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Characterization of phagolysosomal simulant fluid for study of beryllium aerosol particle dissolution

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Abstract

A simulant of phagolysosomal fluid is needed for beryllium particle dissolution research because intraphagolysosomal dissolution is believed to be a necessary step in the cellular immune response associated with development of chronic beryllium disease. Thus, we refined and characterized a potassium hydrogen phthalate (KHP) buffered solution with pH 4.55, termed phagolysosomal simulant fluid (PSF), for use in a static dissolution technique.

To characterize the simulant, beryllium dissolution in PSF was compared to dissolution in the J774A.1 murine cell line. The effects of ionic composition, buffer strength, and the presence of the antifungal agent alkylbenzyldimethylammonium chloride (ABDC) on beryllium dissolution in PSF were evaluated.

Beryllium dissolution in PSF was not different from dissolution in the J774A.1 murine cell line ($p = 0.78$) or from dissolution in another simulant having the same pH but different ionic composition ($p = 0.73$). A buffer concentration of 0.01-M KHP did not appear adequate to maintain pH under all conditions. There was no difference between dissolution in PSF with 0.01-M KHP and 0.02-M KHP ($p = 0.12$). At 0.04-M KHP, beryllium dissolution was increased relative to 0.02-M KHP ($p = 0.02$). Use of a 0.02-M KHP buffer concentration in the standard formulation for PSF provided stability in pH without alteration of the dissolution rate. The presence of ABDC did not influence beryllium dissolution in PSF ($p = 0.35$).

PSF appears to be a useful and appropriate model of *in vitro* beryllium dissolution when using a static dissolution technique. In addition, the critical approach used to evaluate and adjust the composition of PSF may serve as a framework for characterizing PSF to study dissolution of other metal and oxide particles.

Keywords: Alveolar macrophage phagolysosome; Dissolution; Simulant; Chronic beryllium disease

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Abbreviations: ABDC, alkylbenzyldimethylammonium chloride; BAS, Baron and Ahmed solution; BeO, beryllium oxide; ICP-MS, inductively coupled plasma-mass spectroscopy; KHP, potassium hydrogen phthalate; M_D, dissolved beryllium mass; M_0, initial particulate beryllium mass; MEM-α, minimum essential medium-alpha; PBS, phosphate buffered saline; PSF, phagolysosomal simulant fluid; SUF, serum ultrafiltrate; US EPA, United States Environmental Protection Agency

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1. Introduction

There are no studies in the published literature investigating beryllium particle dissolution using a simulant whose composition and pH mimic the phagolysosomal fluid of pulmonary alveolar macrophages. The question of what constitutes phagolysosomal fluid is critical because intraphagolysosomal dissolution is necessary to produce dissolved beryllium (Eidson et al., 1991), the hypothesized exogenous input to the cellular immune response associated with development of chronic beryllium disease (Eidson et al., 1991; Richeldi et al., 1993). Simulant experiments are much easier to perform than cell-based in vitro or in vivo models; thus, identifying a meaningful simulant of phagolysosomal fluid would greatly facilitate beryllium aerosol particle dissolution research. For example, such a biologically relevant simulant would aid in developing in vitro or in vivo models of the role of beryllium particle dissolution kinetics in chronic beryllium disease.

Early simulant dissolution studies focused on the fate of aerosol particles deposited in lung extracellular fluid (composed of blood plasma and interstitial fluid). The most common simulants used were based on measurements of the ionic composition of lung extracellular fluid by Gamble (Ansoborlo et al., 1999) and include serum ultrafiltrate (SUF) developed by Eidson and Mewhinney (1980). SUF is not an actual ultrafiltrate of blood serum but a simulant with ionic composition that matches serum without proteins.

In vitro dissolution studies of uranium oxides (Guilmette et al., 2000), stone wool and man made mineral fibers (Steenberg et al., 2001), curium oxides (Helfinstine et al., 1992), and cobalt oxide (Lundborg et al., 1992) demonstrate that variations in simulant fluid composition can influence observed dissolution rates. For example, Steenberg et al. (2001) found that citric acid but not acetic acid greatly increases dissolution of stone wool and man-made mineral fibers.

A simulant must mimic the chemical properties of the biological compartment being modeled. Extracellular fluid of the lung has near neutral pH (7.2–7.4) while the phagolysosomal environment within the cell serves to dissolve phagocytized materials and has pH $\approx 4.5 - 5$ (Ansoborlo et al., 1999). With evidence that dissolution of insoluble particles occurs predominantly in phagolysosomes (Lundborg et al., 1984), investigators began to adjust the pH of lung extracellular fluid-simulants to match that of the phagolysosome, i.e. pH $4.5 - 5$ (Nyberg et al., 1989a,b; Kreyling et al., 1991; Heilmann et al., 1992).

Many of the enzyme and protein constituents of phagolysosomes are known (Tappel, 1968); however, our literature searches could not find any measurements of the ionic composition of phagolysosomes. Because data describing the ionic composition of phagolysosomes were not available, we assumed that the basic composition was the same as lung extracellular fluid. Therefore, we chose and modified an SUF developed by Guldberg et al. (1998) as the basis for a phagolysosomal simulant fluid (PSF). The purpose of the work described here was to evaluate the suitability of our SUF formulation for use as PSF. By critically analyzing several of the assumptions incorporated into PSF we contribute to an understanding of factors pertinent to the design of simulants of the phagolysosomal environment for the study of beryllium particle dissolution using a static dissolution technique.

