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Biotin- and Glycoprotein-Coated Microspheres as Surrogates for Studying Filtration Removal of *Cryptosporidium parvum* in a Granular Limestone Aquifer Medium

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Members of the genus *Cryptosporidium* are waterborne protozoa of great health concern. Many studies have attempted to find appropriate surrogates for assessing *Cryptosporidium* filtration removal in porous media. In this study, we evaluated the filtration of *Cryptosporidium parvum* in granular limestone medium by the use of biotin- and glycoprotein-coated carboxylated polystyrene microspheres (CPMs) as surrogates. Column experiments were carried out with core material taken from a managed aquifer recharge site in Adelaide, Australia. For the experiments with injection of a single type of particle, we observed the total removal of the oocysts and glycoprotein-coated CPMs, a 4.6- to 6.3-$\log_{10}$ reduction of biotin-coated CPMs, and a 2.6-$\log_{10}$ reduction of unmodified CPMs. When two different types of particles were simultaneously injected, glycoprotein-coated CPMs showed a 5.3-$\log_{10}$ reduction, while the uncoated CPMs displayed a 3.7-$\log_{10}$ reduction, probably due to particle-particle interactions. Our results confirm that glycoprotein-coated CPMs are the most accurate surrogates for *C. parvum*; biotin-coated CPMs are slightly more conservative, while unmodified CPMs are markedly overly conservative for predicting *C. parvum* removal in granular limestone medium. The total removal of *C. parvum* observed in our study suggests that granular limestone medium is very effective for the filtration removal of *C. parvum* and could potentially be used for the pretreatment of drinking water and aquifer storage recovery of recycled water.

Waterborne cryptosporidiosis is mainly caused by *Cryptosporidium parvum* and *Cryptosporidium hominis* in humans (1). *Cryptosporidium* can be found in water contaminated with infected human or animal feces and has a low infectious dose, and ingestion of less than 10 oocysts can lead to infection (2). *Cryptosporidium* oocysts are sometimes detected in drinking water supplies (3) and in potable groundwater (4), causing disease outbreaks. For example, in the 1993 cryptosporidiosis outbreak in Milwaukee, WI, USA, about 400,000 people were infected and more than 100 people died after contamination of drinking water by *C. parvum* (5). More recently, a waterborne outbreak of cryptosporidiosis in Östersund, Sweden, which infected 27,000 people in 2010, was caused by *C. hominis* (6).

C. parvum can survive in surface water and groundwater for a long period of time (7) and is resistant to chemical disinfection, like chlorination (8) and ozonation (9), due to its thick oocyst wall. UV irradiation with low- and medium-pressure lamps has been found to be effective at inactivating *C. parvum* (10). However, the efficacy of UV irradiation as well as that of chemical disinfection is hampered by turbidity in the water. Thus, filtration is often used as an essential primary step for drinking water treatment in the course of a multibarrier treatment system because it is effective and cost-efficient.

Because it is extremely infectious and highly resistant to chlorination, testing for *C. parvum* is often used in risk analysis of drinking water supplies (11). However, due to the high analytical costs involved, monitoring for *C. parvum* in treated water is not routinely performed. Traditionally, water turbidity has been used as a performance measure for *C. parvum* removal from water supplies, but turbidity does not correlate well with the presence of *C. parvum* (12). *Clostridium perfringens*, which is included in the European Drinking Water Directive (13), is a potential surrogate for *C. parvum* due to its low inactivation rate (14), even though *Clostridium perfringens* is five times smaller and may have different filtration characteristics.

Some researchers have used unmodified carboxylated polystyrene microspheres (CPMs) as a surrogate for studying *C. parvum* filtration (15–17), but the results are generally unsatisfactory (18). Although CPMs can mimic the size, buoyant density, and spherical shape of the oocysts, unmodified CPMs are significantly more negatively charged than oocysts (15, 19). In addition, unlike

Received 17 March 2015 **Accepted** 10 April 2015

Accepted manuscript posted online 17 April 2015

Citation Stevenson ME, Blaschke AP, Toze S, Sidhu JPS, Ahmed W, van Driezum IH, Sommer R, Kirschner AKT, Cervero-Aragó S, Farnleitner AH, Pang L. 2015. Biotin- and glycoprotein-coated microspheres as surrogates for studying filtration removal of *Cryptosporidium parvum* in a granular limestone aquifer medium. Appl Environ Microbiol 81:4277–4283. doi:10.1128/AEM.00885-15.

