

Acanthamoeba encystment: multifactorial effects of buffers, biocides, and demulcents present in contact lens care solutions

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Purpose: To determine whether agents which are purportedly capable of inducing encystment of *Acanthamoeba* can recapitulate the signal when tested in differing formulations.

Methods: In accordance with the International Standard ISO 19045, *Acanthamoeba castellanii* ATCC 50370 trophozoites were cultured in antibiotic-free axenic medium, treated with test solutions, and encystment rates plus viability were measured via bright field and fluorescent microscopy. Test solutions included phosphate-buffered saline (PBS), borate-buffered saline, biguanide- and hydrogen peroxide (H₂O₂)-based biocides, propylene glycol (PG) and povidone (POV) ophthalmic demulcents, and one-step H₂O₂-based contact lens disinfection systems.

Results: Only PBS solutions with 0.25 ppm polyaminopropyl biguanide (PAPB) and increasing concentrations of PG and POV stimulated *A. castellanii* encystment in a dose-dependent manner, whereas PBS solutions containing 3% H₂O₂ and increasing concentrations of PG and POV did not stimulate encystment. Borate-buffered saline and PBS/citrate solutions containing PG also did not stimulate encystment. In addition, no encystment was observed after 24 hours, 7 days, or 14 days of exposures of trophozoites to one-step H₂O₂ contact lens disinfection products or related solutions.

Conclusion: The lack of any encystment observed when trophozoites were treated with existing or new one-step H₂O₂ contact lens care products, as well as when trophozoites were exposed to various related test solutions, confirms that *Acanthamoeba* encystment is a complex process which depends upon simultaneous contributions of multiple factors including buffers, biocides, and demulcents.

Keywords: propylene glycol, contact lens care system, hydrogen peroxide disinfecting solution

Introduction

Acanthamoeba protozoa are ubiquitous pathogens found in both soil and aquatic environments, man-made structures and can also reside in healthy organisms.^{1,2} They are capable of causing a variety of human infections, including granulomatous amebic encephalitis in immune-compromised individuals, which affects the central nervous system and is often fatal.¹ *Acanthamoeba* have been shown to serve as a reservoir for *Legionella*, with these bacteria persisting as endosymbionts within the amoebae, leading to the hypothesis that outbreaks of Legionnaire's disease may be directly related to presence of the *Acanthamoeba*.³ In healthy (ie, immune-competent) patients, *Acanthamoeba* spp. can also cause *Acanthamoeba* keratitis (AK), a painful infection of the cornea which can result in severe and permanent visual impairment, including blindness.⁴ The incidence of AK among contact lens wearers in the USA has historically been low, with estimates of one to two cases per million users.⁵ Approximately 80%–90%⁶ of AK infections

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occur among contact lens wearers, however, with additional risk factors associated with noncompliance, such as improper storage and handling, as well as swimming or showering while wearing lenses.⁴ An increase in confirmed AK cases was observed at the beginning of 2004 and ultimately was linked to patients using the multipurpose contact lens cleaning solution Complete® MoisturePlus™ (CMP), resulting in its voluntary recall by Advanced Medical Optics in May 2007.⁷ The association of AK cases with CMP users was proposed to be due to stimulation of *Acanthamoeba* encystment observed following exposure to the CMP solution.⁸

Under conditions of environmental stress such as starvation and desiccation, *Acanthamoeba* trophozoites undergo transformation into a double-walled resistant cyst that is capable of remaining dormant, yet viable, until conditions favorable for growth return. This encystment process is a natural component of the *Acanthamoeba* life cycle, and cyst forms have been shown to resist disinfection by heat, chlorination, and treatment with multipurpose contact lens disinfecting solutions.^{9–16} Kilvington et al showed that over 20% of *Acanthamoeba* trophozoites exposed to CMP solution for 24 hours in lab experiments transformed into immature cysts.⁸ This study concluded that the demulcent propylene glycol (PG) was a causative factor in the CMP solution which promoted encystment, while importantly noting that additional factors, such as the buffering system of the solution, may have also been involved.⁸ The hypothesized influence of other (ie, non-PG) factors is supported by the fact that many currently available ophthalmic products (including a variety of other contact lens disinfecting solutions, contact lens packaging solutions, and lubricating eye drops) contain PG and to date have not been associated with *Acanthamoeba* encystment.

Ophthalmic demulcents are typically water-soluble polymers which may be topically applied to the eye to protect and lubricate mucous membrane surfaces as well as to relieve dryness and irritation.¹⁷ Ophthalmic demulcents are also present in various lens care disinfecting solutions to provide enhanced lubrication. PG is a liquid polyol monographed ophthalmic demulcent that acts as a water-binding moisturizer that can hold nearly three times its weight in water.¹⁸ Similarly, the demulcent povidone (POV) can retain water while also lubricating the surfaces to which it binds. Both are approved for human use as active ingredients in over-the-counter ophthalmic products at concentrations ranging from 0.2% to 1.0% for PG and 0.2% to 2.0% for POV.¹⁷

Previous studies have investigated the contribution of PG in multipurpose solutions on *Acanthamoeba* encystment.

