albicans* cell pellets are shown in Figure 3. When treated with lipo-Ag^+, the amount of Ag^+ in the microbial cell pellet was higher at 15 min compared to 2.5 h post-inoculation. However, when treated with free Ag^+, the amount of cell-bound Ag^+ remained unchanged. However, the reduction in amount of cell-bound Ag^+ at 2.5 h post-inoculation may be due to the release of cell-bound Ag^+ into the broth as a result of disrupted cell permeability in Ag^+ damaged cells.

Scanning electron microscopy (SEM)

SEM photomicrographs were taken to observe the effect of Ag^+ on microbial cell surfaces; images were taken after 1.5 h exposure to free Ag^+ at 2 × MLC level (Figure 4). The SEM results show some distortion to the *C. albicans* cell appearance (''wrinkled'' cell surface)

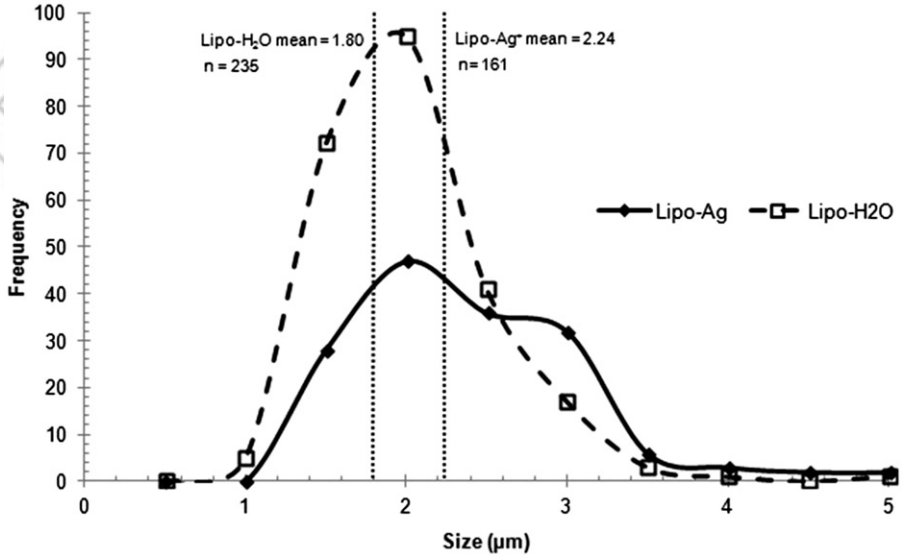

Figure 1. Size distribution (diameter, μm) of liposomes encapsulating silver nitrate (lipo-Ag^+) and a control formulation encapsulating water (lipo-H_2O). Mean diameter for lipo-Ag^+ was 2.24 μm ($n = 161$) and lipo-H_2O was 1.80 μm ($n = 235$).