2. Materials and methods

An array of experiments was developed to test the suitability of our PSF formulation (Table 1) for in vitro studies of beryllium particles:

- To test the assumption that the selected PSF is an appropriate phagolysosomal simulant, beryllium dis-

Table 1
Composition of 0.02-M potassium hydrogen phthalate (KHP) buffered phagolysosomal simulant fluid (PSF)

Constituent	mg/L
Sodium phosphate dibasic anhydrous (Na_2HPO_4)	142.0
Sodium chloride (NaCl)	6650.0
Sodium sulfate anhydrous (Na_2SO_4)	71.0
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	29.0
Glycine ($C_2H_5NO_2$)[a]	450.0
Potassium hydrogen phthalate (1-(HO_2C)–2-(CO_2K)–C_6H_4)	4084.6
Alkylbenzyldimethylammonium chloride (ABDC)[b]	50 ppm

Sodium	Potassium	Calcium	Chloride	Phosphate	Sulfate	Glycine
Concentration of ionic constituent species (mmol/L)						
116.8	25.0[c]	0.2	114.2	1.0	0.5	6.0

[a] Representative of organic acids.

[b] Added as an antifungal agent.

Table 2
Composition of 0.02-M potassium hydrogen phthalate (KHP) buffered Baron and Ahmed solution (BAS) of leukocyte intracellular fluid

Constituent	mg/L
Sodium chloride (NaCl)	2010.0
Potassium chloride (KCl)	8700.0
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	765.0
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	4615.0
Potassium hydrogen phthalate (1-(HO_2C)–2-(CO_2K)–C_6H_4)	4086.0
Glycine ($C_2H_5NO_2$)[a]	450.0
Aldkylbenzyldimethylammonium chloride (ABDC)[b]	50 ppm

Sodium	Potassium	Calcium	Magnesium	Chloride[c]	Glycine
Concentration of ionic constituent species (mmol/L)					
34.4	140.9[d]	5.2	22.7	206.9	6.0

[a] Representative of organic acids.

[b] Added as an antifungal agent.

[c] Concentration as prepared; leukocyte intracellular concentration is 110.5-mmol/L (Baron and Ahmed, 1969).

[d] Includes the contribution from 0.1-M potassium hydroxide used to adjust the 0.02-M potassium hydrogen phthalate (KHP) buffer to pH 4.55.

solution in PSF was compared to that observed using the J774A.1 murine cell line (Ralph and Nakoinz, 1975).

- To determine if variations in ionic concentration influences beryllium dissolution, PSF was compared to a simulant fluid (Table 2) having the same pH but ionic concentrations that match intracellular fluid of leukocytes.
- To ensure that buffer strength was adequate to maintain pH without altering the basic rate of beryllium dissolution, beryllium dissolution in PSF was compared at three buffer concentrations.
- Finally, dissolution was compared in PSF with alkylbenzyldimethylammonium chloride (ABDC) and PSF lacking ABDC to ensure that the presence of this compound, often added to simulants to prevent fungal growth (Finch et al., 1988), does not influence beryllium dissolution.

2.1. Formulation of the phagolysosomal simulant fluid (PSF)

We chose and modified an SUF developed by Guldberg et al. (1998) as the basis for our PSF. Ionic composition of the Guldberg et al. (1998) SUF was the same as that of lung extracellular fluid for phosphate, organic acids, and sulfate, but differed in concentration for many other constituents. Although we hypothesize that ionic composition may not be a factor in dissolution, we adjusted the concentration of several constituents to match Gamble (1967) measurements of lung extracellular fluid, and we eliminated constituents such as ammonium ions that had been added to maintain pH 7.3 (Kanapilly et al., 1973), but were not relevant to the phagolysosome. The Guldberg et al. (1998) SUF used 0.01-M potassium hydrogen phthalate (KHP) buffer to achieve pH 4.5. Our pilot studies suggested that a 0.01-M KHP solution may not be adequate to maintain pH stable at 4.55 under all study conditions, so the buffer concentration of PSF was doubled to 0.02-M KHP to limit variation in pH to less than ±0.10 during dissolution studies.

2.2. Beryllium study materials

Two occupationally relevant forms of beryllium material were selected for use in the dissolution studies to evaluate our PSF: beryllium metal and beryllium oxide (BeO). Details of the source of the study powders, their aerodynamic size classification using a five-stage aerosol cyclone, and determination of specific surface area are described elsewhere (Hoover et al., 1989; Stefaniak et al., 2003). Powders collected in stage-3, aerodynamic diameter 1.7–2.5 μm, or stage-4, aerodynamic diameter 0.9–1.7 μm, of the aerosol cyclone were studied. These sizes are optimum for deposition in the alveolar region of the lung (see for example Foord et al., 1978) and are readily phagocytized by macrophage cells (Hahn et al., 1977). Specific surface area of these powders is 13.7 m^2/g (stage-3 beryllium metal powder), 19.4 m^2/g (stage-4 beryllium metal powder), and 11.1 m^2/g (stage-3 BeO powder). The dissolution behavior of the BeO material has also been evaluated in vitro using the J774A.1 cell line (Day, 2002).

BeO was used in the studies comparing dissolution in PSF to dissolution in the J774A.1 murine cell line. BeO was also used in the experiment comparing the influence of buffer concentration on pH stability of the PSF formulation. Because beryllium metal has a greater intrinsic solubility than BeO (Finch et al., 1988), beryllium metal was used in the evaluation of the influence of a variation of the ionic composition of the solvent. Likewise, beryllium metal was used to evaluate the influence of the presence or absence of the antifungal component of the PSF formulation.

2.3. Static dissolution technique

Acellular dissolution experiments were performed using a static dissolution technique developed by Kanapilly et al. (1973). A static technique was chosen because it is among the easiest and most widely implemented in vitro systems and is not subject to filter clogging, maintaining constant simulant pressure and flow, and production of large volumes of simulant associated with other methods such as the flow-through system (Ansoborlo et al., 1999).

2.3.1. Beryllium suspension preparation

Based on knowledge (Finch et al., 1988) of the low dissolution rate of beryllium metal ($k = 1.5 \times 10^{-9}$ g/(cm^2 day)) and BeO ($k = 3.7 \times 10^{-9}$ g/(cm^2 day)) in neutral pH media, we used a liquid suspension method to place precise amounts of the beryllium materials in the static dissolution chambers. As described below, this method minimized the number of dry powder handling steps. Assuming that phosphate buffered saline (PBS) has dissolution properties similar to the SUF used by Finch et al. (1988), the amount of beryllium potentially dissolved in the liquid suspension medium between preparation of the suspension and placing materials into the chambers was estimated to be less than 0.1%. Note that the suspension method of preparing the chambers would not be suitable for moderately soluble or highly soluble materials.