Although one study concluded that PG was a primary factor in CMP solution-induced encystment,⁸ additional studies showed that solutions containing PG at similar concentrations did not stimulate encystment.¹⁹ Additionally, *Acanthamoeba* encystment has been shown to be influenced by different buffering agents.²⁰ Furthermore, the impact of PG in H₂O₂-based disinfecting solutions has not been thoroughly studied. Given the previous studies showing PG as a causative factor of *Acanthamoeba* encystment plus the lack of similar testing for H₂O₂-based solutions,⁸ a series of model disinfecting solutions and commercially available lens care products were tested to directly assess the individual and combined impacts of different ophthalmic demulcents, chemical disinfectants, and buffering agents on *Acanthamoeba* encystment. The purpose of the study reported here was to determine whether agents which are purportedly capable of inducing encystment of *Acanthamoeba* can recapitulate the signal when tested in differing formulations. Such data have important implications for the development of contact lens disinfecting solutions concerning the types and concentrations of buffering agents and biocidal components to be used when considering *Acanthamoeba* encystment.

Materials and methods

Cultures and media

A. castellanii ATCC 50370 (American Type Culture Collection, Manassas, Virginia, USA) was thawed from cryopreserved ampoules and grown for 3–4 days at 28°C±2°C in semi-defined axenic AC#6 growth media lacking antibiotics. One day prior to testing, cells were passaged to fresh medium and incubated for an additional 20–24 hours at 28°C±2°C. Following outgrowth, trophozoites were dislodged, washed three times in quarter-strength Ringer's solution (Oxoid BR0052G; Thermo Fisher Scientific, Waltham, MA, USA), and centrifuged at 500×g for 5 minutes at room temperature. Cells were counted using a disposable hemocytometer (C-chip Fuchs-Rosenthal DHC-F01, INCYTO; Drive Covington, GA, USA) and adjusted to 1.0×10⁷ trophozoites/mL immediately before testing. Neutralization buffer for H₂O₂-containing solutions (AC#6-C) consisted of AC#6 media supplemented with 0.1% filter-sterilized bovine liver catalase (Sigma C40; Sigma-Aldrich Co, St Louis, MO, USA).

Test solutions

Table 1 provides a complete listing along with key composition details for each test solution. A positive encystment control solution was adapted from both our own studies and previous reported studies: phosphate buffered saline (PBS)

Table 1 Summary of test solutions in this study

Test solution	Key components	Experiment
PBS-PAPB-PG+	PBS, 0.25 ppm PAPB, 0.5% PG	Figure 1
PBS-PAPB-PG-	PBS, 0.25 ppm PAPB	Figure 1
BBS-PAPB-PG+	BBS, 0.25 ppm PAPB, 0.5% PG	Figure 1
BBS-PAPB-PG-	BBS, 0.25 ppm PAPB	Figure 1
H ₂ O ₂ range	PBS, 1% PG, H ₂ O ₂ range	Figure 2
PeroxiClear (PER-1), Bausch & Lomb	PBS/citrate, 1% PG, 3% H ₂ O ₂	Figure 3
PER-1a	PER-1, minus PG	Figure 3
PER-1b	PER-1, minus H ₂ O ₂	Figure 3
PER-1c	PER-1, minus PG and H ₂ O ₂	Figure 3
PG range in PAPB	PBS, 0.25 ppm PAPB, 0%–1% PG	Figure 4
POV range in PAPB	PBS, 0.25 ppm PAPB, 0%–2% POV	Figure 4
PG range in H ₂ O ₂	PBS, 0.25 ppm 3% H ₂ O ₂ , 0%–1% PG	Figure 4
POV range in H ₂ O ₂	PBS, 0.25 ppm 3% H ₂ O ₂ , 0%–2% POV	Figure 4
PER-1	PBS/citrate, 1% PG, 3% H ₂ O ₂	Figure 5
Clear Care (PER-2), Alcon Laboratories	PBS, 3% H ₂ O ₂	Figure 5

Abbreviations: BBS, borate-buffered saline; BBS-PAPB-PG+, solution containing BBS, PAPB and PG; BBS-PAPB-PG-, solution containing BBS and PAPB, without PG; PAPB, polyaminopropyl biguanide; PBS, phosphate-buffered saline; PBS-PAPB-PG+, solution containing PBS, PAPB and PG; PBS-PAPB-PG-, solution containing PBS and PAPB, without PG; PER-1a, PeroxiClear solution lacking propylene glycol; PER-1b, PeroxiClear solution lacking H₂O₂; PER-1c, PeroxiClear solution lacking propylene glycol and H₂O₂; PG, propylene glycol; POV, povidone.

