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# Toxicity and oxidative stress response induced by nano- and micro-CoCrMo particles

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# SUPPLEMENTAL MATERIALS

## 1. Materials and Methods

**Particle Preparation and Characterization:** CoCrMo nanoparticles (nano-CoCrMo) were obtained via mechanical milling of the micro-CoCrMo particles. In brief, micro-CoCrMo particles were milled in 20 g/L of a heat shocked fetal bovine serum (FBS) solution (pH 7.0). The powders were ball milled for 2.5 hr at a ball-to-powder weight ratio of 3:2 in a high energy Spex 8000M mill (Metuchen, NJ) using zirconium oxide ceramic vials and balls. Before milling, the vial was fully filled with an inert gas (Argon) to avoid atmospheric contamination. After collection, samples were centrifuged in a Heraeus Megafuge at 25°C for 10 min with a centrifugal force of 8000G to remove most of the FBS and coarse particles. Fine particles in the dispersion were collected for this study. For cell culture studies, both nano- and micro-CoCrMo particles were prepared using our previous protocol [1] in a phosphate buffered saline (PBS) solution containing 10% FBS as a stabilization agent and sonicated using an Omni International Sonic Ruptor 250 Ultrasonic Homogenizer (Kennesaw, GA). A stock concentration of 5 mg/mL CoCrMo particles was prepared by sonication (2 min, 120 watts power output, frequency 20 kHz) in an ice bath, to minimize heating of the sample during particle dispersion. Dilute particle suspensions, ranging from 0.1 to 1000 μg/mL, were prepared in DMEM containing 10% FBS and used immediately on the day of each experiment. Note that from an occupational standpoint, exposure limits have been set for cobalt particles alone, but limits have yet to be defined for cobalt-containing alloys such as CoCrMo or WC-Co, which have been reported to cause similar types of pneumoconiosis following pulmonary exposure. Further, it is difficult to define an exact *in vitro* dosage parameter due to the variability and extent of MoM orthopaedic implant wear on an individual patient basis. Therefore, in this study, we intentionally encompassed a range of particle exposures, from 0.1 to 1000 μg/mL, to represent the potential range in CoCrMo exposure which may occur in occupational implant manufacturing settings and in orthopaedic patient implant wear particle exposures.

The particle size of nano-CoCrMo was analyzed using transmission electron microscopy (TEM) with a FEI Tecnai F20 field emission gun TEM operated at 200 kV and fitted with an Oxford Instruments ultrathin window ISIS energy dispersive X-ray (EDX) system. A 3.5 μL droplet of particle dispersion in 10% FBS was placed on a glow discharge-treated carbon support film (R1.2/1.3 Quantifoil MicroTools GmBH; measured hole diameter of 1.65 μm), blotted and plunge frozen in liquid ethane [2]. The particle size of micro-CoCrMo particles was characterized using scanning electron microscope (SEM). Secondary electron images were taken using a Hitachi SU8230 Ultimate Cold Field Emission operated at 2 keV, 8.2 mm working distance and employing an in-lens electron detector. Elemental composition was determined via EDX.

**CoCrMo Particle Assay Interference:** Prior to execution of the cell viability and oxidative stress assays, the potential interference of CoCrMo particles was examined under the experimental conditions. To test compatibility with the MTT-based cell viability assay, 200 μL of CoCrMo suspensions (0.1 to 1000 μg/mL in DMEM) was added to duplicate wells in a 96-well plate. The plate was briefly centrifuged (500 × g, 5 min) to pellet the particles at the bottom of the wells. The supernatant was then aspirated and 100 μL of plain (un-supplemented) DMEM was added to each well containing CoCrMo particles, along with 10 μL of MTT reagent. After 2 hr incubation at 37°C, 100 μL of solubilization solution was added to each well and the absorbance was determined at 570 nm. Any auto-reduction of the MTT dye reagent to formazan by the CoCrMo particles

themselves would have been detected as an increase in absorbance compared to the blank wells, containing only media, MTT dye reagent and solubilization solution. Similarly, for the oxidative stress assay, we tested whether the CoCrMo particles caused increased fluorescence of either DCF or DHE under our assay conditions. CoCrMo particle suspensions were plated and centrifuged in duplicate wells of a 96-well plate as described above. The supernatant was then aspirated and replaced with 100 μL of 10 μM DCF or DHE working solution prepared in PBS. Plates were incubated for 15 min in the dark and then the fluorescence intensity of each well was quantified every 5 min, up to one hour, at 520 nm for DCF or 620 nm for DHE, to identify any potential particle/dye interference compared to the blank (dye solution only) wells.