For each experiment, the amount of beryllium powder needed was weighed (Model AX205 microbalance, Metler–Toledo) into a clean, dry 20-mL borosilicate glass scintillation vial (Kimble Glass Inc., Vineland, NJ) with a foil-lined screw-top cap. Approximately 20-mL of 0.0095-M PBS (GIBCO, Grand Island, NY) was added to the vial, and the vial reweighed to determine the total mass. From these measurements the concentration of each suspension was calculated. Based on an assumption that freezing and thawing would not alter the crystal structure or surface properties of the metal and oxide materials, the suspension was stored frozen until just before use.

In our experience, the type of container (plastic or glass), the type of liquid matrix (water or PBS), and the order in which powder and liquid were added to the container were important in preparation of the suspensions. A suspension could be prepared when water or PBS was added to a glass scintillation vial containing BeO powder and when PBS (but not water) was added to a glass scintillation vial containing beryllium metal powder. However, using plastic centrifuge tubes or adding powder to liquid resulted in loss of particles to the container wall that could not be fully dislodged using ultrasonic agitation or shaking.

2.3.2. Static dissolution chamber assembly

Each suspension was thawed, briefly subjected to ultrasonic agitation, and then shaken vigorously prior to dosing filter sandwiches. The volume of suspension needed to achieve an initial beryllium particulate mass (M_0) was pipetted onto a 37-mm diameter cellulose sample filter support pad (stock number AP10, Millipore, Bedford, MA) using a precision micropipetter (Model E2-1000, Rainin, Inc., Emeryville, CA) and the pad inserted between two 0.025-μm pore size 47-mm diameter mixed cellulose ester filters (Millipore) that served as barriers to particle migration into the dissolution medium (Fig. 1). The sample filter can be any convenient filter media provided it is readily digestible for spectroscopic analysis of beryllium. In these studies a 37-mm filter support pad was used because its high porosity was amenable to loading the dissolution chamber. This filter "sandwich" was secured tightly in a static dissolution chamber (Intox Products, Moriarity, NM). Four nylon screws with two O-rings were used to provide a particle-tight seal around the edge of the sandwich.

Dose levels were selected to give dissolved beryllium mass levels (M_D) above analytical detection limits using inductively coupled plasma-mass spectroscopy (ICP-MS). To confirm M_0 in the sandwiches estimated by pipetting, replicate filter sandwiches of each material were prepared at the time of experimental setup and submitted for analysis by ICP-MS. In our experience, the coefficient of variation for a set of replicate samples are generally <5% when using the dosing technique described.

Our dissolution experiments were carried out for 10 days; mixed cellulose ester is not recommended for

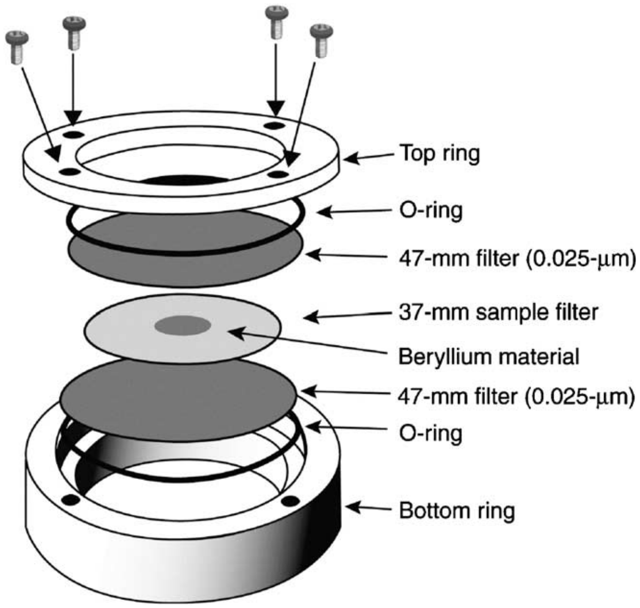

Fig. 1. Static dissolution chamber assembly.

long-term (>30 days) dissolution studies because the filters may become brittle and split, permitting particles to escape into the dissolution medium. In lieu of mixed cellulose ester, investigators have used Tuffryn-HT membrane filters for long-term studies (Guilmette et al., 2000). The mixed cellulose ester filter pore size was selected to be sufficiently smaller than 0.19 ± 0.04 μm, the average diameter of individual BeO primary particles (Stefaniak et al., 2003), to prevent bias in measurements of M_D associated with particle migration into the dissolution medium.

2.3.3. Preparation of phagolysosomal simulant fluid (PSF)

The chemical composition of 0.02-M KHP-buffered PSF is presented as Table 1. Analytical grade chemicals (Fisher Scientific, Fairlawn, NJ) were used to prepare the PSF. As in previous dissolution studies, 50-ppm ABDC was added as an antifungal agent (Finch et al., 1988). The initial pH of the PSF solution was typically 4.0. The pH was raised to 4.55 by addition of 0.1-M potassium hydroxide while monitoring using a calibrated pH electrode connected to a meter (Model AB15, Fisher Scientific) according to United States Environmental Protection Agency (US EPA) Method 9040B: pH electrometric measurement (US EPA, 1996). To verify complete transfer and mixing of representative salt constituents, the chloride concentration of PSF was measured at 37.0 °C according to US EPA Method 9212: Potentiometric determination of chloride in aqueous samples with ion-selective electrode (US EPA, 1996) using a calibrated combination ion-selective electrode (Model 96-17, Orion Research Inc., Beverly, MA) with separate temperature probe connected to a meter (Model 15, Fisher Scientific). Measured chloride concentration, expressed as a percentage of gravimetric weighing, was 100 ± 4% in PSF prepared for the four characterization studies described below.