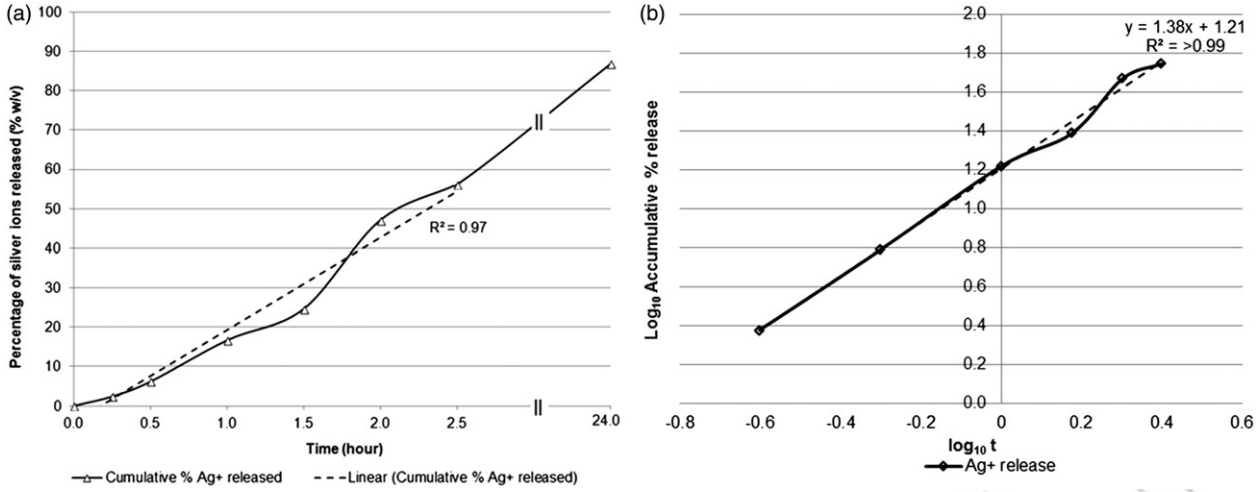

Figure 2. (a) Silver ion (Ag^+) release (% w/v) from phosphatidylcholine-cholesterol (2:1 molar ratio) liposomes into sterile distilled water ($n = 3$) and (b) Ag^+ release data fitted to the Korsmeyer–Peppas equation ($R^2 > 0.99$), indicating release occurs via a diffusion-based mechanism.

Table 1. Mathematical modelling of Ag^+ release kinetics.

Correlation coefficient (R^2)			
Zero order	First order	Higuichi	Korsmeyer-Peppas
0.96	0.97	0.83	>0.99

post-exposure to Ag^+. The percentage of distorted *C. albicans* cells was 27% compared to 19% in the control cells. However, the distortion in the control cells seemed distinctly different to that seen in the treated cells, showing a limited ''dimpling'' of the cells.

The size distributions of Ag^+ treated and control cells were skewed to the lower cell volumes (Figure 5). However, there was a much narrower distribution at the low cell volumes for Ag^+ treated cells. The peak for the control cells was much broader showing a wider variation of cell volumes in this sample. The mean cell volume of Ag^+ treated cells appears to be smaller (15.56 μm^3) than the control cells (21.25 μm^3). This difference is statistically different ($p < 0.005$).

Transmission electron microscopy (TEM)

TEM images for *C. albicans* (Figure 6) show a distorting effect on the cell that is compatible with the ''wrinkled'' effect seen in the SEM. In addition, Ag^+ treated cells show irregularly shaped nuclei when compared to the untreated cells.

Discussion

The Ag^+ exposure experiments were conducted at 37 °C (optimal temperature for some wound infection pathogens), despite the optimum growth temperature for *C. albicans* is approximately 30 °C because the temperature at and around wound sites may be considerably higher than normal skin surface due to inflammation (White, 2009) and the insulating effects of dressings, if present.

The liposomes generated in this study produced multilamellar vesicles (MLV) with diameter ranging between ≈0.5 and 5.0 μm. The presence of an ion gradient between the inner core and exterior environment may drive the active loading/embodiment of ions into the liposomes (Abraham et al., 2002). The observed two subpopulations of lipo-Ag^+ may be due to the incorporation of Ag^+ between the phospholipid bilayer and the retention of Ag^+ within the liposomes, which increases the size of the vesicles when compared to controls.

Mathematical modelling (Korsmeyer–Peppas equation) of the data confirmed that Ag^+ is released from the liposome *via* diffusional means (Figure 2b). The rate of agent release from liposomes depends mainly on the compositional properties of the liposome bilayer membrane. Fusion of liposomes with target cells results in a change in the cell's membrane composition, disturbing the control mechanism for membrane function and fluidity, thereby increasing permeability of substances across the membrane (Furneri et al., 2000). This delivery mechanism is relevant to microbial cultures as shown by the ability of liposomes to attach to the cell surface of *E. coli*, *S. aureus* and *C. albicans* (Reimer et al., 2000).