**Cell Culture and THP-1 Macrophage Differentiation:** BEAS-2B lung epithelial cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and maintained at 37°C and 5% $CO_2$. Human osteoblasts (h.FOB 1.19) were cultured in 1:1 Ham's F12/DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 0.3 mg/mL G418 and maintained at 37°C and 5% $CO_2$. Upon confluency, BEAS-2B and osteoblasts (OB) were rinsed with PBS and trypsinized, transferred to 15 mL tubes and centrifuged at 1200 rpm for 7 min to pellet. Cell pellets were re-suspended in appropriate media at the desired plating density of $1.5 \times 10^5$ cells/mL, transferred to a 96-well tissue culture plate and allowed to adhere overnight prior to the assay(s).

THP-1 monocytes were maintained in suspension culture in RPMI-1640 supplemented with 10% FBS, 1% penicillin-streptomycin and 0.05 mM beta-mercaptoethanol and maintained at 37°C and 5% $CO_2$. Upon confluency, THP-1 cells were transferred to 15 mL tubes and centrifuged at 125 × g for 5 min to pellet. The cell pellet was re-suspended in RPMI-1640 containing 10 ng/mL PMA, which induces THP-1 monocytes to undergo macrophage (M0) differentiation, and plated in a 96-well culture plate at the desired density of $1.5 \times 10^5$ cells/mL. After 24 hr, THP-1 to M0 differentiation was confirmed via examination of cell morphology using a light microscope [3, 4], where M0 cells underwent a signature change in morphology and became adherent to the culture dish.

