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1 **IN VITRO ASSESSMENT OF COBALT OXIDE PARTICLE DISSOLUTION**
2 **IN SIMULATED LUNG FLUIDS FOR IDENTIFICATION OF NEW**
3 **DECORPORATING AGENTS**

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20
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25

26 **Abstract**

27 Inhalation of $^{60}\text{Co}_3\text{O}_4$ particles may occur at the work place in nuclear industry. Their low solubility may
28 result in chronic lung exposure to γ rays. Our strategy for an improved therapeutic approach is to enhance
29 particle dissolution to facilitate cobalt excretion, as the dissolved fraction is rapidly eliminated, mainly
30 in urine.

31 *In vitro* dissolution of Co_3O_4 particles was assessed with two complementary assays in lung fluid
32 surrogates to mimic a pulmonary contamination scenario. Twenty-one molecules and eleven
33 combinations were selected through an extensive search in the literature, based on dissolution studies of
34 other metal oxides (Fe, Mn, Cu) and tested for dissolution enhancement of cobalt particles after 1 to 28
35 days of incubation.

36 DTPA, the recommended treatment following cobalt contamination did not enhance $^{60}\text{Co}_3\text{O}_4$ particles
37 dissolution when used alone. However, by combining molecules with different properties, such as redox
38 potential and chelating ability, we greatly improved the efficacy of each drug used alone, leading for the
39 highest efficacy, to a 2.7 fold increased dissolution as compared to controls. These results suggest that
40 destabilization of the particle surface is an important initiating event for a good efficacy of chelating
41 drugs, and open new perspectives for the identification of new therapeutic strategies.

42

43 **Keywords:** cobalt oxide particles, bioaccessibility, dissolution and chelating agents, lung, *in vitro*.

44 **Introduction**

45 Cobalt (Co) is widely used in industrial processes for its magnetic properties, corrosion resistance, wear
46 resistance, and/or its strength at elevated temperatures (Barceloux, 1999). The γ -emitter ^{60}Co is a neutron
47 activation product formed from structural materials in nuclear reactors. During procedures such as
48 maintenance, ^{60}Co can be released as highly mobile and highly radiant oxide particles. Accidental
49 internal or cutaneous contamination of workers may thus arise (Davis et al., 2007; Le Guen and
50 Ansoberlo, 2005). In addition to the risk of exposure for workers in the nuclear industry, ^{60}Co has been
51 identified as a potential candidate for use in a “dirty bomb” (Rump et al., 2018). Such a device could
52 lead to the contamination of a great number of victims by fallout of radioactive dusts.

53 Cobalt oxide particles Co_3O_4 are poorly soluble compounds, classified as S (slow) by the International
54 Commission on Radiological Protection (ICRP) based on the transfer rate from lungs to blood following
55 pulmonary contamination (ICRP, 2016). Following inhalation of $^{57}\text{Co}_3\text{O}_4$ particles in baboons, only 40
56 % of initially deposited particles were cleared 6 months post-exposure (Kreyling et al., 1991). Short-
57 term clearance resulted from particle transfer to the larynx, whereas, longer-term clearance was due to
58 the translocation of dissociated Co to the blood. Once this dissolved Co fraction reaches the blood
59 compartment, it is rapidly eliminated, with a Co half-life of 12 hours. More precisely, a recent study in
60 rats has shown that 67.6% of cobalt administered as the soluble form, $^{60}\text{CoCl}_2$, is excreted in urine with
61 a half-life of 1.9 h, and 34.5% with a half-life of 45 h (Weber et al., 2012). The chemical toxicity of Co
62 has been found higher for soluble compounds than for Co_3O_4 particles (Ortega et al., 2014). However,
63 following inhalation of insoluble radioactive cobalt particles, long-term consequences such as lung
64 fibrosis or lung cancer could occur as a result of its radiological toxicity.

65 Whatever the route of internal contamination by $^{60}\text{Co}_3\text{O}_4$ particles ($^{60}\text{Co}_3\text{O}_4\text{P}$), the current
66 recommendation for the management of contamination is the intravenous administration of Ca-DTPA
67 (Diethylenetriamine-pentaacetic acid calcium trisodium salt hydrate) or EDTA
68 (Ethylenediaminetetraacetic acid disodium salt dihydrate), although inhalation of DTPA can also be
69 considered following lung contamination (ASN, 2008; REMM). Animal studies aiming at determining
70 the efficacy of various drugs, including DTPA, have been carried out essentially following systemic

