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Dissolution and reactive oxygen species generation of inhaled cemented tungsten carbide particles in artificial human lung fluids

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Abstract. Inhalation of both cobalt (Co) and tungsten carbide (WC) particles is associated with development of hard metal lung disease (HMD) via generation of reactive oxygen species (ROS), whereas Co alone is sufficient to cause asthma via solubilization and haptent formation. We characterized bulk and aerodynamically size-separated W, WC, Co, spray dryer (pre-sintered), and chamfer grinder (post-sintered) powders. ROS generation was measured in the murine RAW 264.7 cell line using electron spin resonance. When dose was normalized to surface area, hydroxyl radical generation was independent of particle size, which suggests that particle surface chemistry may be an important exposure factor. Chamfer grinder particles generated the highest levels of ROS, consistent with the hypothesis that intimate contact of metals is important for ROS generation. In artificial extracellular lung fluid, alkylbenzyltrimethylammonium chloride (ABDC), added to prevent mold growth during experiments, did not influence dissolution of Co (44.0 ± 5.2 vs. $48.3 \pm 6.4\%$); however, dissolution was higher ($p < 0.05$) in the absence of phosphate (62.0 ± 5.4 vs. $48.3 \pm 6.4\%$). In artificial macrophage phagolysosomal fluid, dissolution of Co ($36.2 \pm 10.4\%$) does not appear to be influenced ($p = 0.30$) by the absence of glycine ($29.8 \pm 2.1\%$), phosphate ($39.6 \pm 8.6\%$), or ABDC ($44.0 \pm 10.5\%$). These results aid in assessing and understanding Co and W inhalation dosimetry.

1. Introduction

Cemented tungsten carbide (CTC) refers to a class of alloys containing 80 to 95% tungsten carbide (WC) bound in a matrix of 5 to 20% cobalt (Co), with additives such as nickel, chromium, vanadium, titanium carbide, and tantalum carbide to impart performance-specific properties. Cemented tungsten carbide tools exhibit extreme hardness and wear resistance at the tool and work piece interface during cutting, which is retained at elevated temperatures. As such, applications of CTC include cutting tools for metals; and masonry, concrete, coal, and rock drills and cutters.[1,2]

Inhalation of dusts generated during production of CTC is associated with development of hard metal lung disease (HMD) and occupational asthma. Cases of HMD have been reported among workers exposed to pre-sintered material [3] and among workers exposed to post-sintered CTC material.[4] Using pre-sintered powders, investigators have demonstrated greater generation of reactive oxygen species (ROS) for Co in the presence of WC relative to ROS generation among the individual constituents.[5] Thus, available data suggest that WC particles in association with Co particles, rather than WC or Co particles alone, should be considered a specific toxic entity in

development of HMD. Additionally, workplace studies have identified cases of occupational asthma among employees who handle pre-sintered material (e.g., powder mixers, pressers, and shapers) and among those who grind and manipulate sintered CTC parts.[3-6] To date, a clear toxicological mechanism for cobalt-induced asthma is lacking.[7] Evidence indicates, however, that Co is soluble in artificial lung fluids [8-9] and that ionic cobalt may conjugate with proteins to form a hapten which may elicit an immune response.[6,11]

Using well-characterized particles from representative CTC manufacturing processes, [12] we evaluated the hypothesis that aerosol physicochemical properties are important exposure factors for HMD and asthma. We measured particle solubility in variations of artificial extracellular lung fluid and lung alveolar macrophage phagolysosomal fluid; evaluated solvent effects on observed dissolution rates; and determined particle ROS generation using an in vitro cellular model.

2. Materials and Methods

2.1. Study powders

Five representative bulk powders that span the spectrum of powders used in the manufacture of CTC were chosen for study: feed-stock W, WC, and Co powders, pre-sintered powder from a spray dryer, and dust from a chamfer grinder used for final machining of post-sintered CTC product. The morphology and chemistry of these bulk powders are similar to that observed for airborne particles in the work areas where the powders are handled. Thus, these powders are thought to be representative of aerosols to which workers are potentially exposed during CTC production.[12] A sixth material was prepared as an admixture by mechanically blending aerodynamically size-separated (see section 2.2) WC and Co powders at a ratio of 94:6 by mass. The admixture is not an actual industrial powder, but a mechanical mixture of feedstock powders prepared for the purpose of comparing free radical generation of loosely-associated cobalt and tungsten carbide particles to the individual feedstock powders.