2.3.4. Exposure to simulant

Each chamber was immersed in 80-mL of appropriate simulant contained in a polypropylene plastic beaker and maintained at 37 °C for the duration of the study period using water baths (Model 286, Precision, Winchester, VA; Model G76, New Brunswick Scientific Co., Inc., Edison, NJ). The pH of simulant in each beaker was monitored daily using a calibrated pH electrode connected to a meter (US EPA, 1996). At the end of the study period each dissolution chamber was lifted from its liquid, gently tapped on the inside wall of the beaker to dislodge any residual liquid droplets from the chamber, and then removed from the beaker. The PSF from each beaker was then transferred to separate borosilicate glass sample jars (Qorpak, Bridgeville, PA) and stored frozen at −5 °C until analyzed for dissolved beryllium content using ICP-MS as described below. The volume of liquid transferred to each sample jar was recorded and compared to the original volume placed in the beaker to confirm that no unexplained losses of liquid had occurred and that recovery of the liquid was quantitative (i.e. >95%).

2.4. Study of PSF as an appropriate simulant of phagolysosomal fluid

The first characterization study tested the hypothesis that KHP was an appropriate buffer for modeling phagolysosomal dissolution by comparing BeO dissolution in PSF to that observed using the J774A.1 murine cell line. PSF was prepared as described using the recipe in Table 1. Eleven dissolution chambers were prepared as described and each loaded with a 1077-μg dose of stage-3 BeO powder. Using the molecular weights of beryllium (9.01 g/mol) and BeO (25.01 g/mol), this powder mass provided a beryllium mass dose level of approximately 9/25 × 1077 = 388 μg. Each chamber was placed in a separate beaker containing 80 mL of PSF and the pH was monitored daily using a calibrated pH electrode to ensure it remained at 4.55 ± 0.10. After 10 days, the liquid fractions from each beaker were transferred to separate borosilicate glass sample jars (Qorpak, Bridgeville, PA) as described and stored frozen at −5 °C until analyzed for beryllium content using ICP-MS (described below).

The J774A.1 murine cell line (American Type Culture Collection, Manassas, VA) was selected to study in vitro dissolution because it is an established (Ralph and Nakoinz, 1975), highly phagocytic and adherent cell line that is widely available to researchers. Use of the J774A.1 murine cell line provided for the collection of baseline information about particle uptake and dissolution, which may offer insights to processes in human pulmonary alveolar macrophages. Six 150-mm × 20-mm culture dishes (Falcon, Becton-Dickinson, San Jose, CA) were seeded with 2×10^6 cells in 50-mL beryllium-free minimum essential medium (MEM-α) alpha (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% antibiotics (penicillin–streptomycin, liquid combination, GIBCO). The dish size and initial number of cells were chosen such that the surface area available for growth was sufficient for cells to remain in exponential growth throughout the 10-day study period (data not shown). Initial cell viability was 91% and average final cell viability was 86 ± 3% as assessed by 0.4% trypan blue dye exclusion using a hemacytometer (Reichert Bright-Line, Buffalo, NY). All dishes were incubated at 37 °C under 5% CO_2 and 95% relative humidity atmosphere for two hours to allow cells to adhere. After cells adhered, the MEM-α was removed from each dish and replaced with 50-mL fresh MEM-α containing 57.4 ± 9.1 μg of stage-3 BeO powder (20.7 ± 3.3 μg beryllium as determined from six replicate 50-mL samples of the MEM-α analyzed using ICP-MS). This mass was equivalent to approximately 10 BeO particles per cell and was not expected to cause appreciable cytotoxicity (Eidson et al., 1991) but would result in dissolved beryllium levels above limits of detection for ICP-MS.

Samples of dissolved beryllium were collected on days 2, 4, 6, 8, and 10 using the fractionation protocol presented as Fig. 2. At each time point, first the MEM-α was aspirated from each dish, transferred to separate labeled 50-mL tubes (Falcon), and then subjected to low-speed centrifugation (Model GS-6R, Beckman Instruments, San Jose, CA) at 1100 rpm (250 × *g*) and 4 °C for 6 min to sediment any floating cells into a pellet. Second, the supernatant from each tube was aspirated and transferred to separate labeled 50-mL high-speed centrifuge bottles (Beckman-Coulter, Palo Alto, CA) and subjected to high-speed centrifugation at 10000 rpm (13775 × *g*) and 4 °C for 45 min (Model J2-21, Beckman Instruments, San Jose, CA) to separate dissolved beryllium from free particulate BeO in the MEM-α. From the equation for terminal velocity due to centrifugal force (Hinds, 1999), centrifuging for 45 min was sufficient to sediment a 0.05-μm diameter beryllium oxide particle 4 cm, the height of the liquid in a 50-mL centrifuge bottle. The average diameter of a beryllium oxide primary particle is 0.19 ± 0.04 μm (Stefaniak et al., 2003).

Concurrent with the high-speed spin, 20 mL of PBS (GIBCO) warmed to 37 °C was added to each dish to rinse the cells for 15 min to remove any residual dissolved beryllium on the cells. The rinse PBS was then aspirated and transferred to the centrifuge tube containing the corresponding cell pellet from the low-speed spin. Fifty milliliter of fresh BeO-free MEM-α was added to each dish and then all dishes were returned to the incubator until the next sample collection time point. Each tube was subjected to another low-speed centrifugation; then the supernatant containing dissolved beryllium was transferred to separate labeled borosilicate glass sample jars and stored at −5 °C until analyzed. The resulting cell pellet contained undissolved BeO particles associated with the cells. Upon completion of the high-speed spin, the supernatant, which contained dissolved beryllium in MEM-α, was aspirated and transferred to separate labeled borosilicate glass sample jars and stored frozen at −5 °C until analysis. The remaining pellet contained any undissolved BeO particles free in the MEM- at the time of sample collection. Note that if dissolved beryllium was bound to cellular debris, our centrifugation method might result in an underestimation of the amount of dissolved beryllium.

