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Anti-adherent and antifungal activities of surfactant-coated poly(ethylcyanoacrylate) nanoparticles

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Abstract

Application of non-drug-loaded poly(ethylcyanoacrylate) nanoparticles (NP) to buccal epithelial cells (BEC) imparted both anti-adherent and antifungal effects. NP prepared using emulsion polymerisation and stabilised using cationic, anionic and non-ionic surfactants decreased *Candida albicans* blastospore adhesion, an effect attributable to the peripheral coating of surfactant. Cetrimide and Pluronic® P123 were shown to be most effective, producing mean percentage reductions in blastospore adherence of 52.7 and 37.0, respectively. Resultant zeta potential matched the polarity of the surfactant, with those stabilised using cetrimide being especially positive (+31.3 mV). Preparation using anionic surfactants was shown to be problematic, with low yield and wide particle size distribution. Evaluation of the antifungal effect of the peripheral coat was evaluated using zones of inhibition and viable counts assays. The former test revealed poor surfactant diffusion through agar, but did show evidence of limited kill. However, the latter method showed that cationic surfactants associated with NP produced high levels of kill, in contrast to those coated with anionic surfactants, where kill was not evident. Non-ionic surfactant-coated NP produced intermediate kill rates. Results demonstrate that surfactant-coated NP, particularly the cationic types, form the possible basis of a prophylactic formulation that primes the candidal target (BEC) against fungal adhesion and infection.
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Keywords: *Candida albicans*; Blastospores; Poly(ethylcyanoacrylate) nanoparticles; Adherence; Antifungal

1. Introduction

Although mostly a normal and innocuous commensal organism of various body locations, such as the oral cavity and female genital tract, *Candida albicans* can resort to pathogenicity whilst present in certain compromised states, like those arising from diabetes mellitus and during periods of immunosuppression (Odds, 1988; Coleman et al., 1993; Denning, 1995; Jones et al., 1997; Douglas, 1987). An inexorable rise in resistance to both systemic and topical antifungal agents has meant that effective treatments are becoming elusive, thereby driving the interest in developing new prophylactic and treatment regimens (Jones et al., 1997).

In general, most investigations into infectivity propose an initial mandatory step, namely adherence of causative microorganisms to epithelial host cells. This enables the potential pathogen to evade the protective flushing actions of body fluids, such as saliva and urine (Jones et al., 1997; Douglas, 1987). It has been suggested that microbial adherence occurs in two distinct reversible and irreversible phases, differentiated in part by the distance over which interactions occur (Douglas, 1987; Jones et al., 1995a). The reversible phase can be viewed in terms of the DLVO theory, which considers microbial-host cellular interactions as a function of attractive and repulsive forces that come into play as both cell types approach one another. Hydrophobic interactions are thought to contribute largely to the attractive forces, whereas repulsive forces are due to the net negative charges (zeta potentials) on the surfaces of both the microbial cell and its intended substrate (Jones et al., 1995b). At longer distances, such as those greater than 10 nm, the dominant, but easily disrupted, weak attractive forces allow the microbial cell to approach the substrate. As both approach, a point is reached where electrostatic repulsion will dominate and it is this that must be overcome before adherence occurs.

If a separation distance of less than 1.5 nm can be achieved, attachment may be made irreversible via strong interactions

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between adhesin molecules on the microbial surface and the corresponding receptors on the substrate. *C. albicans* express surface protein adhesins that recognise host extracellular matrix proteins including laminin, collagen, fibrinogen, fibronectin and entactin. The evidence is equivocal as to whether these adhesins are identical (with common or multiple binding sites) or separate (with the ability to recognise discrete domains on target molecules). *C. albicans* also express integrin-like cell surface proteins that can bind to epithelial receptors, such as iC3b and fibronectin (Cannon et al., 1995). Similarly, the adherence of *C. albicans* by lectin-like binding of a protein portion of a mannoprotein to host glycoside receptors has been well established, but it is not the complete picture. Other *C. albicans* lectin-like adhesins also include surface proteins that bind fucosyl or *N*-acetyl-D-glucosamine determinants or galactosides on buccal epithelial cells (Odds, 1988). More recently, four adhesins from a class of proteins termed glycosylphosphatidylinositol-dependent cell wall proteins (GPI-CWP) have been studied in depth. They are capable of attachment to cell wall glucan and work with mutant gene-deleted strains has shown the importance of these proteins in adherence (Mendes-Giannini et al., 1995). Irreversible association is possible at distances greater than 1.5 nm when the microbial cell possess cell surface structures, such as fibrillae, on which adhesion molecules are expressed, which bridge the potential energy partition (Jones et al., 1995a,b; Gristina et al., 1988).