2.2. Aerodynamic size separation of study powders

Each bulk powder was aerodynamically size-separated in the laboratory using a dry powder disperser (Model 175, DeVilbiss, Somerset, PA) and five-stage aerosol cyclone operated at 10 L min^{-1} . [13,14] The 50% aerodynamic cutoff diameters (D_{50}) of the aerosol cyclone were >10 , 4.3, 2.9, 2.0, and 1.2 μm for stages 1 to 5, respectively. For the purposes of this study, we evaluated ROS generation and solubility in artificial lung fluids for the materials collected in stages 1, 2, and 3 of the aerosol cyclone. (There were insufficient masses of materials collected in stages 4 and 5 of the aerosol cyclone to perform the material characterization assays and ROS and solubility studies for all study powders.) Material collected in stage 1 ($D_{50} > 10 \mu\text{m}$) have particle sizes capable of penetrating into the conducting airways (i.e., thoracic-sized particles). Materials collected in stage 2 ($D_{50} = 4.3 \mu\text{m}$) and stage 3 ($D_{50} = 2.9 \mu\text{m}$) have particle sizes capable of penetrating into the alveolar region of the lung (i.e., respirable-sized particles).[15]

2.3. Characterization of study powders

Physicochemical properties of the aerodynamically-size separated powders were characterized using scanning electron microscopy to evaluate the morphology and size of powders [12]; Auger spectroscopy to determine surface elemental composition (down to 100\AA thickness); x-ray diffraction to identify crystalline constituents; helium pycnometry (Multipycnometer, Quantachrome Instruments, Boynton Beach, FL) to determine material density; and nitrogen gas adsorption (NOVA 2000e, Quantachrome) by the Brunauer, Emmett, and Teller (BET) method to determine specific surface area.

2.4. Electron spin resonance (ESR) studies

ESR spin trapping was used to detect both carbon radicals and short-lived free radical intermediates. Hydroxyl radicals were measured using the addition-type reaction of a short-lived radical with a paramagnetic compound (spin trap) to form a relatively long-lived free radical product (spin adduct), which can then be studied using conventional ESR. RAW 264.7 mouse peritoneal monocytes (ATTC, Rockville, MD, USA) were used because they respond to particle exposure in a manner similar to primary alveolar macrophages.[16,17] RAW 264.7 cells were cultured in Dulbecco's modified eagles medium (DMEM) with 10% fetal bovine serum, 2 mM L-glutamine, 100 units mL⁻¹ penicillin, and 0.1 mg mL⁻¹ streptomycin at 37 °C in a 5% CO₂ incubator. For each study powder, a sample of material collected in stage 1, 2, or 3 of the aerosol cyclone was weighed, mixed with artificial extracellular lung fluid, [18] vortexed for 10 sec, and then immediately pipetted into the reaction system of RAW 264.7 cells (1 x10⁶) and DMPO spin trap (200 mM). The final powder concentration in each reaction system was 5 mg mL⁻¹. The cells were incubated for 3 min at 37 °C and then transferred to a flat cell for ESR measurement using a Bruker EMX spectrometer (Bruker Instruments Inc. Billerica, MA). Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K₃CrO₈) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference standards.[19] The relative radical concentration was estimated by multiplying half the peak height by (ΔHpp)², where ΔHpp represents peak-to peak width. The Acquisit program was used for data acquisitions and analyses (Bruker Instruments Inc).

2.5. Artificial lung fluid solubility studies

A known mass of each powder was weighed on a 0.025-μm pore size 47-mm diameter mixed cellulose ester (MCE) filter (Millipore, Bedford, MA) and then covered with a second filter. The filter "sandwich" was secured tightly in a static dissolution chamber (InTox Products, Moriarity, NM) using four nylon screws on the chamber periphery, providing a particle-tight seal around the edge of the sandwich. Each chamber was placed in a polypropylene plastic beaker and immersed in 80 mL of artificial extracellular lung fluid (see section 2.5.1) or phagolysosomal simulant fluid (see section 2.5.2) and maintained at 37 °C for the duration of a static dissolution study. For each dissolution experiment, triplicate samples were prepared for each size fraction of each study powder. At each time point, the chamber was removed from its beaker, the fluid transferred to a borosilicate glass jar, and fresh equilibrated fluid was added to the sample beaker. All samples were stored frozen at -5 °C and then thawed prior to quantification of dissolved Co and W in accordance with U.S. OSHA Method 213 (inductively coupled plasma-atomic emission spectroscopy).