71 contamination with soluble forms of cobalt (Fisher and Dunavant, 1978; Levitskaia et al., 2010a;
72 Levitskaia et al., 2011; Levitskaia et al., 2010b; Llobet et al., 1986, 1988; Zyllicz et al., 1975). Treatments
73 with both DTPA and EDTA increased cobalt excretion and decreased cobalt retention in several tissues
74 (Fisher and Dunavant, 1978; Llobet et al., 1986, 1988; Zyllicz et al., 1975). Some success has also been
75 obtained with molecules selected for their Co^{2+} -binding properties, such as N-acetyl-L-cysteine, D or L-
76 penicillamine, L-cysteine, trientine or glutathione (Levitskaia et al., 2010a; Levitskaia et al., 2011;
77 Levitskaia et al., 2010b; Llobet et al., 1986, 1988). Some of these molecules, namely D-penicillamine
78 and trientine, are also efficient treatments for copper detoxification in Wilson Disease (Delangle and
79 Mintz, 2012). Human cases of cobalt toxicity have been reported following high serum concentrations
80 of Co^{2+} resulting from leaching of Co-containing prostheses. Administration of N-acetyl-cysteine
81 significantly decreased Co^{2+} concentration in blood (Giampreti et al., 2016) whereas EDTA had only at
82 transient efficacy (Pazzaglia et al., 2011). Following inhalation of $^{60}\text{Co}_3\text{O}_4\text{P}$, the most likely scenario at
83 the work place, chelation by DTPA is expected to concern mainly the ionized form of cobalt, Co^{2+} ,
84 suggesting that DTPA treatment would have limited efficacy. Indeed, in case of inhalation of poorly
85 soluble compounds, such as plutonium oxides, only the solubilized fraction present in lung fluids was
86 accessible to DTPA (Gremy et al., 2010). To our knowledge, neither DTPA nor the other potential
87 decorporating agent efficacy have ever been tested after exposure to cobalt oxides.

88 Decorporation aims at increasing excretion and reducing the retention of the contaminant. The main
89 retention site following inhalation of cobalt particles is the lungs. Pulmonary clearance reflects a multi-
90 stage process, integrating the dissolution of the contaminant, the interactions with (biological) ligands
91 and the absorption into fluids. Inhaled particles are rapidly engulfed by macrophages, where they may
92 remain trapped for long periods. The determining process in the transfer of Co from lungs to blood has
93 been shown to be the intracellular dissolution of the Co particles in the macrophages (Kreyling et al.,
94 1993). The Co^{2+} ionic species generated by the dissolution process become bioavailable to chelating
95 drugs. Therefore, enhancing dissolution may represent the first step in the clearance of poorly soluble
96 particles, by increasing the fraction of the contaminant available to chelating drugs. To fulfill the
97 recommendations of the European directive 2010/63/EU, the principles of the “3Rs” (Replacement,
98 Reduction and Refinement) must be considered systematically at all times when animals are used for

99 scientific purposes. These principles were first proposed by Russell and Burch (Russell and Burch,
100 1959). They are currently proposed as the key strategies of a systematic framework aimed at achieving
101 human use of any drugs. A recent paper demonstrates the applicability of the 3Rs in the field of
102 radiotoxicology (Griffiths et al., in press), and particularly the use of adequate dissolution assays to
103 provide information on solubility and bioaccessibility of various contaminants.

104 Whereas extensive literature on the dissolution of other oxide particles exists, only a few studies have
105 been dedicated to the dissolution of $\text{Co}_3\text{O}_4\text{P}$ (Cho et al., 2012; Collier et al., 1992; Lundborg et al., 1992;
106 Ortega et al., 2014). Since the most likely scenario for accidental exposure at the work place is the
107 inhalation of cobalt particles, our approach consisted in the evaluation of various categories of molecules
108 for their efficacy in $\text{Co}_3\text{O}_4\text{P}$ dissolution. We successively tested: 1- molecules for which an efficacy to
109 decorporate soluble Co^{2+} has been previously described, 2- various known Co^{2+} -binding molecules and
110 3- metal-binding sulfur-containing pseudopeptides. We then broadened our tests to molecules known
111 for their ability to increase the dissolution of various metal oxides and to associations of molecules with
112 complementary properties.