Attempts to reduce or inhibit microbial adherence are a viable means to control infection, given the strong relationship between, infectivity, distance of approach and the need to achieve intimate contact. The *in vitro* and *ex vivo* microbial antiadherent properties of sub-minimal inhibitory concentrations of several non-antibiotic, antimicrobial agents have been described, such as chlorhexidine (Gorman et al., 1987; Fowler and Jones, 1992), polyvinylpyrrolidone-iodine (Gorman et al., 1987), cetylpyridinium chloride (Jones et al., 1995a; Fowler and Jones, 1992), cetrimide (Fowler and Jones, 1992), polyhexamethylenebiguanide (Jones, 1995) and hexetidine (Jones et al., 1997). The mechanism of these antiadherent properties appears to be both a disruption of the fungal membrane and, importantly, a steric interference of the approach of the microbial cell to the epithelial cell. However, other factors, including alteration of cell surface hydrophobicity and cell zeta potential, may contribute to this phenomenon (Douglas, 1987; Jones et al., 1995a; McCarron et al., 2004).

As the development of resistance to antimicrobial agents is an ever present problem, other approaches that may physically reduce or inhibit the process of microbial adherence are of interest. It has been shown that polymeric nanoparticles (NP) possess an anti-adherent effect and that poly(propylcyanoacrylate) NP were capable of adhering to *C. albicans* blastospores and reducing their subsequent adherence to buccal epithelial cells *in vitro*. The mechanism by which the NP exerted their effect was thought to be a physical outcome, with the NP sterically hindering a close irreversible attachment (McCarron et al., 2004). NP themselves have attracted considerable interest as colloidal drug delivery systems, capable of delivering a range of therapeutically active entrapped payloads. In the work by McCarron et al. (2004), blastospores were pretreated with NP and as no payload was used, the benefit of the system relied primarily on physical interference. In order to compliment this previous work, an aim of this present study is to investigate the effect of pretreating buccal epithelial cells, as opposed to the *C. albicans*, with nanoparticulate suspensions and evaluating the subsequent adherence of *C. albicans*. NP is prepared generally in a solution of surfactant, which is adsorbed, stabilising the suspension and preventing coalescence. As these surface active agents possess some innate antifungal properties, it is, therefore, necessary to evaluate any antimicrobial activity that is bestowed to non-drug-loaded NP. Thus, a further aim is to evaluate the antifungal properties arising from peripheral release of loosely adsorbed surfactant from the nanoparticulate surface.

2. Materials and methods

2.1. Chemicals

The monomer, ethylcyanoacrylate, was obtained from Loctite Ireland Ltd. (Dublin, Ireland). Cetrimide was purchased from Thornton and Ross Ltd. (Huddersfield, UK). Pluronic® P123 and Tetronic® 904 were gifts from BASF Corporation (Mount Olive, New Jersey, USA). Sodium lauryl sulphate was obtained from Fisons Scientific Apparatus (Loughborough, UK). Tween® 80, benzalkonium chloride, docusate sodium, sodium oleate and crystal violet were obtained from Sigma–Aldrich (Poole, UK). Cetylpyridinium chloride was purchased from BDH Chemicals Ltd. (Poole, UK). Sabouraud liquid medium (SLM) and Sabouraud dextrose agar (SDA) were obtained from Oxoid Ltd. (Basingstoke UK). Potassium dihydrogen phosphate was obtained from Prolabo, VWR International SAS (Fontenay sous Bois, France) and ethanol was from Riedel-de Haën (Seelze, Germany).

2.2. C. albicans strain and growth conditions

C. albicans strain NCYC 1467 (isolated from a case of denture stomatitis) was used in this investigation. It was maintained on SDA slopes at 4 °C. Stationary phase *C. albicans* was obtained by incubation of a sample of the *C. albicans* from an SDA slope in SLM at 37 °C for 16 h. The sample was centrifuged at $3000 \times g$ for 15 min and then re-suspended in SLM to an optical density (OD_{550}) of either 0.5 for the adherence study (see Section 2.8) or 1.0 for the *C. albicans* viability investigation (see Section 2.9). An OD_{550} of 0.5 corresponds to approximately 3×10^7 colony forming units (cfu)/ml (as determined by viable count data). The absence of hyphal forms was confirmed by light microscopy (Jones et al., 1995a; Gorman et al., 1987).

2.3. Preparation of poly(ethylcyanoacrylate) NP

Poly(ethylcyanoacrylate) NP were prepared by the emulsion polymerisation method described previously (McCarron et al., 2004). A defined volume of monomer (400.0 μl) was added dropwise at a rate of 100.0 μl/min to a stirred (100 rev. min^{-1}) continuous phase comprising 20.0 ml phosphate buffer (pH 2.5)

Table 1
Surfactants used in nanoparticle preparation

Anionic	Cationic	Non-ionic
Sodium lauryl sulphate	Benzalkonium chloride	Pluronic® P123
Docusate sodium	Cetrimide	Tetronic® 904
Sodium oleate	Cetylpyridinium chloride	Tween® 80

containing 0.5% (w/v) surfactant. The resulting colloidal suspensions were stirred for 4.0 h to complete polymerisation and then filtered through a Grade 1 sintered glass filter to remove any large particles or aggregates that may have formed. The nine surfactants used in particle preparation are shown in Table 1. Three surfactants were chosen from each of anionic, cationic and non-ionic types, imparting NP with negative, positive and approximately neutral charges, respectively. Due to the poor solubility of the anionic surfactants in phosphate buffer pH 2.5, these particles were prepared using a modified continuous phase of deionised water and ethanol (50:50).