Editor: C. R. Lovell

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00885-15.

doi:10.1128/AEM.00885-15

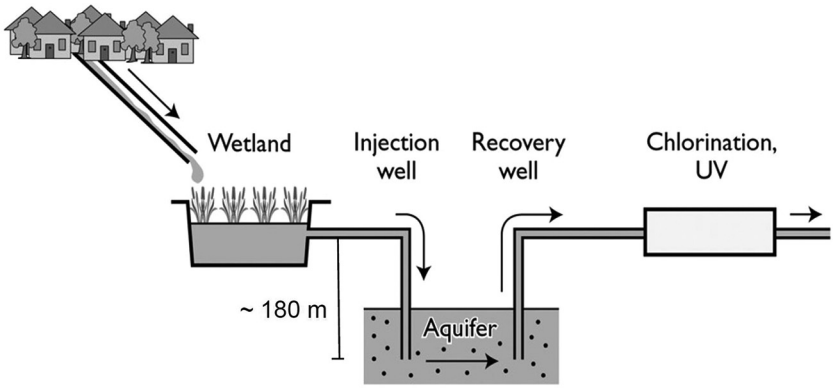

FIG 1 MAR treatment steps for water recycling of urban storm water runoff at Parafield Gardens, Adelaide, South Australia, Australia. Adapted from reference 11 with permission of Elsevier.

oocysts, unmodified CPMs lack a macromolecular structure, which makes them poor surrogates for *C. parvum* (20, 21).

Surface characteristics play an important role in particle retention and transport in porous medium. Thus, for CPMs to be better surrogates, their surface properties need to be modified. A recent study by Pang et al. (19) demonstrated that glycoprotein- and biotin-coated CPMs are superior to unmodified CPMs in predicting filtration removal of noninactivated *C. parvum* oocysts, showing the same $\log_{10}$ reduction in concentration. These biomolecules have a surface charge similar to that of *C. parvum* with an isoelectric point pH of ≈2 (19), and glycoprotein is the major type of protein that *C. parvum* produces on its cell surface (22). However, only transport in alluvial sand medium was validated in their study, and it is unknown whether these modified CPMs are also useful in predicting *C. parvum* filtration in other aquifer media with different chemical properties.

Limestone aquifers supply potable groundwater in many parts of the world and can be very vulnerable to fecal contamination (23), making catchment and water quality management an essential requirement (24) to guarantee drinking water quality. Many managed aquifer recharge (MAR) sites are also developed in limestone aquifers, e.g., in Australia and Mexico (25) and in the southeast United States (26). Thus, investigating *C. parvum* filtration in limestone aquifer medium has important implications for aquifer management and risk analysis of potable groundwater. Harvey et al. (16) previously investigated formalin-inactivated *C. parvum* and CPMs (sizes, 1.6, 2.9, and 4.9 μm) in intact cores of fractured limestone. In their study, CPMs significantly overpredicted *C. parvum* removal, with fractional recoveries of 19% for *C. parvum*, 3% for 4.9-μm CPMs, and 5% for 2.9-μm CPMs. In this study, we aimed to investigate the usefulness of glycoprotein- and biotin-coated CPMs in predicting filtration removal of *C. parvum* in granular limestone aquifer medium and to evaluate the efficacy of granular limestone medium for the removal of *C. parvum* by filtration.

MATERIALS AND METHODS

Aquifer material. The porous medium used in the column experiments was sourced from a tertiary limestone aquifer at the MAR site in Parafield Gardens, Adelaide, South Australia, Australia (25). Figure 1 shows a schematic of the treatment steps used at this MAR site, which recycles urban storm water runoff. Core samples were taken from a depth of from 170 to 180 m below the ground surface at latitude 34.793 and longitude 138.622. The aquifer material was granular and heterogeneous in size, containing some consolidated chunks. The coarse and fine fractions were sieved out so that grains of 0.25 to 2.4 mm (approximately 20% of the original material) were used in the column experiments (mean grain size [d_{50}] = 0.55 mm, uniformity [d_{60}/d_{10}] = 1.94, where d_x is the sieve aperture size when x% passes). The material was then rinsed with deionized water and oven dried at 80°C overnight. X-ray diffraction analysis showed that the aquifer material consisted mainly of calcite (63%) and quartz (31.3%). Other minerals included microcline (1.6%), aragonite (1.5%), albite (0.7%), goethite (0.6%), pyrite (0.5%), ankerite (0.4%), and hematite (0.3%).