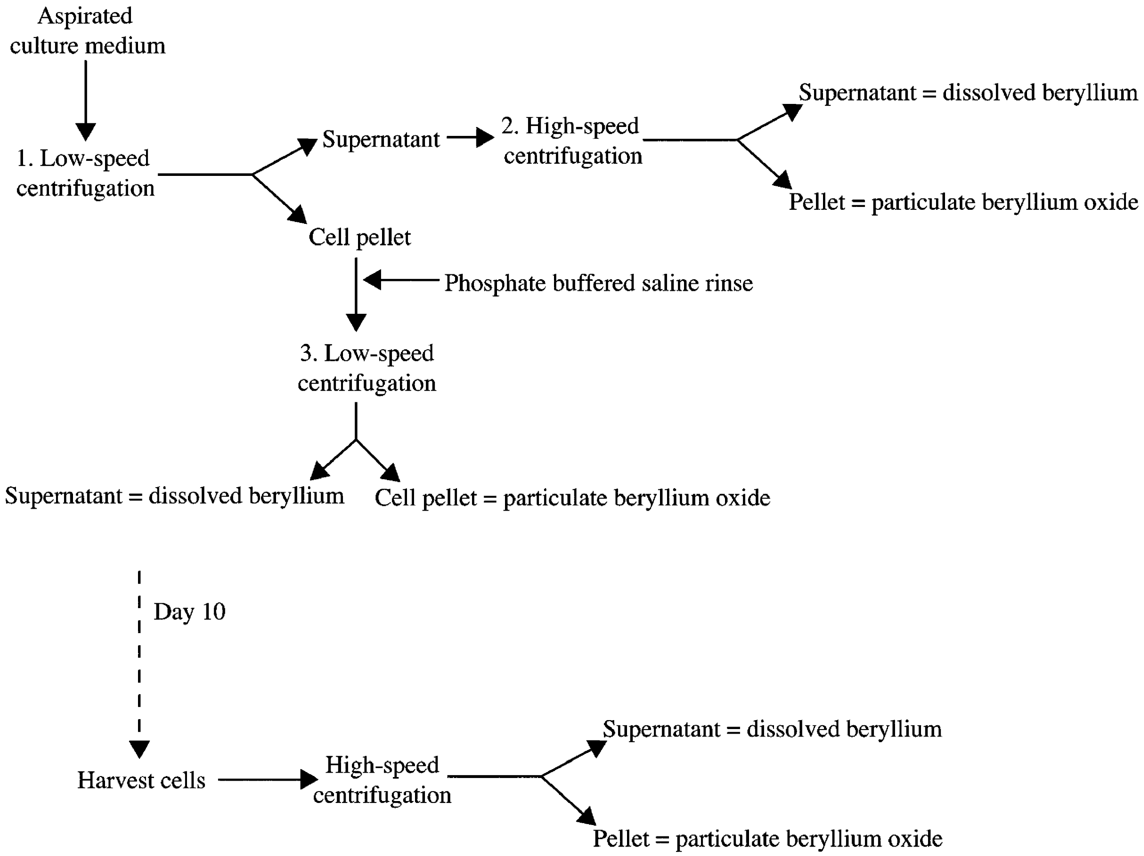

Fig. 2. Protocol used to fractionate dissolved beryllium from particulate beryllium oxide.

On day 10 the MEM-α from each culture was fractionated as described above. In addition, after the PBS rinse step, adherent cells were removed from each dish surface using four sequential 10-mL PBS washings from a syringe. Note that this removal method works well for the J774A.1 murine cell line because they are lightly tethered to dish surfaces. This method also avoids unnecessary losses of cell viability from scraping cells from dish surfaces or alteration of cell properties from exposure to chemicals such as trypsin or other enzymes. A 25-μL aliquot of the cell suspension was then reserved to assess final cell viability. Cell suspensions were transferred to separate labeled 50-mL high-speed centrifuge bottles and subjected to high-speed centrifugation (Fig. 2). The supernatants containing dissolved beryllium were transferred to separate labeled borosilicate glass sample jars and stored frozen at −5 °C until analyzed. The resulting cell pellets contained cell-associated, undissolved particulate BeO. Note that after the initial four × 10-mL PBS wash to suspend cells, the surface of each dish was washed with four ×10-mL PBS twice more to assess the efficiency by which remaining beryllium (in the form of particulate BeO and dissolved beryllium inside cells) was recovered from culture dishes. In this pilot study 100.0 ± 14.8% of beryllium was recovered in the initial washing, 4.9 ± 4.3% in the second washing, and 1.2 ± 1.7% in the third washing. Three cellular controls were handled identically to the experimental dishes and samples collected were also analyzed for beryllium content. The sum of beryllium dissolved on days 2, 4, 6, 8, and 10 for each in vitro cellular dish was normalized by initial dose mass to determine the percent of beryllium mass dissolved.

This cellular experiment was then repeated a second time. In the second trial initial cell viability was 94% and average final viability was 89 ± 2%. An initial dose of 47.6 ± 5.9 μg of stage-3 BeO powder (17.2 ± 2.1 μg beryllium as determined from six replicate samples of dosing MEM-α) was used. This mass was equivalent to approximately 9 BeO particles per cell. Dishes were inspected daily for mold, and in each of the in vitro trials only one experimental culture dish had to be removed due to mold contamination.