In previously described time kill studies using similar conditions against *C. albicans*, results showed that 15 min exposure to lipo-Ag^+ had little effect on cell viability, whereas after 2.5 h of exposure, cell viability was reduced by ≈1.36 $\log_{10}$ cycles (Low et al., 2013). In contrast, 15 min treatment using free silver resulted in a decrease of ≈1.87 $\log_{10}$ cycles, followed by a further reduction of ≈1.18 $\log_{10}$ cycles after exposure of 2.5 h (Low et al., 2011). Both time kill data showed that *C. albicans* viability was reduced to the limit of detection after 24 h of exposure to lipo-Ag^+ or free Ag^+ (Low et al., 2011; Low et al., 2013). In relation to the ICP results (Figure 3), the instant bioavailability of free Ag^+ resulted in a larger reduction in cell viability possibly due to the rapid cell surface weakening effect of the Ag^+ which results in leakages and dissociation of the ions, hence resulting in a lower amount of cell bound Ag^+ at 15 min. In contrast, time kill data indicated that the activity of Ag^+ from those encapsulated in liposome was not as radical, possibly due to the initial retention of Ag^+ in the liposome, that limits its bioavailability. However, the ICP results from this investigation indicated the capabilities of lipo-Ag^+ to also deliver Ag^+ efficiently to the microbial cells (*C. albicans*). This is evident in the increased amount of cell-bound Ag^+ at 15 min post-inoculation when comparing treatment using free Ag^+ and lipo-Ag^+.

The size distribution of control *C. albicans* (Figure 5) indicates the possibility of two sub-populations of cells, that may be due to the budding characteristics of *C. albicans*. It is assumed that the sub-population of smaller sized cells represent recently released buds, whereas the other subpopulation represents the larger, mature yeast cells. Equation 1 is used to calculate cell volumes since a

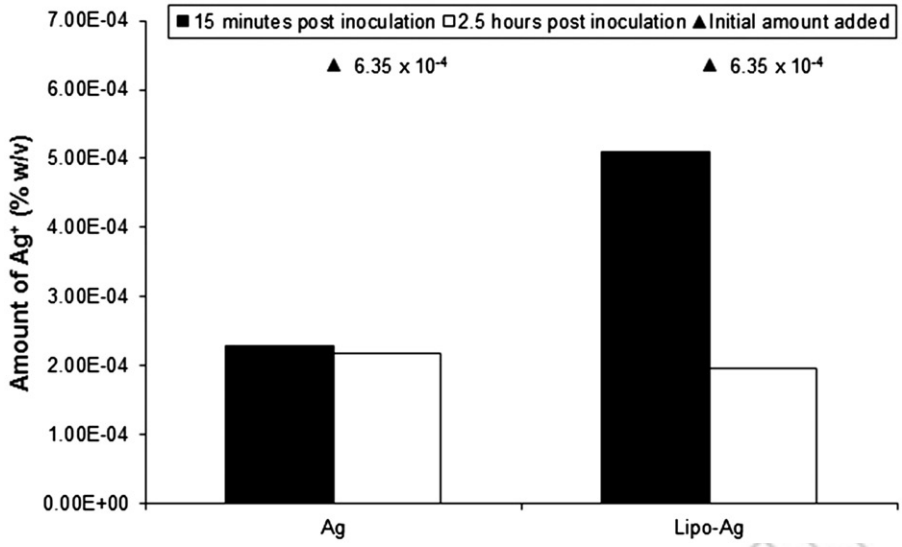

Figure 3. Amount of cell associated Ag^+ (% w/v) in the digested *C. albicans* sample after exposure to free or lipo-Ag^+. *Initial concentration of Ag^+ present in the cell suspensions at time zero was 6.35×10^{-4}% w/v.