2.4. Physicochemical characterisation of NP

Nanoparticle size (Z_{ave}) was determined by adding approximately 1.0 ml of colloidal suspension to 50.0 ml distilled (and 0.22 μm filtered) water. The sample was sized in a Malvern Zetasizer 4 (Malvern Instruments Ltd., Malvern, UK) using light scattering from a laser source (633 nm) determined at a fixed angle (90°). Evaluations were performed on at least three replicate and independent sample formulations. The zeta potentials of NP (0.2 ml) suspended in filtered (0.22 μm) and deionised water (10.0 ml) were determined using a Malvern Zetasizer 4 (Malvern Instruments, Malvern, UK). Aliquots of the NP were placed into a ZET 5104 capillary cell at 25 °C and zeta potential measurements performed at least six times for individual samples (field strength 10–20 V cm^{-1}, electrode spacing 50 mm, dielectric constant 78.54). As above, the zeta potentials of at least three independent formulations were determined. The yield of useable nanoparticulate material was determined by separating larger microparticulate matter and debris from the polymerisation medium using mild centrifugation (3000 × *g*, 10 min) and expressing this mass to the total mass of monomer added, as shown in the following equation:

$$\%\text{yield} = \frac{\text{mass of monomer} - \text{mass of debris/microparticles}}{\text{mass of monomer}} \times 100 \quad (1)$$

2.5. Collection of buccal epithelial cells (BEC)

BEC were removed from the buccal mucosa of healthy volunteers. Ten volunteers used pre-sterilised ampoule files to scrape the inside of both cheeks a total of five times. The files were placed in a conical flask containing sterile PBS pH 7.4 and BEC dislodged with mild shaking. The BEC suspension was centrifuged (3700 × *g* for 20 min) and re-suspended in sterile PBS pH 7.4 to a defined optical density (OD_{550} = 0.4).

2.6. Preparation of nanoparticle suspensions

Before use, the drug-free NP were washed and re-suspended by brief sonication, after centrifugation at 23,000 × *g* for 15 min. For adherence studies, all sets of NP were re-suspended to an OD_{550} of 0.5, thereby ensuring that the number of particles per unit volume for each type of nanoparticulate formulation was approximately equivalent. For antifungal studies, all sets of particles were re-suspended in SLM to defined OD_{550} values to investigate the influence of increasing particle doses on viability.

2.7. Treatment of BEC with nanoparticle suspensions

An aliquot (1.0 ml) of nanoparticle suspension (OD_{550} = 0.5) was added to 1.0 ml BEC suspension (OD_{550} = 0.4). After 5 min of exposure, the suspension was centrifuged at 3700 × *g* for 20 min. The supernatant containing any free NP was removed and the BEC were re-suspended in 1.0 ml sterile PBS (pH 7.4). For the control procedure, 1.0 ml sterile water was used instead of 1.0 ml nanoparticle suspension.

2.8. Adherence of *C. albicans* blastospores to pretreated BEC

An aliquot (1.0 ml) of stationary phase *C. albicans* blastospores, re-suspended to an OD_{550} of 1.0, as described in Section 2.2, was added to 1.0 ml of pretreated BEC and incubated in an orbital incubator (100 oscillations min^{-1}) for 2 h at 37 °C. After 2 h, samples were removed using a bacteriological loop and placed on microscope slides. The cells were flame fixed and then stained with crystal violet. The number of *C. albicans* blastospores adhering to 100 BEC was counted for each type of nanoparticle, as described previously (McCarron et al., 2004). The mean number of blastospores adhering to each BEC for each particle type was calculated. The percentage of cells free from adhering blastospores was also determined and presented as a ratio of (sample)/(control) to allow comparisons. The percentage reduction in adherence was calculated using the following equation:

$$\%\text{reduction in adherence} = \frac{[(\text{control}) - (\text{sample})]}{\text{control}} \times 100 \quad (2)$$

2.9. Determination of the effect of NP on viability of *C. albicans*

Two methods were used to investigate the effect of NP on the viability of *C. albicans* blastospores. First, the agar diffusion method was used to determine whether NP can inhibit growth of the organism. Briefly, seeded plates were prepared by placing 1.0 ml of *C. albicans* suspension (OD_{550} = 0.1) in an empty Petri-dish. A volume (10.0 ml) of molten Sabouraud dextrose agar (SDA), at approximately 50 °C, was poured into the Petri-dish, followed by gentle swirling to mix the inoculum with the agar. Once the SDA had set (approximately 1 h) a 7.0 mm well was cut from the centre of each plate using a glass borer. The

anti-fungal activity of the NP prepared with each of the surfactants was investigated at defined optical densities. Three wells were filled with 50.0 μl of a nanoparticle suspension of a given optical density. After incubating the plates overnight a zone of inhibition of *C. albicans* growth could be observed. The diameter of each zone of inhibition was measured, giving an indication of the ability of a suspension to inhibit *C. albicans* growth.