113 To foresee the use of these molecules in *in vivo* experiments and ultimately in man, FDA approved or
114 non-toxic molecules were preferentially selected. The dissolution of a contaminant will depend on the
115 intrinsic physicochemical properties of this contaminant as well as physicochemical and physiological
116 properties of the biological environment, such as extra- and intra-cellular chemical constituents
117 (Hedberg et al., 2010; Kastury et al., 2017; Pelfrene et al., 2017; Staack et al., 2017; Stefaniak, 2010).
118 Thus, a large number of molecules was tested in media mimicking lung fluids, Gamble's and ALF
119 (Artificial Lysosomal Fluid), using a simple dissolution model in solution. Then, the molecules selected
120 for their efficacy on $\text{Co}_3\text{O}_4\text{P}$ dissolution were tested using a biphasic dynamic assay recently developed
121 to assess the bioavailability of actinides (Van der Meeren et al., 2019). This assay was used to better
122 mimic the transfer of bioavailable species from a retention compartment (agarose gel) to a dynamic
123 phase (transfer compartment), where the drugs were added. Relevant physiological conditions were
124 obtained by introducing various components both in the static and dynamic phases. In addition, the data
125 obtained from this assay with actinides was found to show good correlation between dissolution data
126 and urinary excretion following contamination in rat (Griffiths et al., 2016). In this work, this assay was

127 used as a first assessment to predict the fraction of $\text{Co}_3\text{O}_4\text{P}$ that could potentially be transferred from
128 retention compartments to the blood, as well as the Co^{2+} - fraction available to chelating drugs.
129 $\text{Co}_3\text{O}_4\text{P}$ with sizes representative of those produced in nuclear reactors were chosen (Davis et al., 2007;
130 Ortega et al., 2014). Finally, since a similar chemical behavior can be expected *in vitro* whatever the
131 isotope, the present studies were conducted with the stable isotope, ^{59}Co .
132 Altogether, the use of *in vitro* assays allowed the testing of a great number of molecules thus opening
133 new perspectives for novel therapeutic approaches. This study could represent a benchmark for the
134 development of similar approaches, to test the dissolution of other relevant metal oxide particles.

135

136 **Materials and Methods**

137 **Reagents**

138 Nitric acid and hydrochloric acid of high purity grade (HNO_3 65% Suprapur and HCl 35% Suprapur)
139 were purchased from VWR. Co^{2+} solution for the ICP-MS calibration curves were prepared from
140 standard solutions ($1 \text{ mg} \cdot \text{L}^{-1}$), which were purchased from SPEX (SPEX Certiprep Company).

141 Diethylenetriamine-pentaacetic acid (DTPA Ca), 1-Hydroxyethylidenediphosphonic acid (HEDP),
142 diphenylethylene diamine (DPEN), triethylene tetramine (TRIEN), N-Acetyl-L-cysteine (NAC), L-
143 cysteine, Ethylenediaminetetraacetic acid (EDTA), L-Glutathione (GSH), DL-Dithiothreitol (DTT), L-
144 ascorbic acid, pyrocatechol, L-histidine, Imidazole, o-Phenylenediamine (OPD), 2,6-
145 Pyridinedicarboxylic acid (DIPIC), Oxalate, Deferoxamine mesylate salt (DFOB), Deferiprone (DFP),
146 Ferrichrome, L-ornithine, Thiamine hydrochloride, Enterobactin, Tannic acid and Desferrithiocine
147 (DFT) were purchased from Sigma-Aldrich. Ca-DTPA from PCA (Pharmacie Centrale des Armées,
148 France) was used for the dynamic biphasic assay.

149 To enhance the relevance of the study, whenever possible and for already clinically used molecules, the
150 concentration of molecules were chosen according to the recommendations for human use. As an
151 example the recommended for intravenous daily administration of DTPA is 15-30 $\mu\text{mole/kg}$ depending
152 of the body mass, which corresponds to roughly 500 μM in blood at the time of administration.

153

154 **Cobalt oxide particles preparation and characterization**

155 Cobalt particles ($\text{Co}_3\text{O}_4\text{P}$) were obtained from Sigma-Aldrich. The particle size (370 nm diameter) was
156 chosen to be representative of particles involved in an incident at the work place for which a mean
157 diameter was estimated to be of 410 nm (Davis et al., 2007).

158 $\text{Co}_3\text{O}_4\text{P}$ were suspended in deionized water to prepare stock solutions with a cobalt concentration of 8
159 mg mL^{-1} ; suspensions were then sonicated for 15 min with an Autotune sonicator (Fisher Scientific;
160 Illkirch, France) operated at 750 W, and stored at $-20\text{ }^\circ\text{C}$ until use as previously described (Ortega et al.,
161 2014; Uboldi et al., 2016).

162 $\text{Co}_3\text{O}_4\text{P}$ characterization after 28 days of incubation was performed using Transmission Electron
163 Microscopy (TEM) analysis. Eight μl of particle suspension in ascorbate and DFOB were deposited on
164 carbon film – 200 mesh copper grids, and examined in a Tecnai 12 G² Biotwin scanning transmission
165 electron microscope (Thermo Fisher Scientific, FEI Company, Eindhoven, the Netherlands) using an
166 accelerating voltage of 100 kV and coupled with a Megaview III (Olympus Soft imaging Solutions
167 GmbH, Münster, Germany). Particle size was analyzed using the Image J software
168 (<https://imagej.nih.gov/>, v.1.52n).