2.5. Study of the influence of ionic composition

The second characterization study tested the hypothesis that variability in ionic composition does not influence beryllium dissolution by comparing dissolution in PSF to dissolution in a simulant of intracellular fluid, also having pH 4.55, but differing ionic composition. PSF was prepared as described using the recipe in Table 1. A second simulant fluid termed Baron and Ahmed solution (BAS) (Baron and Ahmed, 1969) was prepared according to the protocol described for PSF but using the recipe in Table 2. The composition of BAS is based on measurements of the intracellular concentration of ions in leukocytes. With the exception of Cl^-, the molar concentrations of all BAS constituents listed in Table 2 match intracellular concentrations. Because all salts used to make BAS contained Cl^-, the concentration of Cl^- exceeded 110.5-mmol/L, its measured value in leukocytes (Baron and Ahmed, 1969). Although a known source of error, this excess Cl^- accentuates the differences in ionic composition between the two simulants and can only help to elucidate the role, if any, of ionic composition in the dissolution of beryllium. The ratio of ionic constituents in BAS relative to PSF ranges from 0.3 for sodium to 26 for calcium.

Twenty static dissolution chambers were loaded, each with a 196-μg dose of stage 4 beryllium metal powder. Next, 10 static dissolution chambers were placed in separate beakers containing 80 mL of PSF and 10 chambers placed in separate beakers containing 80 mL of BAS. The pH of all 20 beakers was monitored daily to ensure it remained at 4.55 ± 0.10. After 10 days, the liquid fractions from each beaker were transferred to separate borosilicate glass sample jars, as described, and stored frozen at −5 °C until analyzed for beryllium content using ICP-MS (described below).

2.6. Study of the influence of buffer strength

The third characterization study was conducted to determine a buffer strength that was adequate to maintain a pH of 4.55 ± 0.10 without altering the basic rate of beryllium dissolution. Although, the buffer strength of the original Guldberg et al. (1998) formulation was 0.01-M, we observed in pilot studies without beryllium that this buffer concentration was not adequate to maintain the pH to within ±0.10 over the 10 day evaluation period. Therefore, beryllium dissolution was studied in PSF prepared at three different KHP buffer concentrations: 0.01, 0.02, or 0.04-M.

Thirty static dissolution chambers were loaded, each with a 452-μg dose of stage 3 BeO powder (163 μg beryllium). Next, 10 static dissolution chambers were placed in separate beakers containing 80 mL of PSF at each of the three buffer concentrations. The pH of all 30 beakers was monitored daily to ensure that each remained at 4.55 ± 0.10. After 10 days, the liquid fractions from each beaker were transferred to separate borosilicate glass sample jars, as described, and stored frozen at −5 °C until analyzed for beryllium content using ICP-MS (described below).

2.7. Study of the influence of the ABDC antifungal agent

The final characterization study tested the hypothesis that the antifungal agent ABDC, an exogenous constituent of PSF, does not influence beryllium dissolution.

This hypothesis was tested by comparing dissolution in PSF that was prepared with and without ABDC. The use of ABDC as an antifungal agent in beryllium dissolution studies is not new (Finch et al., 1988); however, no information is available describing its influence, if any, on beryllium dissolution. Twenty static dissolution chambers were loaded, each with a 496-μg dose of stage 3 beryllium metal powder. Ten were placed in separate beakers containing 80 mL of PSF with 50 ppm ABDC and 10 in separate beakers containing PSF without ABDC. The pH of all 20 beakers was monitored daily to ensure it remained at 4.55 ± 0.10. After 10 days, the liquid fractions from each beaker were transferred to separate borosilicate glass sample jars, as described, and stored frozen at −5 °C until analyzed for beryllium content using ICP-MS (described below).

2.8. Evaluation of dissolution results

We used the traditional dissolution theory of Mercer (1967) to evaluate the dissolution results. Mercer describes an equation for the mass fraction remaining, $\frac{M}{M_0}$, for dissolution of particles with an initial specific surface area and diameters that are log-normally distributed with a geometric standard deviation of σ_g. We have found that Mercer (1967) results for $\frac{M}{M_0} > 0.3$ and $\sigma_g = 1$–2.3 ($\sigma = \ln \sigma_g$ from 0 to 1) can be expressed as a single exponential:

$$\frac{M}{M_0} = \exp\left\{-\lambda kt\mathrm{SSA}\exp -\frac{\sigma^2}{2}\right\} \tag{1}$$

where, M = remaining mass of population of particles, M_0 = initial mass of population of particles, $\lambda = 1.18$, k = chemical dissolution rate constant $\left(\frac{\text{mass}}{\text{area}\cdot\text{time}}\right)$, t = time, SSA = specific surface area at time, $0\left(\frac{\text{area}}{\text{mass}}\right)$, $\sigma = 0.5$.

This equation can be expressed in terms of mass fraction dissolved, $\frac{M_D}{M_0}$:

$$\frac{M_D}{M_0} = 1 - \exp\{-1.04\mathrm{ktSSA}\} \tag{2}$$

where M_D = mass dissolved from population of particles.

2.9. Quality control and analysis methods

Blank samples consisting of PSF and BAS or filters only were prepared to verify the absence of beryllium in reagents. With the exception of the cellular in vitro samples, all blank, replicate dosing, and experimental samples were submitted as a single batch to Carlsbad Environmental Monitoring and Research Center (Carlsbad, NM) for microwave acid digestion and analysis of beryllium content by ICP-MS. Analytical methods follow EPA SW-846 Method 3015: Microwave-assisted acid digestion of aqueous samples and extracts for liquid samples and EPA SW-846 Method 3050B: Acid digestion of sediments, sludges, and soils for filter samples (US EPA, 1996). The method detection limits (standard deviation from analysis of seven replicate aliquots of a standard prepared at three to five times the instrument limit of detection multiplied by 3.14, the Student's t-value for sample size of seven) (40 CFR 136.2) were 0.006 μg beryllium/filter for analysis of filters and 1.56 μg/L for analysis of liquid samples.

Beryllium content of liquid samples from the J774A.1 murine cell line were analyzed using ICP-MS according to EPA Method 3050B (US EPA, 1996) by Battelle Memorial Institute (Columbus, OH). Blank and quality control samples were submitted blind as appropriate. The instrument limit of detection is 0.03 μg of beryllium.