The second method employed was the Miles & Misra technique for viable counts. An aliquot (100 μl) of NP in Sabouraud liquid medium (SLM) was incubated (24 h at 37 °C) in a 96-well microtitre plate with 100 μl *C. albicans* blastospores re-suspended to an OD_{550} of 0.5. Three replicate wells were used for each type of nanoparticle and for each optical density and a control of SLM free from NP was also used. Samples were then removed from each well, serially 10-fold diluted in PBS and plated on SDA plates with the total viable count determined after overnight incubation at 37 °C. In each case, results were expressed as the total viable count for *C. albicans* following treatment with a defined OD_{550} of NP in comparison with the total viable count for the untreated control.

2.10. Preparation of NP labelled with Nile Red

Fluorescently labelled NP were prepared as described previously with slight modification of the method described above. Nile Red was dissolved in ethanol (1.0 mg ml^{-1}) and filtered through a Grade 2 sintered glass filter to remove any insoluble material. This solution (1.0 ml) was added to 19.0 ml of polymerisation medium, be it either phosphate buffer (pH 2.5) or 50:50 aqueous ethanol. BEC were treated with labelled NP and subjected to identical exposure, washing and re-suspending protocols, as described for blank NP above, before viewing using confocal laser scanning microscopy. Fluorescence images were acquired by detection of the emitted light at 630 nm.

2.11. Statistical analysis

For the adherence study, Student's unpaired *t*-test was used to evaluate the significance of the effect of the various types of NP on the adherence of *C. albicans* blastospores to BEC. *p*-values of less than 0.05 were considered significant. The Kruskal–Wallis test was used to evaluate the significance of increasing the optical density of nanoparticle suspensions on the survival of treated *C. albicans* blastospores. The Kruskal–Wallis test was also used to determine if altering the optical density of the nanoparticle suspensions affected the ability of the NP to inhibit growth of *C. albicans* blastospores. A *H*-value > 5.6 was considered significant. If the *H*-value was significant, post hoc analysis was carried out using Nemenyi's test; a *q*-value > 3.31 indicated that two sets of data were significantly different. The Mann–Whitney *U*-test was used to determine whether surfactant solutions had significantly better anti-fungal activity than blank NP prepared in phosphate buffer pH 2.5. Again, $p < 0.05$ denoted significance.

3. Results

The results in Table 2 show that polymerisation media containing non-ionic surfactants as stabilisers produced good yields of NP, with Tetronic 904 being especially effective with recoveries approaching 100%. It is also clear that the cationic surfactants, although able to permit some nanoparticle formation, are not as competent as the non-ionic materials. The anionic materials posed particular problems in this investigation, mostly related to difficulties centred on solubility issues in the phosphate buffer used. Notwithstanding extensive modification where the polarity of the polymerisation medium was altered using a 50/50 (v/v) mixture of aqueous ethanol, production of NP in the presence of anionic surfactants was poor, with typical recoveries around 10%. To achieve useable quantities of NP, elevated amounts of monomer were required. The quality of NP produced using sodium oleate was so poor that further investigations were not pursued.

Mean nanoparticulate size and zeta potential measurements derived from photon correlation spectroscopy are shown in Table 2. The choice of surfactant used to produce the NP significantly affected the physicochemical properties, with no discernable trends in size related to the cationic or anionic natures. Of particular note were the contrary effects of benzalkonium chloride and docusate sodium on particle size, yielding NP of mean diameters of 1.14 and 0.14 μm, respectively. Similarly,

Table 2
Physicochemical characterisation of poly(ethylcyanoacrylate) NP produced using different surfactants (means ± standard deviation, $n = 3$)

Surfactant type	Mean particle diameter (nm)	PI	Zeta potential (mV)	Particle yield (%)
Cationic				
Benzalkonium chloride	1141.5 ± 5.2	0.99	6.7 ± 0.7	38.0 ± 5.9
Cetrimide	391.6 ± 8.9	0.10	31.3 ± 0.5	52.6 ± 2.6
Cetylpyridinium chloride	420.7 ± 11.2	0.11	26.2 ± 0.8	35.3 ± 8.2
Anionic				
Sodium lauryl sulphate	226.1 ± 2.3	0.18	−28.1 ± 1.8	10.1 ± 1.3
Docusate sodium	142.1 ± 7.1	0.21	−28.5 ± 0.9	15.7 ± 2.5
Sodium oleate	ND	ND	ND	ND
Non-ionic				
Pluronic® P123	260.0 ± 6.8	0.23	−13.9 ± 0.7	47.3 ± 8.7
Tetronic® 904	225.1 ± 1.1	0.50	9.8 ± 1.0	98.8 ± 1.5
Tween® 80	182.0 ± 9.7	0.16	−26.2 ± 0.6	63.3 ± 6.3

ND: not determined, PI: polydispersity index.