## 2. Results

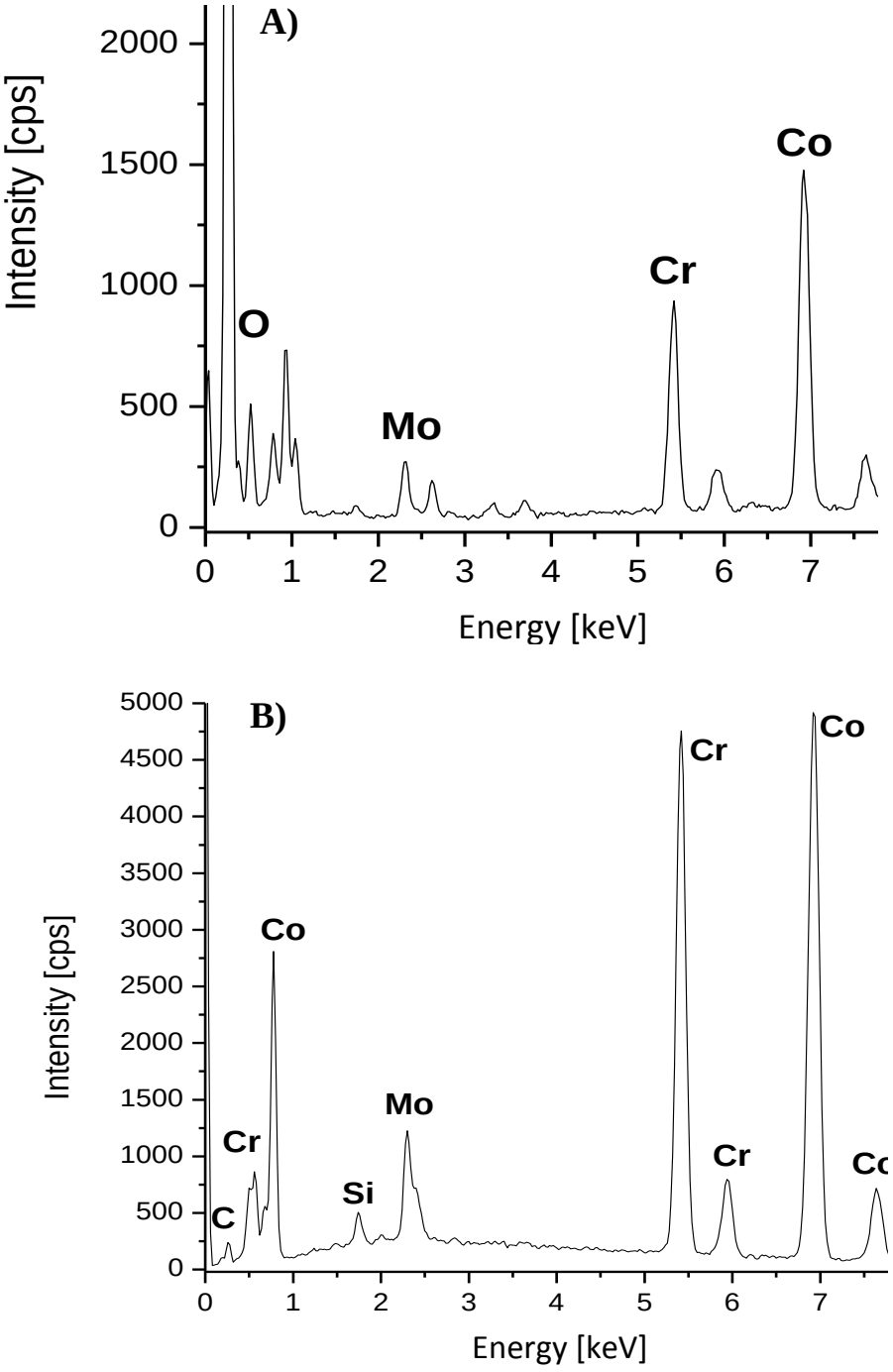


**Figure S1.** Representative EDX spectra of A) nano- and B) micro-CoCrMo particles.

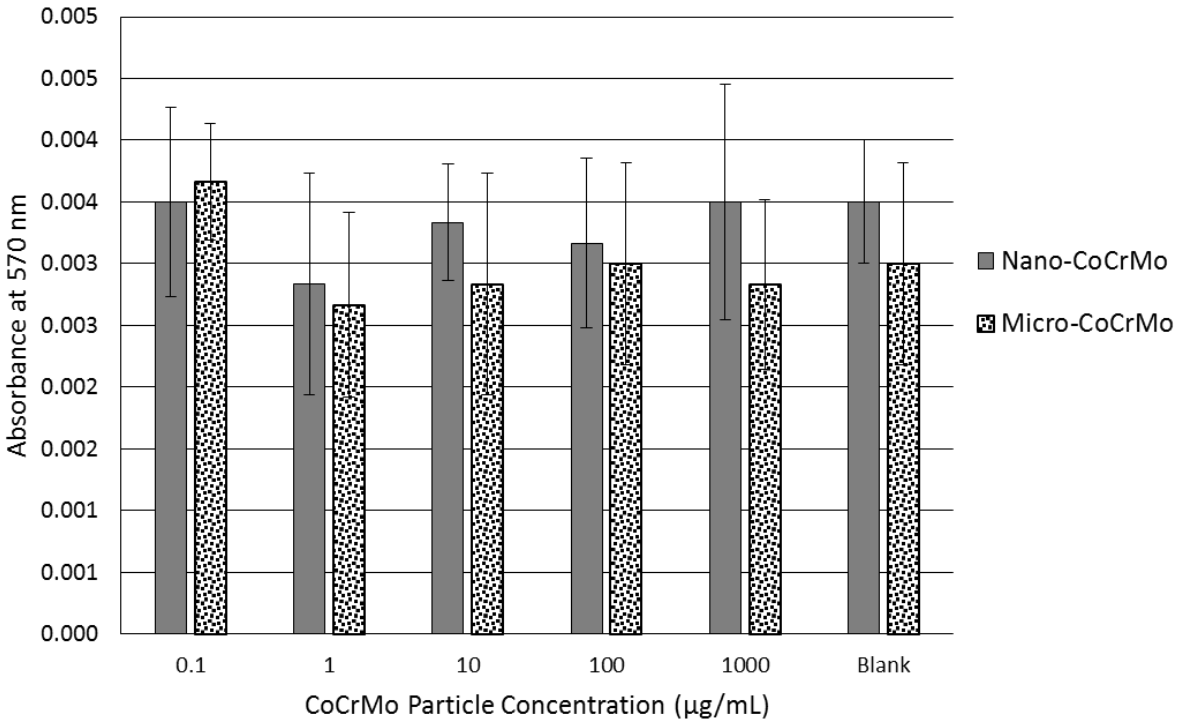


**Figure S2.** MTT assay interference tests in the presence of CoCrMo particles**.