Colloidal particles. *C. parvum* oocysts are spherical or oval and 3.9 to 5.9 μm in diameter (27) and have an isoelectric point pH of 2.0 to 3.3 (19, 28, 29). Two strains of oocysts were used in the experiments. These included fresh human fecal samples from anonymous donors obtained from the PathWest Laboratory Medicine, WA, Australia, from already infected individuals and the Iowa *C. parvum* genotype 2 isolate from Sterling Parasitology Laboratory (University of Arizona, Tucson, AZ).

The stock of the human strains was purified by a delipidation step in diethyl ether, followed by sucrose flotation using a method described by Gobet and Toze (30). The purified stock was then stored at 4°C in diethyl pyrocarbonate-treated phosphate-buffered saline and used within 4 months of receipt. Immediately before making the injection solution, the stock was exposed to a germicidal lamp (UV light at a wavelength of less than 320 nm in a class 2 biohazard cabinet fitted with a 20-W UV lamp) for 30 min to reduce its infectivity.

The stock of the animal strain was purified using discontinuous sucrose gradients and cesium chloride centrifugation gradients. Oocysts were stored in antibiotic solution (0.01% Tween 20, 100 U/ml of penicillin, 0.1 mg/ml of gentamicin) at 4°C and used within 3 months of receipt. The stock suspension of oocysts was diluted with 10 mM NaCl, buffered to a pH of 8.0, to a concentration of 1×10^6 oocysts/ml. Portions of 25 ml of the working suspension were exposed in sterile petri dishes under permanent stirring in a standard laboratory irradiation apparatus (with low-pressure lamps) as described in detail elsewhere (31). The UV fluence applied was 800 J/m^2 254-nm UV. The transmittance of the oocyst suspension (70% in 10 mm) was taken into consideration for the fluence calculation.

To obtain CPMs that mimic the size, spherical shape, and density of the oocysts, 4.5-μm CPMs were purchased from Polysciences Inc. (Warrington, PA, USA). The CPMs were fluorescent yellow-green (YG), yellow-orange (YO), and bright blue (BB). The initial intent was to inject the three types of CPMs together. Thus, the CPMs were purchased in three different colors in order to differentiate them for analysis. The YO CPMs were coated with α_1-acid glycoprotein (Sigma-Aldrich, St. Louis, MO, USA), and the BB CPMs were coated with amine-containing biotin

(Thermo Fisher Scientific Inc., Rockford, IL, USA), following the conjugation of biomolecules by the method described by Pang et al. (19).

The zeta potentials (ζ's), also referred to as surface charges or surface potentials, of the particle solutions were measured using electrophoretic light scattering (Zetasizer Nano ZS; Malvern Instruments Ltd., Worcestershire, United Kingdom). The particle concentrations used for the measurements were 10^4 oocysts/ml and 10^6 microspheres/ml. The low concentration of oocysts used was constrained by the initial concentration of oocysts in the stock solution. A background electrolyte of 10 mM NaCl, pH 8.0 ± 0.2, was consistently used in the study. The zeta potential measurements were carried out in triplicate.

Column experiments. Glass chromatography columns (10 cm long, 1.5 cm internal diameter) were packed under saturated conditions with the aquifer material. In each column, the same amount of water and the same dry weight of aquifer material were used so that the material gave an effective porosity of 0.56 ± 0.02 and a dry bulk density of 1.01 ± 0.06 g/cm^3. A Darcy velocity (U) of 1.21 ± 0.15 m/day, mimicking the typical flow rate measured *in situ*, was applied from the top of the column using a peristaltic pump (Masterflex, Vernon Hills, IL). The background electrolyte of 10 mM NaCl at pH 8.1 ± 0.3 (buffered with $NaHCO_3$) was used throughout the experiments to establish an equilibrium with aquifer material containing predominantly carbonate minerals. Experiments were conducted at room temperature (22 to 23°C), which was similar to the groundwater temperature (23°C) at a depth of 160 to 180 m (25) at the field site where the aquifer material was obtained.