solution containing polyaminopropyl biguanide (PAPB, also referred to as polyhexamethylene biguanide) and PG (PBS-PAPB-PG+).²¹ The non-buffer components of PBS-PAPB-PG+ were also formulated as a borate-buffered saline (BBS) solution containing PAPB and PG (BBS-PAPB-PG+) to investigate the role of different buffering agents. Two different ophthalmic demulcents, PG and POV, were added at their respective US Food and Drug Administration (FDA) monographed ranges¹⁷ into either PAPB or H₂O₂-containing solutions. A series of H₂O₂ concentrations (0%–3%) was also tested in the presence of 1% PG. A new one-step H₂O₂-based contact lens care product, PeroxiClear (PER-1; Bausch & Lomb, Rochester, NY, USA) was tested in parallel with another H₂O₂-based lens care product, Clear Care (PER-2; Alcon Laboratories, Fort Worth, TX, USA). Derivatives of the PER-1 solution which lacked PG, H₂O₂, or both (PER-1a, PER-1b, PER-1c, respectively) were also tested. Neutralization of H₂O₂-containing solutions was confirmed by measuring residual H₂O₂ levels using a Mettler Toledo T50 Autotitrator with a modified 4-hour method adapted from USP 38 monograph. The monograph describes a titrimetric system for assaying the concentration of hydrogen peroxide in a topical solution. The titrant used was 0.1 N potassium permanganate with the endpoint detected using Mettler Toledo LabX light titration, version 3.1.1.0. Each milliliter of the titrant used was equivalent to 1.701 mg of hydrogen peroxide. A modification of the sample size along with programming the software to automatically dispense the titrant at a controlled rate allowed for the detection of hydrogen peroxide at levels between 0 and 30,000 ppm.

Encystment testing

Encystment rates for each solution were measured as described in International Standard ISO 19045:2015: Ophthalmic optics – contact lens care products – method for evaluating *Acanthamoeba* encystment by contact lens care products” (hereafter, “ISO 19045”),²² with modifications as needed to accommodate testing with one-step H₂O₂ disinfecting systems. Assays using solutions containing H₂O₂ were performed in manufacturer-provided neutralizing lens cases containing platinum discs which catalyze the conversion of H₂O₂ to H₂O and O₂. Lens cases were filled with 10 mL of test solution and inoculated to give a final concentration of 1.0×10⁵ trophozoites/mL, then immediately capped and incubated at room temperature (20°C±2°C). At specified time points, the detergent sarkosyl (Sigma 61747; Sigma-Aldrich Co) was added to lens cases to lyse any remaining trophozoites, while the fluorescent stain calcofluor-white (Sigma F3543; Sigma-Aldrich Co), which binds to cellulose within cyst walls,²³ was used to identify cysts. Exposure to the sarkosyl/calcofluor-white mixture (150 mg/10 mg per 1.0 mL in purified water) did not exceed 5 minutes to ensure that only trophozoites, and not cysts, would be lysed. Test solutions which lacked H₂O₂ were tested in multi-well plates containing 3 mL of test solution and inoculated to give a final concentration of 1.0×10⁵ trophozoites/mL, and all other steps were performed as described earlier. Samples were enumerated using an Olympus BX51 upright epifluorescence microscope by comparing manual counts under bright field illumination to epifluorescence (excitation 350 nm/emission 460 nm). Rounded cellular bodies with bright white fluorescence

were counted as cysts. Encystment was expressed as a percentage of cysts observed after solution exposure relative to inoculum control counts, corrected for background cyst count obtained at 0 hour. Final percentages were averaged from three independent assays. For encystment studies that were carried out to 14 days, viable amoebae were also determined as described later in this work.

Viability counts

To enumerate surviving amoebae after 14 days of incubation with test solutions, an agar overlay method²⁴ was used, with modifications. Briefly, 1 mL samples were removed from lens cases after 14 days' incubation and neutralized in AC#6-C for 10 minutes. Samples were again serially diluted (1:10) in AC#6-C, then pelleted by centrifugation at 1,000× *g* for 5 minutes and resuspended into 100 μL of neutralizing buffer. To this, 100 μL of *Escherichia coli* (ATCC 33876, 5.0×10⁸/mL) and 3 mL of molten top agar (10.0 g tryptone; 5.0 g NaCl; 7.0 g Bacto™ agar (BD Biosciences 214010; BD Biosciences, San Jose, CA, USA) held at 45°C were added and vortexed to mix thoroughly. The top agar mixture was then poured onto solidified SM/5 agar plates (2.0 g proteose peptone, 2.0 g yeast extract, 2.0 g glucose, 2.0 g MgSO₄, and 15.0 g Bacto agar in 1.0 L of purified H₂O) and incubated at 28°C±2°C for 5 days. Viable amoebae were enumerated by counting visible plaques formed against a background of *E. coli* growth within the top agar, and recorded as plaque-forming units.