For each of the static dissolution chambers a mass balance was performed. Three of 81 samples were excluded from the data analysis because the measured M_D exceeded M_0 by more than 10% (one from each treatment group in the study of the influence of simulant ionic composition on dissolution and one from the ABDC treatment group in the study of the influence of an antifungal agent on dissolution). Dissolved beryllium levels in treatment groups were compared using the nonparametric Wilcoxon rank-sum test. A significance level, $\alpha = 0.05$, was used for statistical comparisons. A Bonferroni-correction to α was used when comparing more than two groups (Pagano and Gauvreau, 1992). Statistical analyses were performed using Microsoft Excel (Microsoft Corp., Redmond, WA). Note that excluding the discarded sample values from each of the treatment groups slightly changed calculated *p*-values but did not alter the conclusions of the studies.

3. Results and discussion

Fig. 3 plots the dissolution results by treatment group for each of the four characterization studies described above. No beryllium was detected in the cellular control samples or in the liquid and filter blank samples from the acellular studies.

3.1. PSF as a phagolysosomal simulant

In previously reported work (Andre et al., 1987) it was concluded that a cellular in vitro model is a better predictor of beryllium solubility in the lungs than a simulant of lung extracellular fluid. However, the lung extracellular fluid used by Andre et al. (1987) had neutral pH while the phagolysosomal environment has pH 4.5–5 (Nyberg et al., 1989a,b; Kreyling et al., 1991; Heilmann et al., 1992), thus a comparison of dissolution in these two environments with distinctly different pH values may not be appropriate. In the current study the comparison of beryllium dissolution in PSF and in the J774A.1 murine cell line (Fig. 3a) illustrated that median levels were similar. The median percent of beryllium mass dissolved

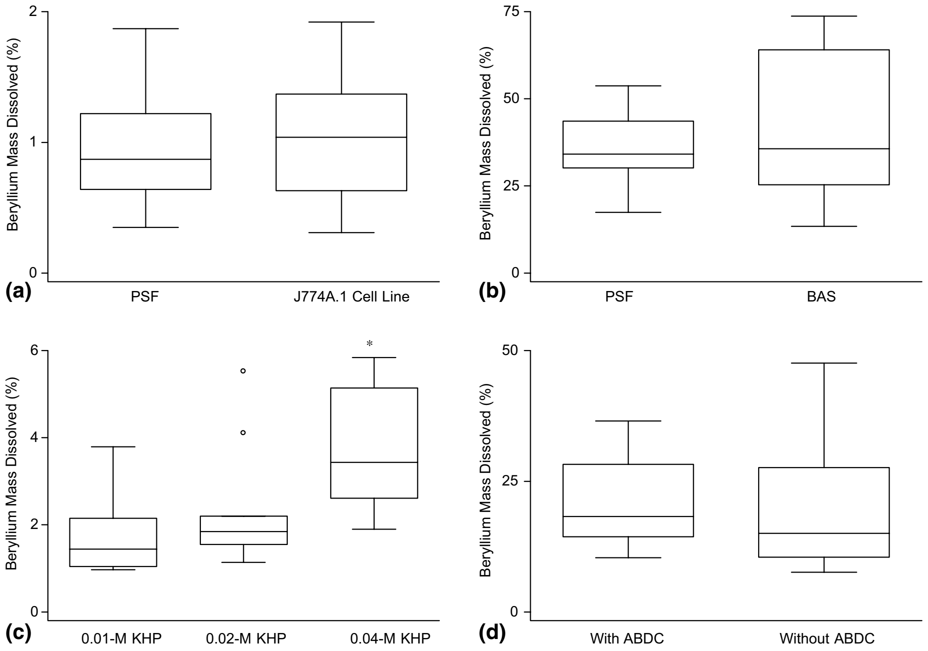

Fig. 3. Plots comparing dissolution results for (a) beryllium oxide in phagolysosomal simulant fluid (PSF) and in the J774A.1 murine cell line; (b) beryllium metal in PSF and in Baron and Ahmed solution (BAS); (c) beryllium oxide in PSF prepared at buffer strengths of 0.01, 0.02, and 0.04-M potassium hydrogen phthalate (KHP); and (d) beryllium metal in PSF with the antifungal agent alkylbenzyldimethylammonium chloride (ABDC) and in PSF without ABDC. The center of each box is the median, the top and bottom of each box are the 75th and 25th percentile, respectively; ends of the whiskers are 1.5 times the interquartile range; and values beyond the whiskers ("outliers") are shown as open circles. Within each experiment, dissolved beryllium levels in treatment groups were compared using the nonparametric Wilcoxon rank-sum test ($^{*}p < 0.05$).

from stage-3 BeO powder in PSF, 0.9 ± 0.5%, was not significantly different ($p = 0.78$) from 1.0 ± 0.5%, the median percent of beryllium mass dissolved by the J774A.1 murine cell line for the pooled trials ($n = 10$). The data suggest that PSF may be a useful and appropriate model of in vitro beryllium dissolution when using a static dissolution technique. In addition, quantification of beryllium dissolution behavior in PSF provided an opportunity to assess the total rate of ionic beryllium bioavailability, which may assist in improving our understanding of how beryllium ions may become available or bind within the cellular environment of macrophages, and subsequently within the lung.

3.2. Influence of simulant ionic composition on dissolution

As shown in the plot of beryllium dissolution in Fig. 3b, the median percent of beryllium mass dissolved from stage-4 metal powder in PSF was 34.1% and in BAS it was 35.7%; this difference in medians was not statistically significant ($p = 0.73$).