To characterize the hydraulic conductivity and dispersivity of the porous medium in the packed columns, an experiment was first conducted using 1 mM sodium nitrate ($NaNO_3$) as a conservative tracer. The concentration of NO_3^- in the column effluent was measured with a UV-visible spectrophotometer (Cary series UV-Vis; Agilent Technologies, Santa Clara, CA) at a wavelength of 210 nm.

For the experiments with the particles, the columns were first flushed with at least 18 pore volumes using the background electrolyte, and 4 pore volumes of tracer solution were injected. Test runs were done using one type of particle at a time, and the columns were repacked with fresh material for each test run except in one experiment, when uncoated and glycoprotein-coated CPMs were injected together. Whenever possible, column tests were done in duplicate. The influent concentrations of the colloids ranged from 10^4 to 10^7 particles/ml. The influent concentrations of the coated CPMs and the oocysts were relatively lower due to particle loss during the coating procedure and the low initial oocyst concentration in the stock.

Enumeration of particles. Initially, enumeration of the human strain oocysts was attempted with epifluorescence microscopy using the dye staining method with 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide, as outlined by Campbell et al. (32). However, no typical oocysts could be identified from the microscopic analysis, even though positive controls yielded quantifiable results. Therefore, another experiment was carried out using animal strain oocysts, and the oocysts in samples were enumerated using a more sensitive enumeration method that is appropriate for samples with very low concentrations of oocysts.

The animal strain oocysts, uncoated YG CPMs, and glycoprotein-coated YO CPMs were enumerated using solid-phase cytometry (ChemScan RDI; AES Chemunex, bioMérieux, Marcy l'Etoile, France), according to the method outlined by Stevenson et al. (33), which allows a low sample limit of detection (SLOD) of 3 particles per filtered volume. *C. parvum* was stained with EasyStain antibody (BTF Pty. Ltd., bioMérieux, North Ryde, Australia) following the instructions provided by the manufacturer. The calibration curve (see Fig. S1 in the supplemental material) for the enumeration of the oocysts and more detailed information about the dying procedure can be found in the supplemental material. Calibration curves for the microspheres in different water matrices can be found in the work of Stevenson et al. (33). To differentiate between the YG and the YO CPMs, different photomultiplier tubes (PMTs) were used. The green PMT detects an emission wavelength of from 500 to 530 nm, and the yellow-orange PMT detects a wavelength of from 540 to 570 nm. As the emission and excitation of BB CPMs were outside the range of the solid-phase cytometer, the BB CPMs were enumerated using a Nikon Eclipse 8000 epifluorescence microscope. Montemayor et al. (34) found that solid-phase cytometry and an epifluorescence microscope produce equivalent results. The microscope was equipped with an automatic stage, which enabled scanning of the entire filtration area, thereby providing the same SLOD provided by solid-phase cytometry. All samples were filtered onto 25-mm black polyester 0.4-μm-pore-size filters (bioMérieux) before the enumeration. Three replicates were analyzed for each sample, and the filtered sample volume was 1 ml; thus, the SLOD for all samples analyzed was 3 particles/ml (33).

Data analysis. The filtration efficiency of the particles was described using peak breakthrough attenuation [$\log_{10}(C_{max}/C_0)$] (where C_{max} and C_0 are the peak [maximum] effluent concentration and input solution concentration, respectively) relative mass recovery (RB), and collision efficiency (α). RB was estimated by integrating the area under the breakthrough curve of the colloid and normalizing the value to that for the conservative tracer nitrate. The collision efficiency (α) is determined from the following formula (35):

$$\alpha = \frac{d\left[\left(1 - 2\frac{\lambda}{L}\ln(RB)\right)^2 - 1\right]}{6(1-\theta)\eta\lambda}$$

where d is the mean grain size (also known as d_{50}), λ is the dispersivity, L is the transport distance, θ is the effective porosity, and η is the single-collector efficiency. The η and α values were calculated on the basis of the equations of Tufenkji and Elimelech (36). Hamaker constants of 1.00×10^{-20} J for glass-water-polystyrene (37) and 6.50×10^{-21} J for oocysts (20) were used. A buoyant density of 1.05 g/cm^3 was applied to represent the density of the CPMs (Polysciences Inc.) and the oocysts (27). Darcy velocity (U) and λ are required for the calculation of η and α. The measured flow rates were used to calculate the U values, while λ was optimized by simulating the nitrate data using the CXTFIT model.