Table 3
Effect of prior treatment of BEC with poly(ethylcyanoacrylate) NP, prepared using different surfactants, on subsequent adherence of blastospores of *Candida albicans* (means ± standard deviation, $n = 3$)

Surfactant type	Mean number of adherent blastospores per BEC	Mean % reduction in adherence	% BEC free from adherent blastospores
Cationic			
Control[a]	2.83 ± 0.25	–	16.00
Benzalkonium chloride	1.84 ± 0.17	35.0	30.00
Cetrimide	1.34 ± 0.13	52.7	36.00
Cetylpyridinium chloride	1.84 ± 0.17	35.0	22.00
Anionic			
Control[a]	3.32 ± 0.28	–	12.94
Sodium lauryl sulphate	2.17 ± 0.21	34.6	26.00
Docusate sodium	2.08 ± 0.22	37.3	32.00
Sodium oleate	ND	ND	ND
Non-ionic			
Control[a]	2.54 ± 0.22	–	15.00
Pluronic® P123	1.60 ± 0.15	37.0	30.00
Tetronic® 904	1.75 ± 0.15	31.1	26.00
Tween 80	1.64 ± 0.16	35.4	28.00

ND: not determined.
[a] Treatment using sterile water instead of a nanoparticle suspension.

the choice of surfactant affected the zeta potential of the NP under investigation, with predictable results. The zeta potential of NP produced using cationic surfactants (cetrimide, cetylpyridinium chloride and benzalkonium chloride) had adsorbed a positive charge and ranged between +6.7 to +31.3 mV. Conversely, the zeta potential of NP that had been prepared using anionic surfactants (docusate sodium and sodium lauryl sulphate) were negative and ranged from −28.1 to −28.5 mV. Interestingly, the zeta potentials of NP prepared using the non-ionic surfactants were dependent on the nature of the surfactant and both positive (Tetronic® 904,) and negative (Pluronic® P123, Tween® 80) zeta potentials were observed.

The effects of treatment of human buccal epithelial cells (BEC) with NP that had been produced using a range of surfactants on subsequent adherence of blastospores of *C. albicans in vitro* are shown in Table 3. Interestingly, treatment of BEC with NP, independent of the surfactant used to manufacture these systems, significantly reduced the mean number of adherent *C. albicans* per BEC compared to control. In this study, the mean number of adherent blastospores per untreated BEC was 2.9, whereas, following treatment with NP, the mean number of adherent blastospores per buccal epithelial cell was reduced by up to circa 53%. The lowest adherences (1.34 and 1.60 blastospores per BEC) were observed with BEC that had been pretreated with NP prepared using cetrimide and Pluronic® P123, respectively. However, results obtained with all types of NP were similar. Furthermore, in many cases, the mean percentage of BEC free from adherent blastospores was significantly greater for those BEC that had been pretreated with NP. For example, following treatment of BEC with sterile water, the percentage of BEC that were devoid of *C. albicans* was approximately 14.6, whereas following treatment with NP, this percentage increased to approximately 30% in each case. Pretreatment of BEC with NP prepared using cetrimide resulted in the largest percentage (36%) of BEC to be free from adhering blastospores.

As can be seen from Table 4, NP prepared using cationic and non-ionic surfactants were capable of reducing the growth of *C. albicans* significantly. NP re-suspended to an OD_{550} of 0.5 or less had no observable effect on growth, regardless of

Table 4
Influence of increasing optical density of nanoparticle suspensions on zones of inhibition measured on Sabouraud dextrose agar plates inoculated with *C. albicans* and treated with nanoparticle suspensions (means ± standard deviation, $n = 3$)

Surfactant type	Zone of inhibition OD_{550} 1.00 (mm)	Zone of inhibition OD_{550} 1.50 (mm)	Zone of inhibition OD_{550} 1.75 (mm)
Deionised water	0.00	0.00	0.00
Benzalkonium chloride	1.67 ± 0.14	2.00 ± 0.00	2.00 ± 0.50
Cetrimide	3.83 ± 0.13	2.83 ± 0.29	3.70 ± 0.29
Cetylpyridinium chloride	1.67 ± 0.29	5.67 ± 0.29	4.20 ± 0.76
Sodium lauryl sulphate	0.00	0.00	0.00
Docusate sodium	0.00	0.00	0.00
Sodium oleate	ND	ND	ND
Pluronic® P123	0.00	1.17 ± 0.20	1.00 ± 0.17
Tetronic® 904	1.30 ± 0.13	1.50 ± 0.50	0.00
Tween 80	2.00 ± 0.17	3.67 ± 0.12	4.00 ± 1.00

ND: not determined.

Table 5
The effect of treatment of blastospores of *C. albicans* (24 h at 37 °C) with nanoparticle suspensions of different optical densities on blastospore viability, defined as surviving colony forming units per ml (cfu/ml) (means ± standard deviation, $n = 3$)