2.5.1. Artificial extracellular lung fluid. Artificial extracellular lung fluid termed serum ultrafiltrate (SUF) was prepared using the recipe described by Finch et al.[18] The pH of SUF was maintained at 7.3±0.1 for the duration of the dissolution experiments by blanketing the headspace in each sample beaker with CO₂ (5%) and air (95%). SUF contains 5 mmol L⁻¹ of glycine, an important constituent in the dissolution of Co [20] and 1.2 mmol L⁻¹ phosphate, which may react with dissolved cobalt to form insoluble cobalt phosphate.[21] Although endogenous to extracellular lung fluid, 50 ppm of ABDC was added to the SUF to prevent mold growth [16] during this short-term experiment and to evaluate whether this constituent could be included in long-term (i.e., 30 day) studies. To evaluate whether these fluid constituents influenced dissolution, experiments were performed using only bulk feedstock powder to evaluate the dissolution of Co in SUF (with and without glycine or phosphate) and to evaluate the dissolution of W, WC, and Co in SUF (with and without ABDC). Samples were collected at 1, 4, 8, 12, 18, 24, 36, 48, and 72 hr to measure the mass of dissolved Co or W.

2.5.2. Artificial macrophage phagolysosomal fluid. Artificial lung alveolar macrophage phagolysosomal fluid (PSF) was prepared using the recipe described by Stefaniak et al.[22] The pH of the PSF solution was maintained at 4.55±0.10 for the duration of the dissolution experiments. PSF contains 6 mmol L⁻¹ of glycine, which is intended to represent the organic acid constituents present in

phagolysosomal fluid, 1 mmol L⁻¹ phosphate, and 50 ppm ABDC. As with SUF, experiments were performed using bulk feedstock powders to evaluate whether these fluid constituents influenced dissolution. Samples were collected at 1, 3, 6, 12, and 24 hr to measure the mass of dissolved Co or W.

3. Results and Discussion

As summarized in Table 1, feedstock powders were homogeneous, whereas the pre-sintered powder and the post-sintered CTC dust were heterogeneous mixtures of WC/Co powders. Cobalt was not identified on the surfaces of pre-sintered spray dryer powder, whereas W, C, and Co were each present on the surface of post-sintered chamfer grinder powder.

Table 1. Physicochemical properties of aerodynamically size-separated feedstock powders, process intermediary powders, and post-sintered cemented tungsten carbide powders.

Powder	Aerodynamic Size (µm)	Surface Constituents ^a	Crystalline Constituents ^a	Density (g/cm ³)	Specific Surface area (m ² /g)
Tungsten	>10	O, W, C	W	18.9±0.2	0.61±0.04
	4.3	O, W, C	W	19.8±0.3	0.87±0.05
	2.9	O, W, C	W	18.9±0.2	1.04±0.11
Tungsten carbide	>10	O, W, C	WC, W ₂ C	15.1±0.1	0.55±0.02
	4.3	W, O, C	WC, W ₂ C	15.6±0.1	0.65±0.03
	2.9	O, W, C	WC, W ₂ C	14.8±0.1	0.82±0.06
Cobalt	>10	O, Co	Co	7.7±0.0	6.05±0.87
	4.3	O, Co	Co	7.6±0.2	6.19±0.61
	2.9	O, Co	Co	7.7±0.1	6.02±0.35
Admixture	>10	O, W, C, Co	WC, W ₂ C, Co	13.8±0.1	0.74±0.08
	4.3	O, W, C, Co	WC, W ₂ C, Co	14.4±0.1	0.84±0.10
	2.9	O, W, C, Co	WC, W ₂ C, Co	13.9±0.1	1.14±0.05
Spray dryer	>10	C	WC, W, W ₂ C, Co	10.4±0.1	0.79±0.09
	4.3	C	-- ^b	--	0.79±0.11
	2.9	C, O	--	--	1.43±0.08
Chamfer grinder	>10	C, O, W, Co	WC	12.9±0.1	0.49±0.07
	4.3	O, C, W, Co	--	--	0.61±0.09
	2.9	C, O, W, Co	--	--	1.11±0.05

^a The order of constituents is from most to least abundant; O = oxygen, W = tungsten, C = carbon, WC = tungsten carbide, W₂C = tungsten carbide

^b Insufficient mass of aerodynamically size-separated material available for this analysis