RESULTS AND DISCUSSION

Surface charge. The surface charge of the human UV-treated *C. parvum* oocysts measured in this study (ζ = −35.3 ± 1.3 mV) was similar to the ζ values of viable *C. parvum* oocysts (ζ = −37 to −42 mV in $NaClO_4$ at pH 7.0, electrical conductivity = 0 to 2,200 μS/cm) reported by Hsu and Huang (28). The unmodified YG CPMs were markedly more negatively charged (ζ = −107.0 ± 6.1 mV) than the *Cryptosporidium* oocysts, which is similar to what was found by Pang et al. (19) for unmodified BB CPMs (ζ = −111 mV in 1 mM NaCl, pH 7). In contrast, the surface charge of the glycoprotein- and biotin-coated CPMs mimicked the surface charge of the oocysts relatively well. Although glycoprotein and biotin have similar surface charges (19), the charge of glycoprotein-coated YO CPMs in our study (ζ = −13.3 ± 2.1 mV) was lower than that of biotin-coated BB CPMs (ζ = −37.3 ± 0.9 mV). This is most likely due to the fact that, prior to coating, the unmodified YO CPMs were much less charged (e.g., ζ = −44.6 mV in 2 mM NaCl at pH 7) than the unmodified BB CPMs (−111 mV, as mentioned above), resulting in a lower charge after coating. For the same reason, the glycoprotein-coated YO CPMs synthesized in this study were less charged than the glycoprotein-coated BB CPMs synthesized in the study by Pang et al. (19). The reason that we coated YO CPMs instead of BB CPMs with glycoprotein was that the YO CPMs could be counted using solid-phase cytometry but the BB CPMs had to be manually counted using microscopy, as mentioned above.

Filtration efficiency. Peak breakthrough attenuation [$\log_{10}(C_{max}/C_0)$], relative mass recovery (RB), and collision efficiency

TABLE 1 Summary of column experiments[d]

Tracer	Expt	Run no.	C_0 (no. of particles/ml)	Note	U (cm/min)	RB	η	α	T_o (min)	pH_{in}	pH_{out}	$\log_{10}(C_{max}/C_0)$
Nitrate[e]		1			0.07	9.23×10^{-1}			290	7.85	8.24	0.0
C. parvum human strain		1	1.00×10^5	[a]	0.08	0.00×10^{0}[c]	2.62×10^{-2}	[c]	224	7.78	8.23	[c]
C. parvum animal strain		1	9.30×10^5	[b]	0.09	0.00×10^{0}[c]	2.45×10^{-2}	[c]	258	7.99	7.91	[c]
Glycoprotein-coated YO CPMs in mix	T2A	1	1.60×10^6	[b]	0.09	1.30×10^{-5}	2.57×10^{-2}	4.16	220	8.21	8.33	−5.2
Glycoprotein-coated YO CPMs	T6	1	6.30×10^4	[b]	0.08	0.00×10^{0}[c]	3.06×10^{-2}	[c]	288	7.92	8.42	[c]
Glycoprotein-coated YO CPMs	T7	2	3.30×10^5	[b]	0.11	0.00×10^{0}[c]	2.16×10^{-2}	[c]	211	7.86	8.07	[c]
Biotin-coated BB CPMs	T8	1	7.80×10^6	[a]	0.08	2.53×10^{-7}	2.75×10^{-2}	5.41	250	8.03		−6.3
Biotin-coated BB CPMs	T11	2	6.50×10^5	[a]	0.09	3.71×10^{-5}	2.66×10^{-2}	3.63	250	7.84	8.09	−4.6
Uncoated YG CPMs in mix	T2A	1	1.60×10^7	[b]	0.09	2.40×10^{-4}	2.57×10^{-2}	3.02	220	8.21	8.33	−3.7
Uncoated YG CPMs	T3	1	8.10×10^6	[b]	0.07	3.02×10^{-3}	3.18×10^{-2}	1.67	260	8.10	8.46	−2.6
Uncoated YG CPMs	T9	2	3.40×10^7	[b]	0.08	1.56×10^{-3}	2.84×10^{-2}	2.09	320	7.89	7.99	−2.6

[a] Counted by epifluorescence microscopy.
[b] Counted by solid-phase cytometry.
[c] Breakthrough below sample limit of detection.
[d] C_0, input solution concentration; U, measured Darcy velocity; RB, relative mass recovery; η, single-collector efficiency; α, collision efficiency; T_o, pulse duration; pH_{in}, influent pH; pH_{out}, effluent pH; C_{max}, peak (maximum) effluent concentration; mix, simultaneous injection of uncoated and glycoprotein-coated microspheres.
[e] The injection concentration of nitrate was 1 mM (85 mg/liter).