Stand-alone biocidal efficacy

Testing of one-step H₂O₂ disinfecting solutions was adapted from previously described methods.^{11,19} Independent duplicate assays were performed in new lens cases containing neutralizing platinum-coated discs. Lens cases were filled with 10 mL of either PER-1 or PER-2 and inoculated to give a final concentration of 5.0×10⁴ trophozoites/mL, then immediately capped. At each time point, samples were neutralized with AC#6-C for 10 minutes, then serially diluted (1:10) in tubes containing AC#6-C. Aliquots from the dilutions were added to the wells of a 96-well plate and incubated at 28°C±2°C. Wells were then microscopically inspected over a 14-day outgrowth period for evidence of trophozoite growth and replication. Log₁₀ reduction values were averaged from independent duplicate assays as determined by the most-probable number method using Spearman–Karber computations.²⁵

Statistical methods

All analyses were run using Minitab® 17.2.1 Statistical Software (Minitab Inc., State College, PA, USA); Bonett's method was used to determine any statistical differences in

variances between two-sample sets using an alpha =0.05. Any statistical differences in the variances were accounted for in the comparison of the mean values. Two-sample *t*-tests were used for comparing the means between the control and test sample sets at 95% confidence interval. If the *P*-value was less than or equal to 0.05, the means were considered to be significantly different between the two-sample sets.

Results

Across multiple experiments, the positive encystment control solution PBS-PAPB-PG+ (containing 0.5% PG and 0.25 ppm PAPB in a phosphate-buffered system) consistently yielded encystment rates of ≥50% (Figures 1 and 2). To assess the influence of different buffering systems on *A. castellanii* encystment, the non-buffer system components of PBS-PAPB-PG+ were reformulated into a borate-buffered system – BBS-PAPB-PG+. After incubation with BBS-PAPB-PG+ for 24 hours, *A. castellanii* trophozoites did not encyst (Figure 1). The mean encystment rate of the control solution PBS-PAPB-PG+ was significantly higher than each of the test solutions (solution containing PBS and PAPB, without PG [PBS-PAPB-PG-], BBS-PAPB-PG+, and solution containing BBS and PAPB without PG [BBS-PAPB-PG-]) (*P*<0.05).

Next, a series of PBS/citrate-buffered solutions containing a concentration of 1.0% PG and a range of H₂O₂ concentrations from 0% to 3% were tested, none of which induced encystment of *A. castellanii* (Figure 2). The mean encystment rate of the positive control (PBS-PAPB-PG+) was

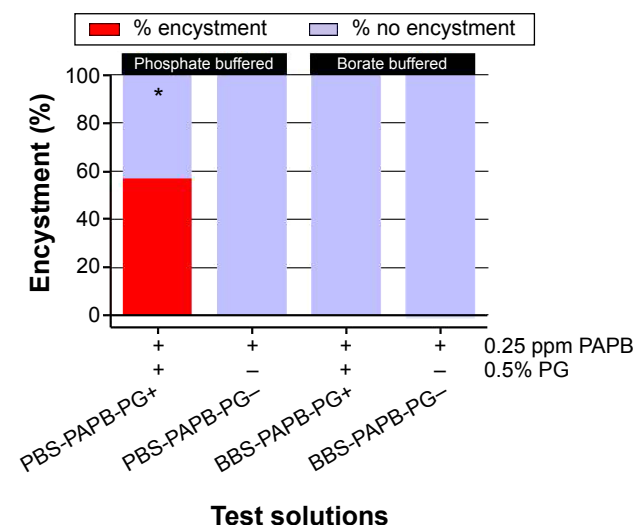


Figure 1 Impact of buffer agents on *Acanthamoeba castellanii* encystment rates. **Note:** Red bar represents percentage of trophozoites that encysted after 24 hours' incubation, while blue bars represent percentage of population that did not (**P*<0.05). **Abbreviations:** BBS, borate-buffered saline; BBS-PAPB-PG+, solution containing BBS, PAPB and PG; BBS-PAPB-PG-, solution containing BBS and PAPB, without PG; PAPB, polyaminopropyl biguanide; PBS, phosphate-buffered saline; PBS-PAPB-PG+, solution containing PBS, PAPB and PG; PBS-PAPB-PG-, solution containing PBS and PAPB, without PG; PG, propylene glycol; POS, positive.

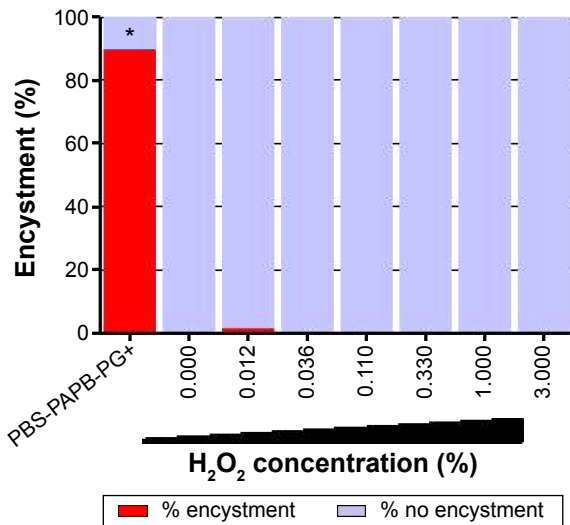


Figure 2 Encystment rates of *Acanthamoeba castellanii* exposed to PBS-PAPB-PG+ control and to 1% PG with varying concentrations of H₂O₂.