In the presence of Cl^-, centimeter-size polished beryllium metal specimens are subject to high rates of localized "pitting" or electrochemical dissolution between pH 2 and 12.5 (Hill et al., 1998; Venugopal et al., 2000). These prior findings suggest that, in aqueous conditions with pH that matches the phagolysosome, Cl^- concentration may be an important parameter to consider when designing a simulant of biological fluid to study dissolution of beryllium metal particles. However, in the current study, the Cl^- concentration in BAS was twice as high as in PSF, but dissolution in these two simulants was not significantly different. Data reported were consistent with results from a study performed in our laboratory to investigate the potential role of Cl^- in dissolution (data not shown). Dissolution from an equal initial mass of beryllium metal was compared in five simulant fluids using the same static dissolution technique. Four of the simulant fluids, intended to mimic the phagolysosome, were prepared using an acetic acid-sodium acetate buffer and had pH 4.6 with 0 (control), 0.01, 0.05, or 0.1-M Cl^-. The fifth simulant, intended to mimic extracellular lung fluid, was prepared using sodium hydroxide-boric acid buffer and had pH 7.2 and contained 0.1-M Cl^-. At pH 4.6 no linear relationship between increased chloride concentration and beryllium dissolution was observed. Significantly more beryllium dissolved at pH 4.6 with or without Cl^- than at pH 7.2 with 0.1-M Cl^- ($p < 0.001$), consistent with the finding that the dissolution rate of beryllium increases as simulant pH decreases (Finch et al., 1988). Collectively, these data suggest that when modeling phagolysosomal dissolution of beryllium using a simulant, it may be more important to control pH than ion concentration. As a result, PSF may also be appropriate for investigating phagolysosomal dissolution of other materials that exhibit pH-dependent dissolution such as americium oxide (Taya and Mewhinney, 1992; Taya et al., 1992), cobalt oxide (Kreyling et al., 1990; Collier et al., 1992; Lundborg et al., 1992), curium oxide (Helfinstine et al., 1992), lead arsenate (Marafante et al., 1987), and manganese dioxide (Lundborg et al., 1984, 1985).

Note that recent thermodynamic modeling indicates that ionic composition may be important in the speciation of beryllium in simulants of biological fluids (Sutton and Burastero, 2003). Thus, identifying and matching ionic composition of phagolysosomal fluid may be important for simulant studies depending on the specific aims, i.e. to determine dissolution rate, beryllium speciation, or both.

3.3. *Influence of buffer strength on dissolution in PSF*

In the PSF treatment group with 0.01-M KHP, pH increased from 4.54 at time zero to 4.60 by day 1 and remained within ±0.01 of this value for the remainder of the study. For the PSF treatment group with 0.02-M KHP, pH was constant at 4.54 ± 0.01 throughout the study period. In the PSF treatment group with 0.04-M KHP, pH decreased from 4.54 at time zero to 4.50 on day 1 and remained within ±0.01 of this value for the remainder of the study. A plot of percent of beryllium mass dissolved in PSF prepared at the three buffer strengths is shown in Fig. 3c. Using stage-3 BeO powder, the median percent of beryllium mass dissolved was 1.5% in PSF with 0.01-M KHP, 1.8% in PSF with 0.02-M KHP, and 3.4% in PSF with 0.04-M KHP. The median percent of beryllium mass dissolved in PSF with 0.01-M KHP was not statistically different from the median in PSF with 0.02-M KHP ($p = 0.12$). However, the median percent of beryllium mass dissolved in PSF with 0.04-M KHP was statistically greater than the median dissolved beryllium in PSF with 0.01-M KHP ($p = 0.001$) and 0.02-M KHP ($p = 0.02$), suggesting that increased buffer strength altered the dissolution behavior of PSF. Thus, incorporation of the 0.02-M buffer concentration into the standard formulation for PSF provided the desired stability in pH (i.e., ±0.10 of initial) without alteration of the dissolution rate.

3.4. *Influence of antifungal additive on dissolution in PSF*

The percent of beryllium mass dissolved in PSF prepared with and without ABDC are presented as Fig. 3d. Median percent of beryllium mass dissolved from stage-3 metal powder was 18.3% in PSF containing 50-ppm ABDC ($n = 9$) and 15.1% in PSF without ABDC ($n = 10$). The difference in median levels of dissolved beryllium between these treatment groups was not statistically significant ($p = 0.35$), validating the assumption that ABDC does not influence beryllium dissolution in PSF.

3.5. *Influence of study material on dissolution*

Finch et al. (1988) found that k for beryllium metal powder is faster than for beryllium oxide in hydrochloric acid (pH 1) and SUF (pH $\approx$ 7.3). Similarly, we found that dissolution of metal powder (Fig. 3b and d) was higher than BeO powder (Fig. 3a and c) in PSF (pH 4.55).

In PSF, dissolution was higher for stage-4 metal powder (Fig. 3b) than stage-3 metal powder (Fig. 3d). Because k is a constant unique to a chemical form of material, $\frac{M_D}{M_0}$ should increase as specific surface area increases (Eq. (1)). Specific surface area was 19.4 m^2/g and 13.7 m^2/g for stage-4 and stage-3 metal powder, respectively. Thus beryllium dissolution data reported here were consistent with Mercer (1967) dissolution theory.

4. Summary and recommendations

Characterization of any simulant is necessary to demonstrate that results have practical significance relative to cellular in vitro or in vivo models. In the current study, beryllium dissolution in PSF with pH 4.55 was similar to dissolution levels observed in vitro using the J774A.1 murine cell line. Thus, PSF appears to be useful and appropriate for studying phagolysosomal dissolution of beryllium using a static dissolution technique.

Variations in ionic composition and the presence of 50-ppm ABDC does not appear to bias observed beryllium dissolution in PSF. However, increasing the strength of the KHP buffer in PSF beyond 0.02-M was not needed to provide pH stability and significantly increased the amount of beryllium dissolved. We recommend use of the PSF formulation given in Table 1, in conjunction with daily monitoring of pH. It will also be prudent to monitor and assess any effects on the stability of pH and consistency of dissolution results if other physicochemical forms or masses of beryllium study materials are used.

It is hoped that PSF will aid in understanding the physicochemical basis of beryllium dissolution and contribute to modeling dissolution of beryllium in vitro and in vivo. The critical approach used to evaluate and adjust the composition of PSF may also serve as a framework for characterizing PSF to study dissolution of other metal and oxide particles.

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