3.1. Results of electron spin resonance studies

On an equal powder mass basis, ROS generation in the RAW 264.7 cell line increased as particle size decreased for all powders except Co powder, which did not generate radicals (see figure 1). When dose was normalized to surface area, ROS generation was independent of particle size, which suggests that surface chemistry may be important for radical generation (see figure 2). On both a mass and surface area basis, chamfer grinder particles generated the highest levels of radicals. This observation may be explained as follows: during sintering, the Co binder phase becomes liquid and, upon cooling, the WC grains become embedded in the binder to form CTC. Thus, the more intimate chemical bonds among surface metals in heterogeneous post-sintered alloy material relative to the weaker and less extensive physical bonds encountered among particles in pre-sintered material appears important for ROS generation.

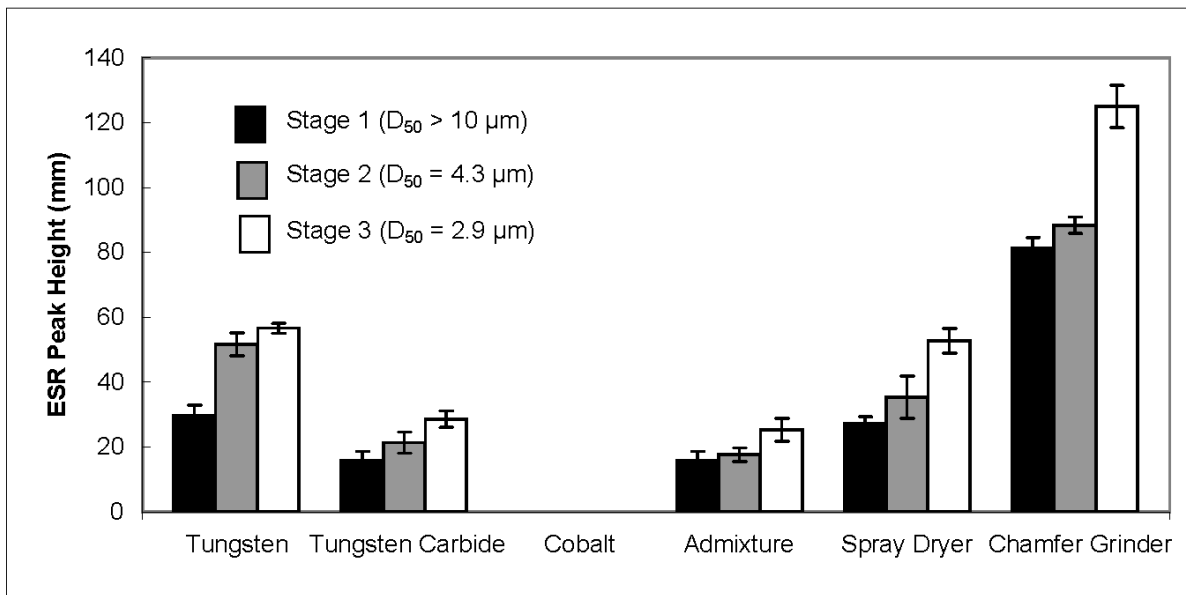


Figure 1. Plot of the influence of powder type on electron spin resonance (ESR) peak height in artificial extracellular lung fluid+RAW 264.7 cell line system for feedstock and process sampled powders. On a mass basis, all powders except cobalt metal exhibited a trend of increased radical production with decreased particle size. Cobalt metal powder alone did not generate radicals.