Note: Red bar represents percentage of trophozoites that encysted after 24 hours' incubation, while blue bars represent percentage of population that did not (**P*<0.05).

Abbreviations: PAPB, polyaminopropyl biguanide; PBS, phosphate-buffered saline; PBS-PAPB-PG+, solution containing PBS, PAPB and PG; PG, propylene glycol.

significantly higher than all varying concentrations of H₂O₂ (*P*<0.05), while among the varying concentrations of H₂O₂, no significant differences were observed (*P*>0.05).

To assess *A. castellanii* encystment with regards to the addition of ophthalmic demulcents to model disinfecting

solutions, phosphate-buffered solutions that contained either 0.25 ppm PAPB or 3% H₂O₂ were prepared. Both POV and PG were added to each at dose levels spanning their respective FDA monograph concentration ranges (POV: 0.0%–2.0%, PG: 0.0%–1.0%; monograph ophthalmic demulcent concentration ranges defined by the FDA Code of Federal Regulations for use in over-the-counter ophthalmic drug products).¹⁷ After 24 hours' incubation, encystment rates for solutions containing 0.25 ppm PAPB increased in a dose-dependent manner for both PG and POV (88% and 14%, respectively, at their highest test concentration). For both PAPB and H₂O₂ test solutions, the mean encystment rates for different concentrations of POV and PG were compared to their respective base solution (ie, 0.0% POV or PG) (Figure 3). For the PAPB test solutions containing POV, only the formulation of 2.0% POV was significantly greater than the solution containing no (0.0%) POV (*P*<0.05). Mean encystment rates of PAPB test solutions containing PG were significantly greater than that of the PAPB solution without PG (0.0%) for all concentrations tested (*P*<0.05). Solutions containing 3% H₂O₂ did not induce encystment (ie, ≤1%) regardless of the presence or absence of demulcents. For all concentrations of POV with the H₂O₂ test solutions, the mean encystment rates were not significantly different from the solution

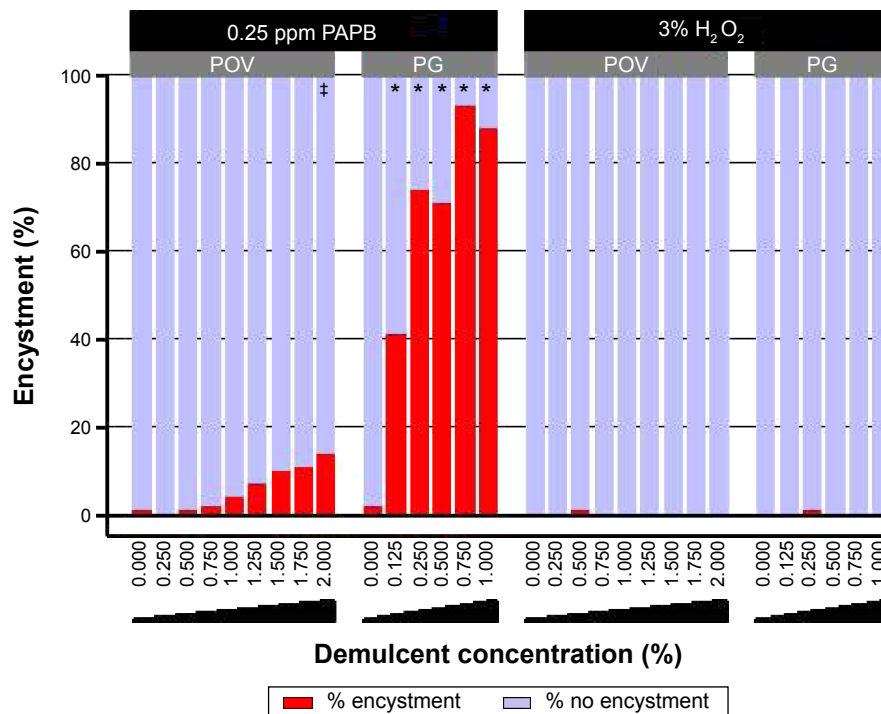


Figure 3 Encystment rates of *Acanthamoeba castellanii* exposed to phosphate-buffered solutions that contained either 0.25 ppm polyaminopropyl biguanide (PAPB) or 3% H₂O₂ with increasing concentrations of povidone (POV) and propylene glycol (PG).