**

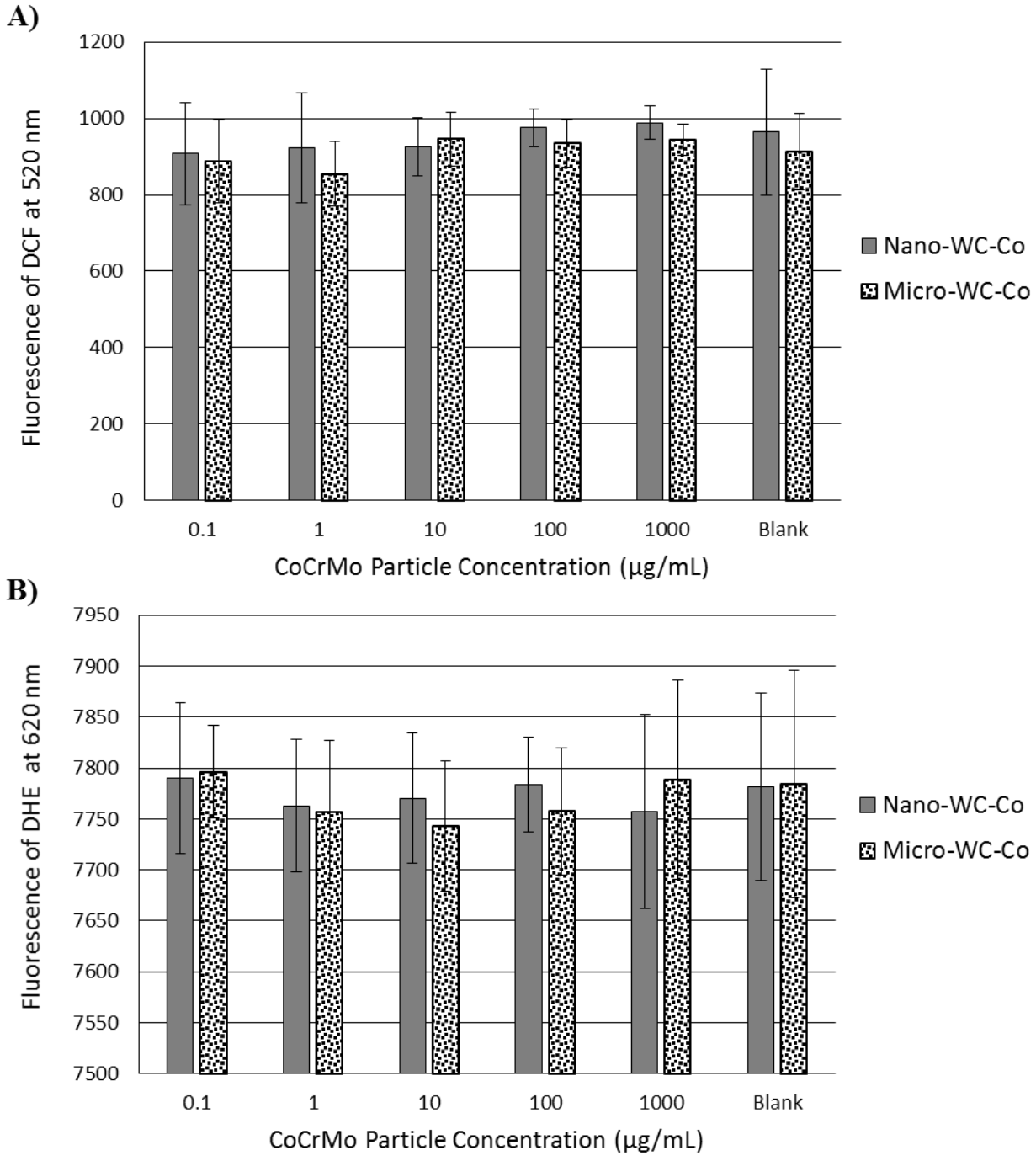


**Figure S3.** (**A**) DCF and (**B**) DHE assay interference tests in the presence of CoCrMo particles.

References:


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