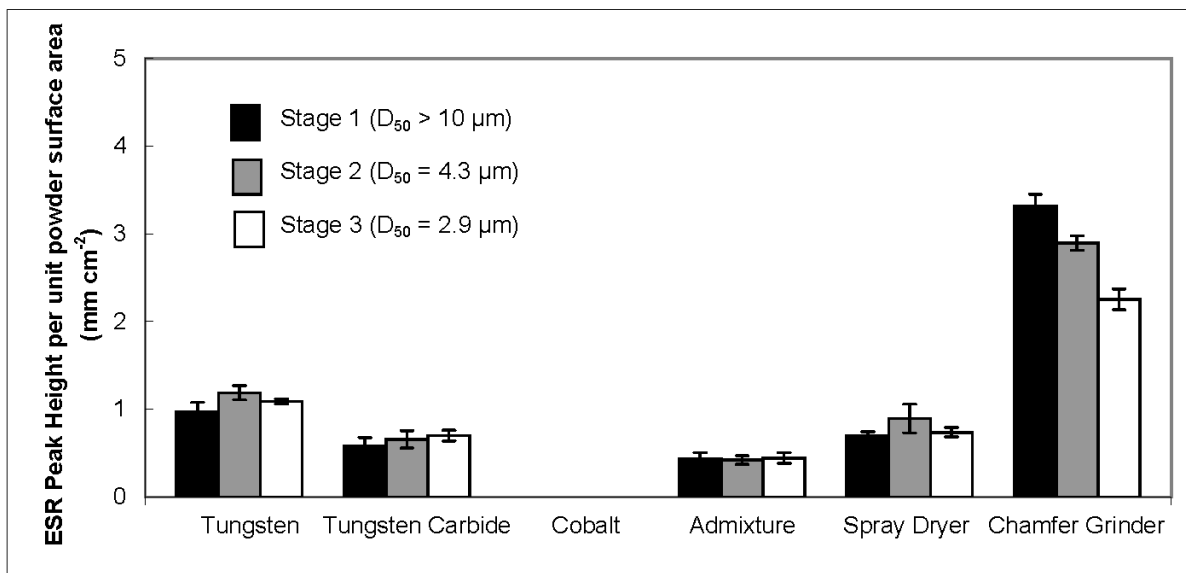


Figure 2. Plot of the influence of particle type on electron spin resonance (ESR) peak height in artificial extracellular lung fluid+RAW 264.7 cell line system for feedstock and process sampled powders. On a surface area basis, radical production was independent of particle size. Cobalt metal powder alone did not generate radicals.

3.2. Results of artificial lung fluid solubility studies

At the time of this writing, quantification of dissolved Co and W in samples of size-fractionated powders was in progress; therefore, only results from studies of bulk powders are reported herein. Figure 3 summarizes the total masses of metals dissolved in SUF without ABDC, glycine, or phosphate relative to SUF during a 72 hr exposure period. Dissolution of cobalt was higher ($p < 0.05$)

in the absence of phosphate suggesting that cobalt ions may have reacted with phosphate to form cobalt phosphate $\text{Co}_3(\text{PO}_4)_2$, a very insoluble salt.[21]

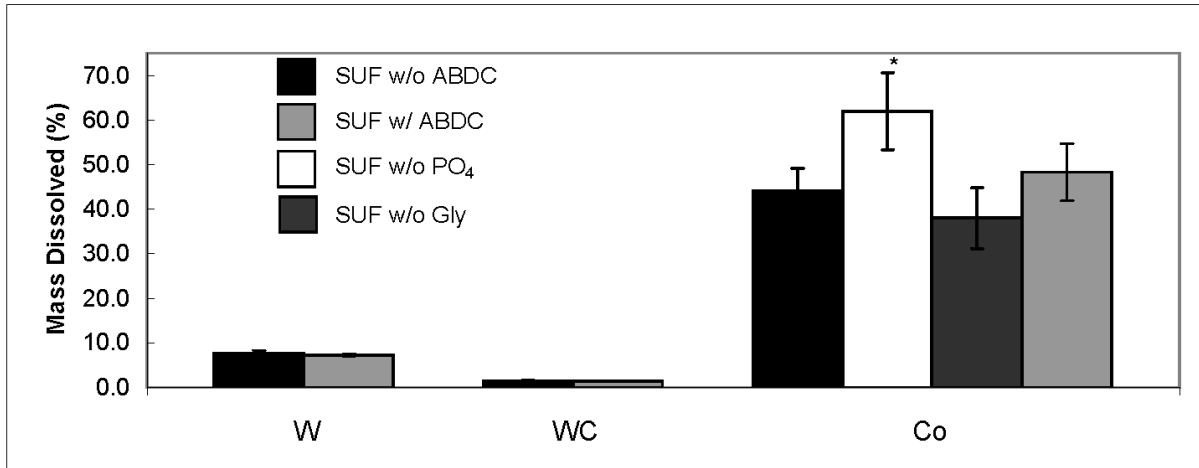


Figure 3. Total mass fraction of tungsten, tungsten carbide, and cobalt dissolved from artificial extracellular lung fluid (SUF) during a 72 hr exposure to solvent. * = mass fraction dissolved was significantly different ($p < 0.05$) from SUF.