Notes: Red bars represent percentage of trophozoites that encysted after 24 hours' incubation, while blue bars represent percentage of population that did not (†*P*<0.05 for PAPB-2.0% POV vs PAPB-0.0% POV; **P*<0.05 for all PAPB-PG containing solutions vs PAPB-0.0% PG).

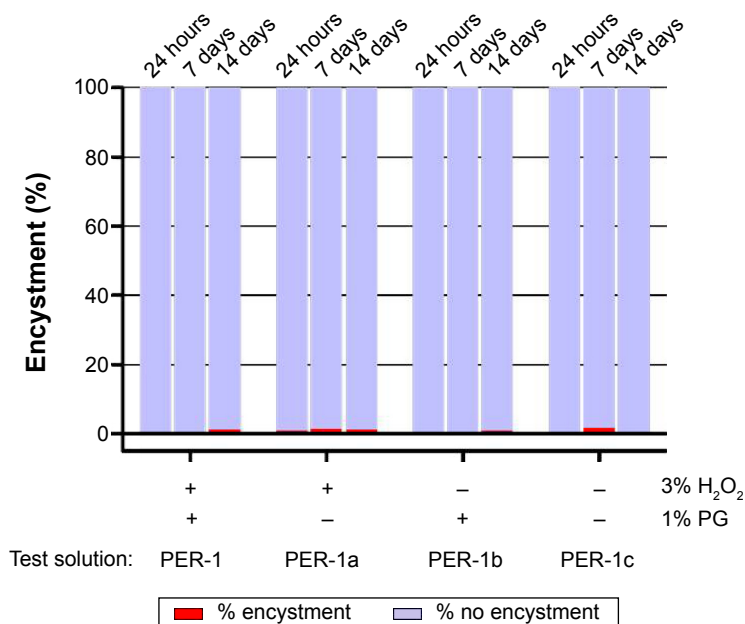


Figure 4 Encystment rates of *Acanthamoeba castellanii* trophozoites exposed to PeroxiClear (PER-1), PeroxiClear solution lacking propylene glycol (PER-1a), PeroxiClear solution lacking H₂O₂ (PER-1b), and PeroxiClear solution lacking propylene glycol and H₂O₂ (PER-1c) for 24 hours, 7 days, and 14 days. No significant differences were observed across any of the solutions tested regardless of the time point ($P>0.05$).

Notes: Red bars represent percentage of trophozoites that encysted, while blue bars represent percentage of population that did not. The + and - symbols indicate whether or not the 3% H₂O₂ and/or the 1% PG were included within the solution.

Abbreviation: PG, propylene glycol.

containing no POV ($P>0.05$). Similarly, for the H₂O₂ test solutions containing PG, the mean encystment rate of the test solution containing a 0.0% concentration of PG was not significantly different than the test solution with increasing concentrations of PG ($P>0.05$).

Label instructions for the new one-step H₂O₂ disinfecting solution (PER-1) tested included simultaneous disinfection and neutralization with a minimum of a 4-hour soak time followed by storage of disinfected contact lenses for up to 7 days. Considering these storage instructions, the potential for encystment during a simulated long-term storage condition was tested as follows. When *A. castellanii* was added to PER-1 and its derivatives, PER-1a, PER-1b, and PER-1c (Table 1), and incubated for 24 hours, 7 days, or 14 days, encystment rates for all solutions at each time point were $\leq 2\%$, which was significantly less ($P<0.05$) than the positive control (Figure 4). Surviving cells from inoculated lens cases stored for 14 days were also enumerated with no recovery of *Acanthamoeba* observed for PER-1 and PER-1a solutions (both of which contained 3% H₂O₂), whereas 2,200 and 1,100 amoeba/mL, respectively, were recovered from PER-1b and PER-1c (both of which did not contain H₂O₂).

Finally, both of the one-step H₂O₂ lens care products – PER-1 and PER-2 – were evaluated for encystment effects as well as for stand-alone biocidal efficacy against *A. castellanii*

trophozoites. Encystment rates for both products were $<1\%$ after 24 hours' incubation, significantly ($P<0.05$) lower than the positive control solution (Figure 5A). Average log₁₀ reduction results at 4, 6, and 24 hours were 4.3, 4.3, and 4.3, respectively, for PER-1, and 4.1, 4.2, and 4.1, respectively, for PER-2 (Figure 5B).

Discussion

While standardized performance criteria are lacking for biocidal efficacy and encystment testing of *Acanthamoeba*, better understanding of the factors that contribute to the organism's ability to evade treatment are needed for the development of improved contact lens care systems. The study presented here demonstrated that stimulation of *A. castellanii* encystment by model contact lens disinfecting solutions can be dependent upon multifactorial contributions from various solution components. Among the known factors, the buffering system and biocidal agents used in a solution were shown to directly influence encystment rates under some (but not all) test conditions. In addition, exposure of *A. castellanii* trophozoites to a variety of test solutions which contained PG (including a new H₂O₂-based contact lens disinfecting solution) did not stimulate encystment.