Surfactant type	OD_{550} 1.00		OD_{550} 1.50		OD_{550} 1.75	
	Surviving (cfu/ml)	Mean % kill	Surviving (cfu/ml)	Mean % kill	Surviving (cfu/ml)	Mean % kill
Benzalkonium chloride	0.00	100.00%	0.00	100.00%	0.00	100.00%
Cetrimide	0.00	100.00%	0.00	100.00%	0.00	100.00%
Cetylpyridinium chloride	0.00	100.00%	0.00	100.00%	00.00	100.00%
Sodium lauryl sulphate	$(2.28 \pm 0.14) \times 10^7$	0.00%	$(1.88 \pm 0.27) \times 10^7$	0.00%	$(2.19 \pm 0.32) \times 10^7$	0.00%
Docusate sodium	$(2.44 \pm 0.28) \times 10^7$	0.00%	$(2.38 \pm 0.35) \times 10^7$	0.00%	$(2.17 \pm 0.29) \times 10^7$	0.00%
Sodium oleate	ND	ND	ND	ND	ND	ND
Pluronic® P123	$(1.03 \pm 0.15) \times 10^7$	38.13%	$(8.11 \pm 0.87) \times 10^6$	51.17%	$(7.04 \pm 0.47) \times 10^6$	57.61%
Tetronic® 904	$(2.34 \pm 0.35) \times 10^6$	85.93%	$(8.86 \pm 0.91) \times 10^5$	94.67%	$(2.93 \pm 0.39) \times 10^6$	82.37%
Tween 80	$(6.00 \pm 0.89) \times 10^6$	63.88%	$(7.06 \pm 0.83) \times 10^6$	57.53%	$(5.94 \pm 0.56) \times 10^7$	64.21%

ND: not determined.

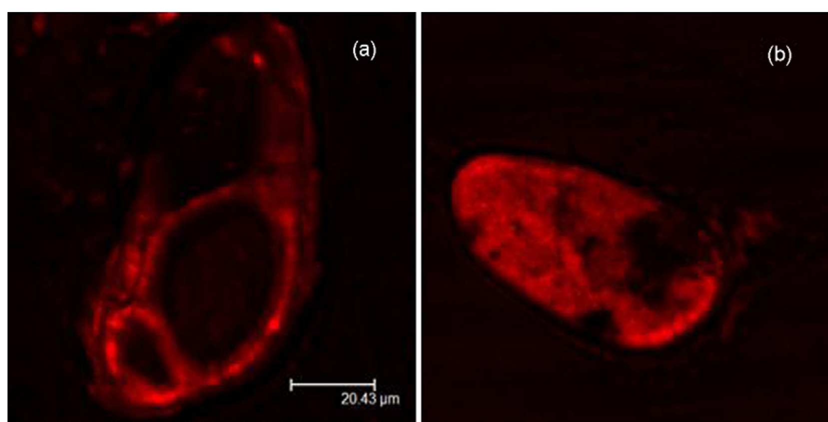

Fig. 1. BEC treated with Nile Red-loaded NP made with (a) Tetronic® 904 and (b) cetrimide as surfactant in the polymerisation medium.

the surfactant used in preparation (data not shown). In addition, NP prepared using anionic surfactants also had no significant and observable effect on growth. No clear pattern was discernable with respect to the influence of increasing optical density on growth of *C. albicans*. In fact, increasing optical density only caused a significant increase in growth reduction when NP were prepared using cetylpyridinium chloride ($H = 7.32$). Indeed, even in this case an increase in growth reduction was only significant for an increase in OD_{550} from 1.00 to 1.50 ($q = 3.78$).

Due to the poor effect on growth caused by NP re-suspended to an OD_{550} of 0.5 or less, an OD_{550} of 1.0 was the lowest optical density used for the fungicidal study. As can be seen from Table 5, incubating *C. albicans* blastospores for 24 h at 37 °C with NP prepared using cationic surfactants resulted in total kill of the organism, regardless of the optical density to which the particles had been re-suspended. In contrast, incubating blastospores with NP prepared using anionic surfactants resulted in no kill, regardless of optical density. NP prepared using non-ionic surfactants caused appreciable reductions in viable counts of *C. albicans* blastospores. However, no pattern of increasing rates of kill was observed in any case when optical density was increased. For example, increasing the optical density of Pluronic® P123 nanoparticle suspensions decreased the number of surviving organisms in a non-significant fashion ($p = 0.0582$).

Fig. 1 shows the fluorescent images of BEC demarcated with adherent labelled NP. All surfactants used produced evidence of surface adherence, but the images produced using cetrimide and Tetronic® 904 were particularly strong and detailed. It was interesting to note that internal cellular structures were made visible during illumination, a finding that is somewhat surprising given that adherent NP were expected to reside on the external cellular membrane.

4. Discussion

The non-drug-based approach taken in this work is based on an acceptance that adherence of microorganisms to epithelial cells is the initial step in the process of infection (Jones et al., 1997; Douglas, 1987). Whilst the treatment of infection normally involves the use of antimicrobial agents, an interest has arisen in the identification, development and utilisation of strategies that will prevent infection in susceptible patients. In such cases, resolution of candidal infection is frequently difficult (Jones et al., 1997; Douglas, 1987). Concerns have been raised about the topical usage of antibiotics for the treatment of oral candidosis, for example, due to the possible emergence of

resistant strains (Jones et al., 1995a, 1997) and, accordingly, an interest has developed in the use of alternative strategies that rely less on drug-based approaches for the prevention or treatment of candidosis.