(α) (Table 1) were calculated for the unmodified CPMs and the modified CPMs (excluding the glycoprotein-coated CPMs injected alone). The oocysts and the glycoprotein-coated CPMs (injected alone) exhibited no breakthrough, and therefore, $\log_{10}(C_{max}/C_0)$, RB, and α could not be calculated. Interestingly, when the uncoated and glycoprotein-coated CPMs were injected together (mix), the concentration and filtration efficiency [$\log_{10}(C_{max}/C_0)$, RB] of the uncoated CPMs were reduced by 1 order of magnitude compared to the values when they were injected alone (Fig. 2; Table 1). This is probably due to enhanced particle-particle collision, resulting in clumping and, therefore, a stronger straining effect. The breakthrough of the glycoprotein-coated CPMs was below the SLOD when they were injected alone. Presumably, when they were injected together, the less negatively charged gly-

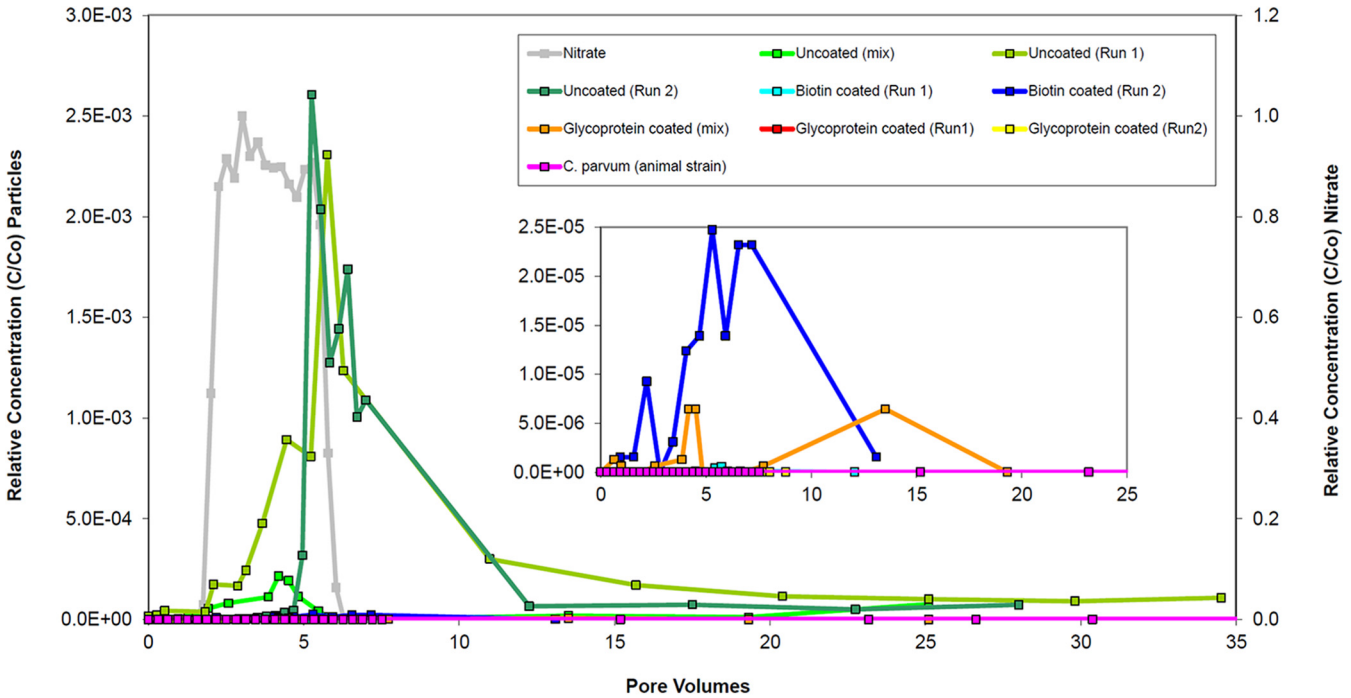

FIG 2 Relative concentrations of nitrate, *C. parvum* oocysts, and unmodified and modified CPMs with biotin and glycoprotein in column experiments with granular limestone aquifer medium in 10 mM NaCl, pH 8. (Inset) A graph with an expanded scale shows the relative concentrations of modified CPMs and the oocysts.