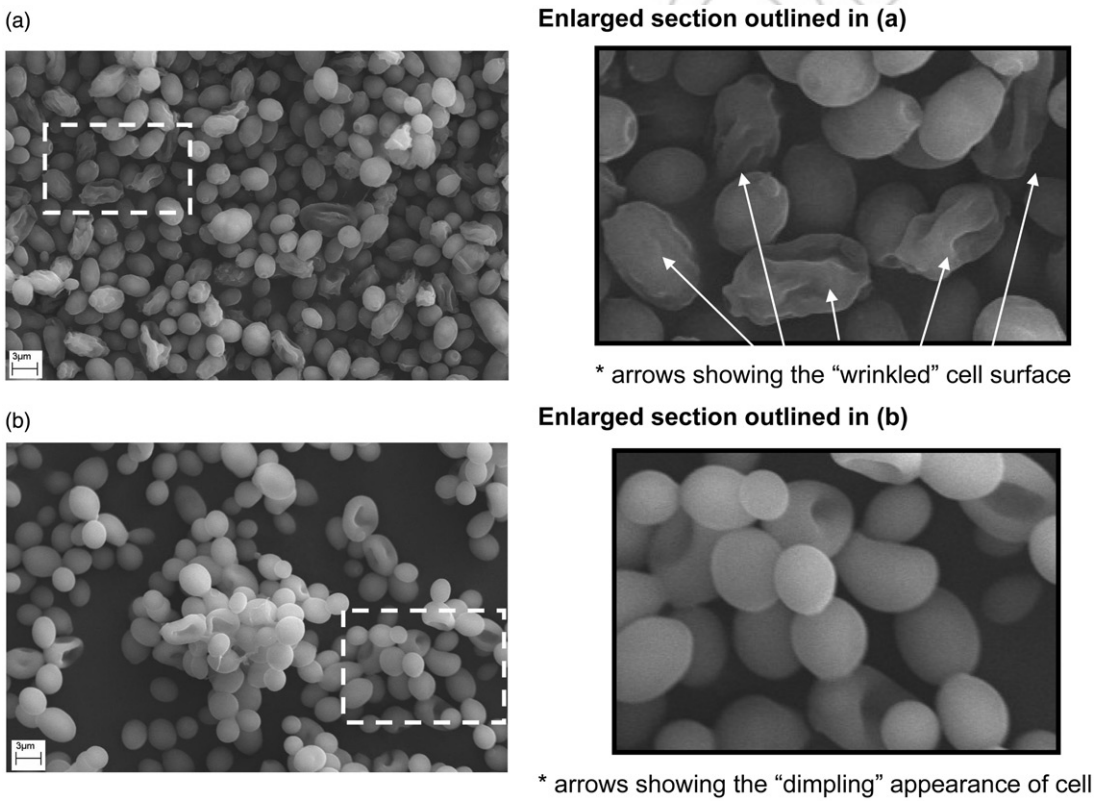

Figure 4. SEM of (a) *C. albicans* exposed to liposome encapsulated Ag^+ (lipo-Ag^+) and (b) untreated *C. albicans* showing the effects of free Ag^+ on cell membrane integrity. Scale bar = 3 µm.

prolate spheroid (a sphere stretched along its polar axis) most closely approximates to the volume of elliptical yeast cells. The values for length and width in Equation 1 were measured from photographs of SEM images. Although not all cells align to the plane of the photograph, we have assumed the proportion of aligned to non-aligned cells would be similar in all preparations studied. Therefore, the difference in the cell size distribution between control and treated cells will be minimally affected by the variation in planar orientation of the cells.

The SEM results did not show sufficient detail to illustrate extensive damage to the cell by Ag^+. However, the SEM of *C. albicans* did show a population of Ag^+ treated cells having a distorted (wrinkled) appearance. This may be due to the activity of Ag^+ weakening the cell structure, thereby increasing the sensitivity

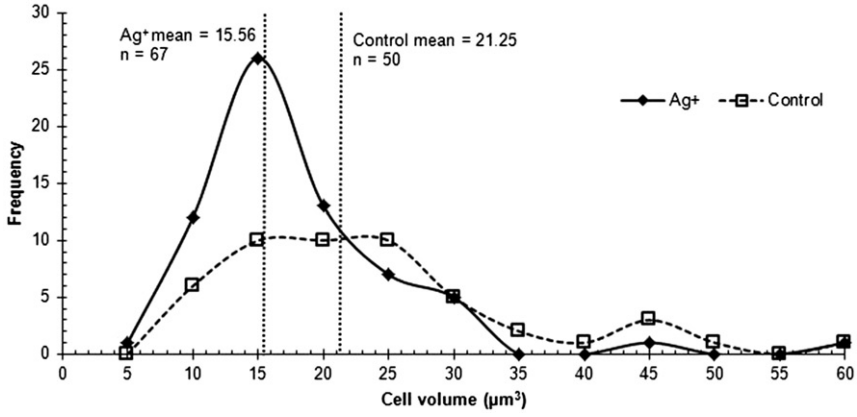

Figure 5. Cell volume distribution (μm^3) of Ag^+ treated and control (untreated) *C. albicans*. Mean diameter for Ag^+-treated *C. albicans* was 15.56 μm ($n = 67$) and untreated *C. albicans* was 21.25 μm ($n = 50$).