The findings of McCarron et al. (2004) showed that pretreatment of *C. albicans* blastospores with nanoparticle suspensions significantly reduced their subsequent adherence to human buccal epithelial cells (BEC) (McCarron et al., 2004). Evidence based upon increasing blastospore size and alteration of zeta potentials confirmed that effective peripheral adherence of NP had occurred. However, the anti-adherent effect observed was deemed to be due to steric hindrance of preliminary line of attack initiated by blastospores towards the BEC. The outcome is one were both blastospore and BEC are unable to achieve the intercellular distance (approximately 1.5 nm) required to instigate irreversible binding as the nanoparticulate diameter is in the region of 100–300 nm and would be expected to form a protective barrier of this thickness around the fungal cell. One obvious difficulty with this approach, however, is that it is not possible to pretreat *C. albicans* in a clinical situation. Thus, pretreating the BEC with NP instead of *C. albicans* blastospores was an obvious extension to the work and the aim of this present investigation.

A further extension to the work of McCarron et al. (2004), and studied in this present investigation, was the role played by the adsorbed surfactant (McCarron et al., 2004). Two important points should be made about this surfactant layer. First, as it is adsorbed, its attachment is tenuous and sustained surface release is possible. Second, it is with this layer that impending blastospores will interact and not the underlying solid nanoparticulate core. This delegates the core to a mostly adjuvant role and one that is unlikely to influence the findings on this study. Attention is very much focused on the peripheral characteristics, both in terms of its anti-adherent and antimicrobial effects. Although the NP used in this work did not contain an antimicrobial agent, the peripheral surfactant coat could perform this role.

Surfactants are used frequently as steric stabilisers during the preparation of poly(alkylcyanoacrylate) NP and are added at low concentration, typically around 0.5–1.0% (w/v), to the continuous phase. Alkylcyanoacrylate NP prepared without surfactant in the continuous phase are predisposed to coalescence and aggregation, forming an unstable suspension. As the alkylcyanoacrylate chains enmesh into a nanoparticle matrix during polymerisation, the surfactant molecules adsorb onto the surface. Preliminary studies in this work showed that it was difficult to obtain any one consistent value for a surfactant concentration across all the surfactant types used that would result in good nanoparticulate yields. In the end, 0.5% (w/v) was shown to be most applicable in terms of yield and was kept constant for all formulations. Furthermore, the type of surfactant used determines final size, a finding commonplace in the literature and supported during this work (Table 2). For example, the non-ionic surfactants, such as the poly(oxethylene-oxpropylene) derivatives, are widely used for stabilisation purposes and preparation methods using them result in NP with a size range of approximately 100–600 nm (McCarron et al., 1999).

The NP examined in this study exhibited a wide range of zeta potentials and particle sizes that were dependent on the surfactant used in their production. Typically, polymerisation in the presence of cationic surfactants resulted in good yields of nanoparticulate material (Table 2), but benzalkonium chloride particles exhibited large diameters (>1.0 μm). All particles prepared using cationic surfactants possessed positive zeta potentials, this being attributed to the presence of the surfactant on the nanoparticle surface. The sizes of NP produced in the presence of either anionic or non-ionic surfactants were all within the nanometer size range. In fact, it was notable that, in each case, the NP produced in the present study using ethylcyanoacrylate as monomer were smaller in size than those previously prepared (McCarron et al., 2004) using the same surfactants and propylcyanoacrylate as monomer. This may be attributable to the additional bulk and ensuing size occupied by the propyl group over that of the ethyl residue once chains has enmeshed into a polymeric core. Yields of nanoparticulate material were poor for anionic surfactants, due to the elevated pH of the water/ethanol mix used to circumvent solubility difficulties. It is accepted widely that NP formed from alkylcyanoacrylates are not formed well at above pH 5, due to potentiation of the anionic polymerisation mechanism of these monomers. As a result, anionic NP were not viable.

Table 2 lists the polydispersity index (PI) for the eight formulations that produced useable amounts of NP. PI is a unitless expression for the tightness of the particle size distribution and has a meaning distinctive to the term more normally associated with spread in polymeric molecular weight. When $PI < 0.08$, a tight monomodal distribution is the key feature of the size distribution, while $PI > 0.08$ represents a wider spread or a probable multimodal distribution. It is clear from Table 2 that there is no correlation between surfactant type and PI. NP made from benzalkonium chloride and Tetronic® 904 do not have a tight size distribution, although the reason for this is different for both. The former surfactant is a poor stabiliser, giving a mixed population of microparticles, NP and a collection of coalesced particles. The particle size distribution stabilised by the latter surfactant type revealed the presence of small NP, below 80 nm, which was notably smaller than anything produced by the other surfactants. Therefore, when selecting an optimal nanoparticulate suspension, size, PI and yield must be considered together.

