Journal of Environmental Chemical Engineering 1 (2013) 504–509

Contents lists available at SciVerse ScienceDirect

Journal of Environmental Chemical Engineering

journal homepage: www.elsevier.com/locate/jece

Aggregation kinetics of *Cryptosporidium parvum* oocysts

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ARTICLE INFO

Article history:
Received 5 March 2013
Accepted 9 June 2013

Keywords:
Oocyst
Cryptosporidium parvum
Surface charge
Zeta potential

ABSTRACT

Cryptosporidium parvum (*C. parvum*), an organism of increasing importance in drinking water, spreads to the environment through faeces of infected animals and humans. *C. parvum* produces infective chlorine-resistant oocyst that are removed from contaminated raw waters primarily using physical methods such as entrapment in surface or depth bed filters and coagulation–flocculation. In this paper, the aggregation properties of gamma-irradiated *C. parvum* oocysts in dilute concentrations, as a function of stirring time in the presence of bivalent and trivalent cations were studied. The size and surface charge of *C. parvum* oocysts were characterized before they were used for aggregation experiments. The surface charge of the oocysts, measured using a zeta potential analyzer were in the range of -57 ± 2 mV, which increased to -31 ± 2 mV in presence of bivalent electrolytes. Aggregation of oocyst in dilute suspensions was monitored using confocal laser scanning microscope (CLSM) in presence of bivalent or trivalent electrolytes. Aggregation was not visible in the diluted *C. parvum* oocyst suspension in Milli Q water system in the time frame of the experiment. Addition of a bivalent or trivalent salt with stirring condition induced aggregation due to the suppression of the surface charge of oocysts, resulting in a decrease in the energy of interaction between oocysts, and a decrease in double layer thickness around the oocysts. Predictive models could be built to understand *C. parvum* oocyst aggregation in raw waters, which could significantly improve the removal of oocysts from untreated (raw) drinking water.

Introduction

The third edition of the WHO Guidelines for Drinking Water Quality (2004) introduced the concept of reference pathogens for drinking water [1]. *Cryptosporidium* a species of parasitic protozoa that is transmitted as oocyst through drinking water is considered as one such reference pathogen [2]. *C. parvum* and *C. hominis* are the two species of *Cryptosporidium* largely implicated in the waterborne and faecal-orally transmitted disease cryptosporidiosis in humans. Oocysts, produced by *Cryptosporidium* as part of its life cycle, are very persistent in the environment, resistant to chemical disinfectants like chlorine, and are difficult to filter due to their small size (4–6 μm). *Cryptosporidium* is widespread in waters with high levels of exposure and high rates of asymptomatic carriage (10–30%) than illnesses, with a mortality rate of 1:100,000 or lower in healthy adults while the risk is much higher in children, old and immune compromised individuals [2]. *C. parvum* oocysts have been studied using imaging techniques like scanning electron microscopy (SEM), optical, fluorescence, confocal and more recently atomic force microscopy [3–6].

Coagulation is a process by which very fine dispersed colloids are destabilized in liquid systems, leads to formation of larger agglomerates that allow their sedimentation or filtration. While sedimentation is possible if the particle size is at least 1 μm and a density more than that of water, coagulation/flocculation is commonly achieved by adding various types of chemicals (coagulants, salts of Al and Fe, etc.) to water that promotes destabilization of colloid dispersion and agglomeration of the resulting individual colloidal particles. Most work on characterization of colloids have focused on the structure formed as a function of pH, electrolyte concentration and hydrodynamics and have tried to correlate them to the strength of the resulting structure. In this context, video microscopy has been extensively to study aggregation of particulate matter in real time [7–15]. A comprehensive review of the optical techniques on concentrated colloidal suspensions [16] and a critical review of confocal microscopy for static colloidal suspensions in confined geometries are available [15,17]. Traditionally, in these systems wherein the aggregation is monitored, the number of particles are typically ten million particles/L and involve a very long time for aggregation [14,15,18]. Oocyst and its surrogate (polystyrene latex beads or microspheres) have been characterized in terms of their surface charge (zeta potential) and electrophoretic mobility. The surface charge is a strong function of solution chemistry and has been observed to be negative under most study conditions. [19–25].

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http://dx.doi.org/10.1016/j.jece.2013.06.009

The objective of this work was to look at aggregation kinetics of *C. parvum* oocysts in dilute concentrations (as per ANSI/NSF standard [26]) as a function of stirring time in the presence of divalent or trivalent cationic salts. Physical characterization of *C. parvum* oocyst was carried out to ensure that the oocysts were conforming to the values reported in literature. Imaging of aggregation in such dilute suspensions using CLSM is being reported for the first time.

Materials and methods

Materials

Gamma irradiated *C. parvum* (IOWA Strain) oocysts were obtained as Custom EasySeed from BTF (Australia, http://www.btfbio.com). The master stock had a concentration of 4×10^5 oocysts/ml in standard phosphate buffered saline solution (pH = 7.5, TDS = 5860 ppm). *C. parvum* oocyst was visualized by staining with a fluorescein-tagged monoclonal antibody (Easy Stain kit, BTF, Australia), that binds to the oocyst wall components. Fluorescent carboxylated polystyrene-based latex microspheres, which were used as a surrogate for oocyst, were procured from Polysciences Inc. as a 2.5 wt% aqueous suspension ($\sim 1.68 \times 10^9$ microspheres/ml, average size 4.5 μm). LR grade sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), aluminium chloride ($AlCl_3$) and Tween 20 were procured from Sigma Aldrich.