TABLE 2 Comparison of α values for *C. parvum* derived from various porous media under comparable experimental conditions

Study	*C. parvum* activation	Porous medium	*U* (m/day)	*d* (mm)	Column length (cm)	Solution	pH	α
This study	UV inactivated	Limestone sand	1.3	0.55	10	10 mM NaCl	8	>3.93[a]
Pang et al. (19)	Noninactivated	Alluvial sand	~1	0.78	22	1 mM NaCl	7	0.05–0.06
Abudalo et al. (44)	Formalin inactivated	Ferric oxyhydroxide-coated quartz sand	0.76	0.92	10	0.1 mM NaCl	5.6–5.8	0.26
Park et al. (45)	Noninactivated	Ottawa sand	0.71	0.53	20	3 mM NaCl	7	0.36
Hijnen et al. (46)	Noninactivated	Fluvial gravel (NOM,[b] Fe-ox content)	0.9	0.5	50	River Meuse water[c]	8.0–8.4	0.40[d]
Harter et al. (42)	Noninactivated	Alluvial sand	0.7	0.42	10	Degassed tap water[e]	7.9	0.56[f]

[a] Theoretical value for breakthrough below sample limit of detection (3 oocysts/ml).
[b] NOM, naturally occurring organic matter.
[c] Electrical conductivity, 569 to 574 μS/cm.
[d] Correction by Hijnen et al. (47).
[e] Electrical conductivity, 220 μS/cm.
[f] Calculated by Park et al. (45) using the method of Tufenkji and Elimelech (36).

coprotein-coated CPMs attached to the more negatively charged unmodified CPMs and were carried along. The α values calculated were >1 and similar to those found for granular activated carbon (38), showing that the material is very effective at removing colloids. Low α values represent low levels of removal, and conversely, high α values represent high levels of removal.

Figure 2 demonstrates that the unmodified CPMs overpredicted the oocyst concentrations in the flowthrough column experiments, while the concentrations of the oocysts and modified CPMs were of the same order of magnitude (minimal to no breakthrough at all). This finding is consistent with the findings obtained in the study of Pang et al. (19). The plausible explanation is that there was a greater tendency for electrostatic repulsion between the aquifer medium and the more negatively charged unmodified CPMs than between the aquifer medium and the less negatively charged oocysts or modified CPMs, as suggested by their ζ values. Our results suggest that the tendency of particles to attach to the aquifer medium (Table 1) is related to the overall surface potential (ζ value) of particles. The most negatively charged particles (YG uncoated CPMs) displayed the lowest level of attachment and lowest removal, while the least negatively charged particles (YO glycoprotein-coated CPMs) showed the highest level of attachment and the most removal. Similar to what was found in the work of Pang et al. (19), biotin-coated CPMs slightly overpredicted oocyst concentrations and, thus, are more conservative than glycoprotein-coated CPMs. Column test run 1 with the biotin-coated CPMs had lower levels of breakthrough than run 2. This may have been due to ripening or a buildup of colloids encouraging more attachment caused by the higher influent concentration used for run 1 (39).

The size ratio of oocysts or CPMs to sand grains (φ/d_{50}) was 0.9% (where φ represents the diameter of the colloid), which is above the threshold of 0.5% for straining to occur (40). Independently of the particle size-to-grain size ratio, the shape irregularity of the porous medium may also cause straining due to smaller pore spaces being present after packing of irregularly shaped medium (41). Upon visual examination of the porous medium used in our study, the material was highly irregular in shape. Thus, straining may have played an important role in the removal of the oocysts and CPMs in the aquifer medium investigated here. This is reflected in their extremely low level of recovery by mass (Table 1), indicating that irreversible attachment (including straining) was more dominant than reversible attachment during the experiments. The irreversible attachment seen at some sites may be due to the short period of the experiment (<30 pore volumes), and with a continuous flush or a change in solution chemistry, the previously strongly attached particles may detach from the porous medium. For example, Harter et al. (42) and Mohanram et al. (17) found that initial oocyst attachment is reversible, even in soils with a high iron oxide content (17). However, we did not run the experiments long enough or change the solution chemistry to see a detachment of the oocysts and the surrogates, although the uncoated CPMs exhibited some degree of reversible attachment, as suggested from their tailings in the breakthrough curves.