Figure 4 summarizes the total masses of metals dissolved in PSF during a 24 hr exposure period. Dissolution of bulk W and WC powders were higher in the absence of ABDC. The phosphate concentration of PSF is similar to that of SUF; however, $\text{Co}_3(\text{PO}_4)_2$ did not form in PSF ($p=0.30$) which may be due its equilibrium solubility in acid.[23] The mass fraction of tungsten dissolved from W and WC powders was lower in PSF (24 hr exposure) relative to SUF (72 hr exposure). Some glass fibers are also known to be less soluble in macrophages relative to extracellular lung fluid.[24] These dissolution data for W and WC are consistent with results from microanalyses of lung tissue samples collected from CTC workers up to four years after cessation of exposure which demonstrated the presence of W but not Co in lung tissues.[25-27] Thus, W and WC particles that are deposited in lung and engulfed by macrophages may exhibit slower dissolution than if exposed to extracellular lung fluid alone for the same period.

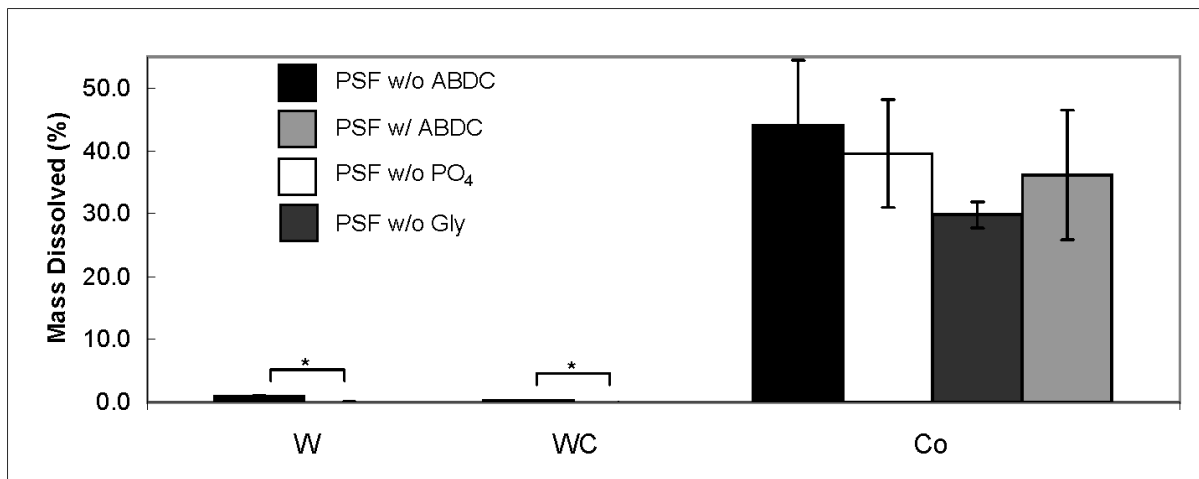


Figure 4. Total mass fraction of tungsten, tungsten carbide, and cobalt dissolved from artificial lung alveolar macrophage phagolysosomal fluid (PSF) during a 24 hr exposure to solvent. * = mass fraction dissolved was significantly different ($p < 0.05$) from PSF.

Collectively, the implication of these data for HMD is that the total exposure of the lung to ROS may be enhanced by retention of particles in macrophages. The extent of this effect will depend on the rate of physical clearance of engulfed particles from the lung by transport of macrophages up the muco-ciliary escalator, as compared to chemical dissolution and clearance of the particle constituents from the extracellular lung environment.

4. Summary

Size-dependent differences in ROS generation were observed for all study powders, except Co, which did not generate radicals in the cellular model. Chamfer grinder particles generated the highest levels of ROS in the RAW 264.7 cell line. Collectively, these results suggest an important role for particle physicochemical properties such as surface chemistry and bonding contact of Co and WC (i.e., pre- or post-sintered material) in generation of ROS. In artificial extracellular lung fluid and lung alveolar macrophage phagolysosome models, the rank order of dissolution from bulk feedstock powders was: Co > W > WC. The mass fractions dissolved were slower in PSF compared to SUF, suggesting that particles may persist longer in the lung if phagocytized and undergo dissolution than if cleared by chemical dissolution in the extracellular airway lining fluid. Results from studies of artificial lung fluid composition on observed dissolution rates indicate that careful attention to endogenous lung fluid constituents (e.g., phosphate) and exogenous model constituents (e.g., ABDC) is necessary when performing dissolution studies of W, WC, and Co powders.

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