Previous studies have demonstrated that multipurpose solutions containing PG may stimulate *Acanthamoeba*

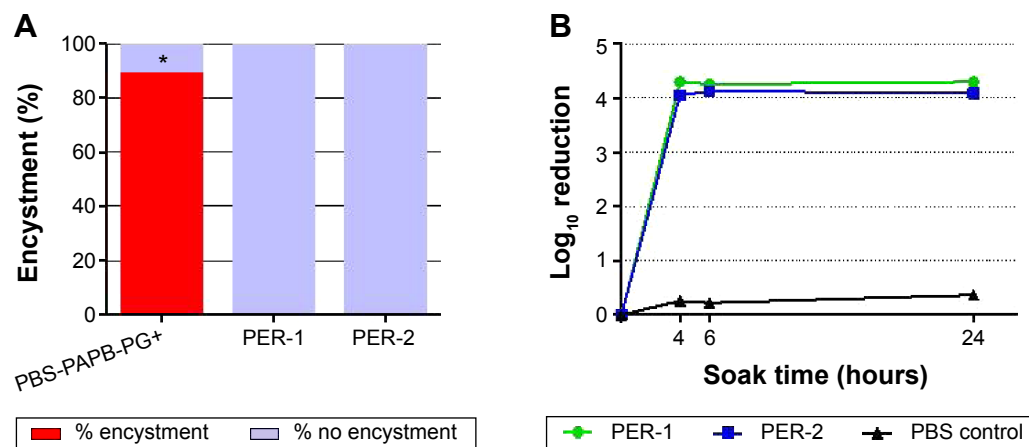


Figure 5 (A) Encystment rates of *Acanthamoeba castellanii* exposed to PBS-PAPB-PG+ control, PeroxiClear (PER-1), and Clear Care® (PER-2) solutions. Encystment rates for both PER-1 and PER-2 were <1% after 24 hours' incubation, significantly ($*P<0.05$) lower than the positive control solution. **(B)** For stand-alone biocidal efficacy testing, \log_{10} reduction values of *A. castellanii* exposed to PER-1, and PER-2 solutions reached complete kill at 6 and 24 hours, while PBS control did not. **Note:** Red bar represents percentage of trophozoites that encysted after 24 hours' incubation, while blue bars represent percentage of population that did not. **Abbreviations:** PAPB, polyaminopropyl biguanide; PBS, phosphate-buffered saline; PBS-PAPB-PG+, solution containing PBS, PAPB and PG.

encystment to varying degrees. In one study, CMP solution was reported to induce encystment at a rate of 20.8% in *A. castellanii*, while Menicare Soft and Epica Cold (both also containing PG) yielded only 1.2% and 2.4% cysts, respectively.⁸ Additionally, when several multipurpose solutions were supplemented with 0.5% PG, encystment rates were unaffected for some solutions, while for others, encystment rates slightly increased. Yet, in all instances, the rates remained much lower than those reported for the CMP solution.⁸ It has been suggested that these increases in encystment rates were due to increased osmolality resulting from the addition of PG to the solutions.¹⁹ Later studies by Lonnen et al confirmed the low levels of encystment previously observed in Menicare Soft and Epica Cold.¹⁹ However, a multipurpose solution which lacked PG was also shown to induce *Acanthamoeba* encystment. The authors hypothesized that this encystment in the absence of PG was potentially due to the phosphate buffer component of that test solution.¹⁹

A number of factors have been proposed to contribute to *Acanthamoeba* encystment associated with a solution, including osmolality,^{19,26} intrinsic biocidal activity,²⁷ and buffering agents.^{8,19} Indeed, various multipurpose solutions that contain PG either do not induce encystment or result in much lower encystment rates than those observed with CMP solution.⁸ The phosphate-buffered positive control solution (PBS-PAPB-PG+) used in the present study, which contained PG, was compared to a model solution composed of the same ingredients at identical concentrations in a borate-buffered positive control solution – BBS-PAPB-PG+. Encystment was not observed after 24 hours' incubation with BBS-PAPB-PG+, suggesting that the encystment signal observed in PBS-PAPB-PG+ can be

attributed to the combined effects of the phosphate buffer and PG. In addition, the PER-1c solution (containing 1.0% PG in a PBS/citrate-buffered formulation without a biocidal agent) also did not induce encystment, even with prolonged incubation up to 14 days (Figure 5). Further, studies have also shown that *Acanthamoeba culbertsoni* encystment was inhibited by citrate buffer, while Tris buffer lysed trophozoites.²⁰ Taken together, these results clearly demonstrate that encystment of *Acanthamoeba* spp. is mediated by the overall composition of a solution, including its buffering system in conjunction with other components.