In this study, it was shown that treatment of BEC with NP significantly decreased subsequent *C. albicans* blastospore adherence *in vitro*. Whilst complete inhibition of microbial adherence was not observed, the extent of the reduction was impressive and was similar to that associated with pretreatment of BEC with sub-minimum inhibitory concentrations of non-antibiotic antimicrobial agents (Jones and Gorman, 1997). It should be noted, however, that the reduction in adherence, both in terms of mean number of adherent blastopores per BEC and the percentage of BEC free from adherent blastopores, was not as pronounced when blastopores were pretreated rather than BEC (McCarron et al., 2004). A similar phenomenon was observed in previous work using non-antibiotic antimicrobial agents, where either blastopores or BEC were treated and the results of adherence studies compared by analysis of areas under the frequency distribution curves (Jones and Gorman, 1997). There could be several explanations for the differences in adherence reduction

in this and the complimentary study of McCarron et al. (2004). First, it should be noted that these studies sourced BEC from different human donors. Several authors have recognised that the composition of the donor pool of epithelial cells significantly affects the subsequent number of adherent blastospores (Sandin et al., 1987; Schep et al., 1994). Therefore, the observed differences in the adherence of control blastopores between this and the previous study (approximately 14.6% and 40.7% of BEC free from adherent blastospores) reflect the relative variations in microbial affinities of epithelial cells from different volunteer pools. Notwithstanding these possible variations, it is feasible that the differences in adherence observed arises from a greater affinity of NP to the blastospore surface and a greater ability of these NP to block adhesin molecules on the surface of blastopores than to occupy sites on the surface of BEC. It is also important to note that blastopores are much smaller in size that BEC, so the same number of attached NP occupies a much greater proportion of their surface area than they would if they were attached to a BEC. This may mean that fewer NP would be required per blastopore to reduce their ability to adhere to epithelial cells.

In the clinical situation, pretreatment of *C. albicans* blastopores is not possible. Therefore, if this approach is to be employed clinically, BEC must be treated using the NP in a mouthwash-type formulation. This study has shown that pretreatment of BEC with nanoparticle suspensions is successful in reducing adherence of *C. albicans*. In addition, and importantly, it was also shown that nanoparticle suspensions are capable of reducing growth and killing *C. albicans*. The agar procedure is a useful way to separate free drug that has been released from its nanoparticle carrier as the latter is unable to diffuse through the agar network. In this study, distinct zones of inhibition were observed, which can only arise from released surfactant. However, one drawback with this method is that large molecular species show low diffusivity and the method is not particularly sensitive. The alternative assay, based upon direct exposure, did reveal an observed antifungal affect, which was particularly pronounced with the cationic surfactants. These are known antimicrobial agents. This may mean that *C. albicans* blastopores evading the initial anti-adherent effect of NP on the surface of BEC may be killed or prevented from multiplying by NP eliciting surface release of an antimicrobial agent in close proximity to the site of blastopore attachment. This is a departure from the more commonplace means of drug delivery from colloidal systems, where it is more usual to find drug release originating from an entrapped payload within the nanoparticle matrix or core. There is a clear duality of function attributable to the NP in this work; an anti-adherent effect induced by surface surfactant that in itself is active and released slowly in close proximity to the target cell.

The images in Fig. 1 reveal an interesting outcome. Nile Red is particularly suitable for labelling NP suspended in an aqueous environment. Data produced in this work (not shown) show clearly that this fluorescent label remains ensconced within the core and does not release into the field of view. Although all surfactant types confirmed surface attachment visually, the emissive images produced by Tetronic® 904 and cetrimide were particularly strong. It is also clear that nanoparticulate attachment is not uniform over the BEC surface, with regions illuminating poorly. The membrane adjacent to the nuclear regions of the BEC appears to bind NP weakly. It is possible that cellular uptake via endocytosis has occurred, but this is unlikely as cells were not stored or used in media that would promote the continuation of metabolic processes. NP stabilised with cetrimide are especially interesting. The results in Table 2 indicate that these NP will carry a positive zeta potential, thereby promoting attachment to the BEC and explaining the findings shown in Fig. 1. The results in Table 3 then indicate that these NP are effective at reducing blastospore attachment. Moving to Table 4, the results indicate that cetrimide release from the NP surface is occurring as evidenced by the zones of inhibition. NP *per se* are unable to diffuse through agar. The effectiveness of this cetrimide diffusion is confirmed by results in Table 5, where effective kill is seen when BEC and blastospores come into direct contact. A similar reasoning can be made for NP stabilised with Tetronic®, but the attachment to NP could be stronger, as shown by the smaller zone (Table 4), and microbial activity may be inherently less (Table 5).

In conclusion, this work has shown that non-drug-loaded NP have both anti-adherent and antifungal properties that are attributable to the peripheral coating of surfactant. This system may form the basis of a prophylactic formulation, possibly mouthwash based, that primes the potential candidal target, the BEC in this case, against adhesion and infection. This is done by coating the epithelial layer with adherent NP that fend off attack by preventing close approach and simultaneously releasing microbial materials to those blastospores that do succeed in securing attachment. This should form the basis of an alternative strategy in the prophylaxis of candidosis of the oral cavity in susceptible patients without the possibility of the emergence of microbial resistance or other topical antimicrobial agent related side-effects, like staining of teeth. Due to the colloidal size range of the nanoparticulate systems under examination, with the exception of those particles that were produced using benzalkonium chloride, administration to the oral cavity would be straightforward and no grittiness would be experienced by the patient. NP stabilised using cetrimide would be particularly effective in this regard.

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