In this study, we observed that the recovery of unmodified CPMs in granular limestone material was markedly higher than that of *C. parvum*, for which total removal (zero recovery) was demonstrated. However, the opposite was observed in the column experiment (10 mM artificial groundwater; pH 8; $U \approx 23$ m/day) of Harvey et al. (16) with an intact karst limestone core (16.5 cm long) and formalin-inactivated *C. parvum*. In their study, the recovery of *C. parvum* was 4 and 6 times greater than that of 2.9- and 4.9-μm unmodified CPMs. The total removal of the oocysts observed in this study suggests that granular limestone medium is more effective than karst limestone material for filtration removal of *C. parvum*. This is to be expected, as, unlike karst limestone medium, granular limestone medium contains fewer preferential flow paths and is more uniform in conducting particle transport, which most likely resulted in sieving of the oocysts.

The collision efficiencies for CPMs (α = 1.67 to 5.41) in the granular limestone medium (d_{50} = 0.55 mm) calculated for this study (Table 1) are 2 orders of magnitude greater than those of the corresponding particles (α = 0.03 to 0.06) derived from alluvial sand (d_{50} = 0.78 mm) packed in 22-cm-long columns reported by Pang et al. (19), although the Darcy velocities (~1 m/day) and surface charges of porous medium were similar in the two studies. The high α values calculated in this study reflect the high level of removal of colloids in granular limestone medium. This supports our untested hypothesis that granular limestone medium contains pockets of positive charge that could be effective in removing *C. parvum* oocysts. Table 2 compares the collision efficiencies (α values) of *C. parvum* in sand and gravel medium in a number of

studies, including fluvial gravels with natural organic matter and ferric oxyhydroxide. The comparison suggests that granular limestone medium is more effective in filtration removal of oocysts than other porous media, on the basis of a theoretical collision efficiency calculated from the SLOD for our study. Thus, it appears to be a good candidate for use in a pretreatment step for drinking water supplies and removal of *Cryptosporidium* oocysts by MAR.

Our results have demonstrated that glycoprotein- and biotin-coated CPMs are superior to unmodified CPMs as surrogates for studying the filtration removal of *C. parvum* in granular limestone medium, with biotin-coated CPMs being slightly more conservative. This finding is consistent with that from the study of alluvial sand by Pang et al. (19). The lack of breakthrough of *C. parvum* observed in our study suggests that granular limestone medium can be used in sand filtration for water treatment and a granular limestone aquifer is suitable for water recycling through MAR.

Our study further establishes that glycoprotein- or biotin-coated CPMs are promising surrogates for studying the filtration removal of *C. parvum* in porous medium. They are easy to work with and can be rapidly detected by using automated counting techniques (e.g., flow cytometry, spectrofluorimetry, and the highly sensitive solid-phase cytometry used in this study). The low cost of producing the modified CPMs is another advantage (43), making these surrogates cost-effective. Further validation in other porous media and pilot trials for water treatment would allow a more extensive investigation of the usefulness of these newly developed surrogates.

ACKNOWLEDGMENTS

This work was supported by the Austrian Science Fund (FWF) as part of DK-Plus 1219-N22 (Vienna Doctoral Program on Water Resource Systems). Additional support came from the project Groundwater Resource Systems Vienna (GWRS—Vienna), funded by Vienna Waterworks as part of the (New) Danube-Untere Lobau Network Project (Gewässervernetzung [Neue] Donau-Untere Lobau [Nationalpark Donau-Auen]); the Government of Austria (Federal Ministry of Agriculture, Forestry, Environment & Water Management); the Government of Vienna; and the European Agricultural Fund for Rural Development (project LE 07-13). Further support came from the EU-FP7 project AQUAVALENS (project no. 311 846). The research work was also supported by the CSIRO Water for a Healthy Country flagship program (Australia).

This study is a joint publication of the Vienna University of Technology and the Medical University of Vienna through the Interuniversity Cooperation Centre Water & Health (www.waterandhealth.at).

We acknowledge the support of Karen Barry (CSIRO) for providing the aquifer material used in this study. We also thank PathWest Laboratory Medicine, WA, Australia, for supplying *Cryptosporidium*-positive fecal samples.

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