PG is classified by the FDA as a liquid polyol demulcent, safe for use in over-the-counter products at concentrations ranging from 0.2% to 1.0%.¹⁷ Its primary function in contact lens disinfecting solutions is to provide enhanced lubrication and wettability of contact lenses. POV, another demulcent often present in eye drop formulations, is similarly approved for use at concentrations ranging from 0.2% to 2.0%.¹⁷ While neither demulcent has been shown to confer toxicity within its monograph concentration range, demulcents such as PG are capable of raising the osmolality of a solution. Indeed, hyperosmolality has been shown to cause encystment in pathogenic strains of *Acanthamoeba*.²⁸ This was taken into consideration in the present study by controlling for osmolality and adjusting all test solutions to a specified osmolality target of 290–300 mOsm/kg, within the physiologically biocompatible range found in most commercially available contact lens care solutions. Here, the PG and POV demulcents showed a dose-dependent stimulation of encystment only when present in PBS-based solutions which also contained 0.25 ppm PAPB. When the same concentrations

of PG and POV were instead present in PBS solutions containing 3% H₂O₂, neither demulcent stimulated encystment. While the intrinsic biocidal activity of H₂O₂ is typically very strong against *Acanthamoeba* trophozoites, it is important to note that the H₂O₂ experiments were carried out in neutralizing lens cases, where after 4 hours of incubation, the H₂O₂ levels were <100 ppm (data not shown). For encystment testing in this study, all solution exposures were for at least 24 hours, so unless complete kill had occurred during the initial 4 hours, there would still have been viable amoebae present and exposed to the PBS containing PG and POV for the remainder of the assay. Moreover, when the concentration of PG was held at a constant 1% and the concentration of H₂O₂ was dosed from 0% to 3%, again no encystment was detected for any concentration of H₂O₂ tested. This is particularly relevant for H₂O₂ concentrations substantially below 3% where biocidal activity is expected to be reduced. Specifically, if encystment stimulation by PG is dependent on a sublethal insult, it is possible that exposure to low (ie, less effective) levels of H₂O₂ could result in stimulation of encystment. However, this was not observed, thus further supporting the hypothesis that encystment is dependent on more than just individual components of a solution.

Labeling instructions for the H₂O₂-based products PER-1 and PER-2 indicate that contact lenses may be stored for up to 7 days following the initial 4-hour (PER-1) or 6-hour (PER-2) disinfection period. Therefore, we also examined the influence of prolonged storage up to 14 days on *Acanthamoeba* encystment using a variety of formulations with and without both 3% H₂O₂ and 1.0% PG components. No encystment was observed for any of these solutions after 24 hours', 7 days', or 14 days' storage in H₂O₂-neutralizing lens cases (Figure 4). This is particularly notable given that one of these solutions lacked any H₂O₂ while still containing 1.0% PG. Similarly, no encystment was detected with other commercially available one-step H₂O₂ solutions also incubated for 24 hours, 7 days, and 14 days (data not shown). Enumeration of surviving *Acanthamoeba* after 14 days' incubation in the lens case demonstrated that, as expected, the H₂O₂-containing solutions were highly effective (ie, near complete kill), while in contrast, substantial numbers of viable amoebae were recovered from solutions that lacked H₂O₂. Given that prolonged starvation is commonly used to create mature cysts in the laboratory, the recovery of viable amoebae with no evidence of encystment after 14 days is somewhat unexpected and warrants future investigation.

The ability of pathogenic *Acanthamoeba* to undergo encystment presents a particular challenge to lens care disinfecting systems because the cyst forms survive longer and are

less susceptible to a variety of antimicrobial treatments.^{9,11-15} While the contribution (if any) of cyst forms to initiating AK infections remains unclear, a contact lens disinfecting solution that enhances encystment could potentially be problematic. Recent reports have suggested pseudocyst formation as an alternative stress response to encystment.²⁹ Of note, pseudocyst formation was suggested to be caused by PG,³⁰ although the testing and detection methods differed from those reported here. Per the ISO 19045 encystment standard,²² the cysts observed in our studies were not characterized as either pseudocysts or immature cysts; however, this distinction merits further study. In particular, it would be valuable to better understand the onset of these two reported cyst forms in order to delineate what differences may influence their respective formation, as well as any risk factors associated with these forms for contact lens-associated AK infections. Changes of trophozoites observed in the present study were shown to be greatly influenced by both buffering and biocidal components of the challenge solutions and demonstrate the importance of a whole system, rather than individual components, on *Acanthamoeba* encystment.

Conclusion

Overall, the results reported here confirm that encystment associated with PG exposure is dependent on multiple factors, including buffering agents and biocidal components, and that these additional factors should be considered as a whole when investigating encystment effects. Further examination of the biocidal agents commonly used in contact lens disinfecting solutions and their interactions with other ingredients (including multicomponent buffering systems) would provide a more comprehensive understanding of *Acanthamoeba* encystment. This knowledge could facilitate the rational invention and development of new contact lens care solutions with improved safety and efficacy.

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Disclosure

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