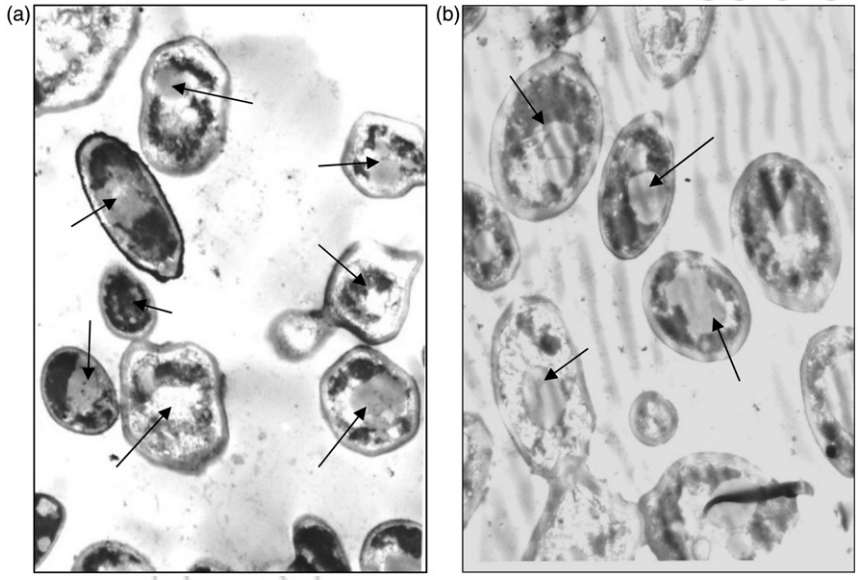

Figure 6. TEM of (a) liposome encapsulated Ag^+ (lipo-Ag^+) treated *C. albicans* and (b) untreated *C. albicans* at magnification 6000 × showing the deleterious effects of free Ag^+ on gross cell and nucleus structure. *Cell nuclei indicated by arrows in the photomicrograph.

of Ag^+ treated cells to fixing and drying (alcohol dehydration) procedures during SEM sample preparation, which resulted in the lack of ability to maintain cell shape. Corresponding TEM sections of *C. albicans* also showed irregular cell shapes, possibly due to the activity of Ag^+ weakening the cell structure. In addition, the observed irregular nuclei of Ag^+ treated cells (Figure 6) are consistent with the effect of Ag^+ on the nuclear regions of *E. coli* and *S. aureus* (Feng et al., 2000).

Liposomes have the ability to interact with many different cell types to deliver encapsulated agents across the cell membrane (Detoni et al., 2009), thus making them an ideal carrier for an enhanced delivery mechanism. These attributes are particularly useful for the delivery of antimicrobial agents to pathogenic microorganisms where poor permeability of the free drug through the cell envelope results in low activity. As discussed earlier, silver ions are highly reactive moieties. Hence, their activity can be quenched by binding to anions, which are usually abundant in the cell environment, for example the wound fluid. This makes it difficult to deliver and maintain an effective concentration of antimicrobial agents without the danger of overdosing (Mooney et al., 2006). The results from this research indicated the feasibility of using liposomes as an effective controlled release delivery system to provide a steady concentration of Ag^+ to *C. albicans* in the presence of a complex milieu, thereby potentially mitigating toxicity issues due to over-dosing.

Conclusion

The results provide the basis for exploring the use of liposomes as a controlled delivery system of Ag^+ to *C. albicans*. Additionally, a controlled release delivery system providing optimal antimicrobial concentration may reduce the likelihood of developing localised over-dose, thereby avoiding localised side effects. This controlled release delivery system could have an application to a practical and safe approach to treating topical slow/non-healing wounds.

Declaration of interest

The authors report no declarations of interest.

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