Methods

Size distribution of oocysts by image analysis

The oocysts from the master stock were diluted in Milli-Q water and mixed vigorously. The suspended oocysts were then filtered through a 0.45 μm black filter (HTBP, Millipore), washed with 0.01% Tween 20 and Milli-Q water, stained using fluorescein-labelled monoclonal antibodies (Easy Stain, BTF Australia) and washed with the supplied wash buffer post incubation. The filters were then mounted in the mounting medium (provided in the Easy Stain kit) and observed. The oocysts stained apple green with bright edges and a relatively less bright oocyst body. The slide was mounted on the platform of Olympus BX40 Optical microscope and the images captured and analyzed using Image-Pro Plus software. The digital images of oocyst were acquired using a 40× objective in fluorescent mode, converted to grey scale and after thresholding, the mean diameter of oocyst was measured using the image analysis software.

Size distribution of oocysts by light scattering

The particle size distribution of oocyst was measured using Malvern Master Sizer 2000, Hydro 2000S. The tank that held the sample and dispersant was filled with de-ionized double distilled water. The speed of the circulation pump was kept around 2000 rpm during the experiment. The laser intensity was >75% before starting the experiment. 10 ml of oocyst master stock suspension was poured in the sample tank and the readings recorded.

Surface charge of oocysts

Zeta potential measurements of the aggregates at various stirring time points were carried out using a Zeta Plus zeta potential and particle size analyzer from Brookhaven Instruments Corporation, Holtsville, NY. The oocyst master stock was diluted 10 times and 100 times respectively by adding Milli-Q water and mixed well. The cell of the zeta potential meter was filled with this suspension and the electrodes (Molybdenum as an anode & Platinum as cathode) were fitted to the cell. The electrodes were energized at 150 V and the oocyst movement was tracked. The same process was followed to measure the surface charge of oocysts in presence of $CaCl_2$ and $NaHCO_3$ in Milli-Q water. The same procedure was followed to measure the surface charge of microsphere (surrogates of oocysts).

Aggregation of oocyst

Aggregation experiments were performed with dilute concentrations (50,000 oocysts/L) of irradiated *C. parvum* oocysts. These were tagged with antibody-FITC dye and a suspension was prepared with Milli-Q water. The colloidal suspensions were stirred at 100 rpm for one hour to ensure that only monomers were present in the stock. Presence of monomers was verified by viewing the sample under fluorescence microscope. The resultant pH of the solution was 7.5. To this monomer suspension, destabilizing salts (either $CaCl_2$ or $AlCl_3$) of specified molar concentration was added and stirred further. Aliquots were taken at 30 min time intervals from the stirred suspension into 5 ml cuvettes and imaged with CLSM at 20× magnification.

Imaging of aggregated and non-aggregated oocysts

C. parvum oocyst from stock vial was diluted to a concentration of 10,000 oocysts/ml and washed with saline and Milli-Q water at 1 ml volume in an Eppendorf tube. The final *C. parvum* oocyst pellet was re-suspended in 50 μL of Milli-Q water, incubated with 1% glutaraldehyde or 10% formaldehyde overnight, washed, sequentially dehydrated in ethanol (50–100%), then spotted on HTBP membranes under negative pressure and left for drying. Alternately, the processed samples were spotted on clean glass cover slips for imaging. The filters and cover slips were mounted on SEM supports, coated with a gold layer and observed.

Field Emission Scanning Electron Microscopy (FE-SEM, Hitachi FE-4500) was used to image irradiated *C. parvum* oocysts. Images were recorded with typical gun voltage(s) ranging between 10–20 kV and magnifications up to 50,000×. CLSM, Leica TCS SP5 was used to capture images of the suspensions at various time frames. Five ml aliquots from the stock were taken into plastic cuvettes which were of 4.5 cm in length and 1 cm in width. Imaging was done using 499 nm excitation, 488/543 dichroic and 500–600 nm emission slit widths. The cuvette was tightly closed so that there was no water leakage. Multiple samples were prepared each time and the images were acquired using CLSM. Since the cuvette was transparent, particles at various depths were imaged easily. Fig. 1 shows the schematic of the set-up used for imaging.

Since *C. parvum* oocysts in suspension were in constant Brownian motion, the images were acquired at higher capture frequency. By varying the focal plane, oocysts at various depths were imaged. For each of the time intervals, multiple aliquots of sample were taken into the cuvette and images acquired. Approximate number of oocysts present in 5 ml of suspension in the cuvette was determined by the initial suspension

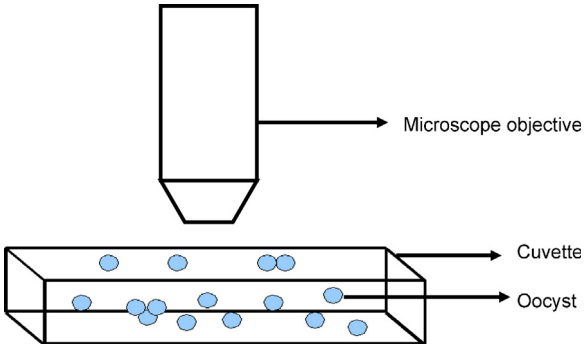

Fig. 1. Schematics of the set-up for image analysis of *C. parvum* oocysts.