169

170 **Metal-binding sulfur pseudopeptides**

171 Three metal-binding sulphur pseudopeptides were tested in the study. Their structure is based on
172 polyaminocarboxylate scaffolds (NTA, EDTA or DOTA) grafted by 3 (for NTA) or 4 (for EDTA and
173 DOTA) cysteine amide (Cys-NH_2) residues (See Scheme S1). The synthesis of $\text{NTA}(\text{CysNH}_2)_3$ is
174 reported in the literature (Pujol et al., 2011) and the two other derivatives, specifically designed for the
175 study, $\text{EDTA}(\text{CysNH}_2)_4$ and $\text{DOTA}(\text{CysNH}_2)_4$ were obtained using similar synthetic procedures.

176 Their affinity for Co^{2+} was measured in competition experiments with NTA following the decrease of
177 the intense UV-Vis absorption of the Co^{2+} -thiolate complexes upon NTA addition. (See Supplementary
178 Material).

179

180

181

182 **Preparation of incubation media**

183 All the solutions were prepared with deionized ultrapure water. Four media were used: 140 mM NaCl,
184 5 mM KCl (pH 5.6), HEPES 20 mM, NaCl 100 mM (pH 7.4), Gamble's solution (pH 7.4) and Artificial
185 Lysosomal Fluid (ALF, pH 4.5). Gamble's and ALF were prepared according to Marques et al.
186 (Marques et al., 2011). Buffers were filtered through a 0.22 µm cellulose membrane filter and degassed
187 half an hour before use.

188

189 **Dissolution assay in solution**

190 1.2 mL of Co₃O₄P suspensions (1 mM Co) were prepared in medium containing various ligands at 500
191 µM concentration leading to ligand/cobalt ratios of 0.5, unless mentioned, and incubated at 37°C under
192 stirring (37 rpm) for 1 to 28 days according to previous studies (Ortega et al., 2014; Weber et al., 2012).
193 At each collection time, an aliquot of 500 µL of each sample was centrifuged 10 minutes at 16 000 g
194 and the supernatant was filtered through a 0.1 µm syringe filter to eliminate remaining particles (Ortega
195 et al., 2014). 100 µL of supernatant were diluted in HNO₃ 5% and analyzed by inductively coupled
196 plasma mass spectrometry (ICP-MS, NexION 350X, PerkinElmer) equipped with an auto sampler and
197 a crossflow nebulizer. The instrument was calibrated using 0.1, 0.5, 1, 2, 5, 10 and 20 parts per billion
198 (ppb) of cobalt. To determine the amount of total cobalt in each sample, an aliquot of 100 µl of particles
199 solution was collected at the beginning of the incubation, and analyzed by inductively coupled plasma
200 mass spectrometry (ICP-MS after acid digestion with 30% HCl followed by 65% HNO₃, as previously
201 described (Ortega et al., 2014). Each experiment was performed at least in triplicate, each replicate being
202 prepared from a different cobalt solution. The results were expressed as % of solubilized cobalt
203 (solubilized cobalt vs total cobalt).

204

205 **Dissolution using the biphasic dynamic assay**

206 Agarose gels were prepared by dissolution of agarose powder type IIA (Sigma) as a 2.5% solution in
207 physiological saline (140 mM NaCl, 5 mM KCl, pH 5.6), ALF or Gamble's solutions as described (Van
208 der Meeren et al., 2019). The agarose solution was melted by heating in a microwave, and Co₃O₄P were
209 added under gentle stirring at the final Co concentration of 0.13 mM in gels. A ligand:cobalt ratio of 3.8

210 was used whatever the molecules, alone or in combination. The agarose solution was then distributed in
211 24 well-culture dishes, representing the static phase. After the gel had hardened, the dynamic phase was
212 added in the wells. The plates were then incubated at 37°C in a 5% CO₂ atmosphere on an orbital shaker.
213 At the end of the experiment, the samples were collected, digested with concentrated ultrapure
214 chlorhydric and nitric acid. Digested samples were diluted in HNO₃ 1% and analyzed using ICP-MS
215 (NexION 350X, PerkinElmer or ICAPO Thermo). The calibration was conducted with 0.1, 0.5, 2, 10,
216 20, 50 µg/L standards prepared in HNO₃ 1%. Gallium (Analab) was used as internal standard. All
217 conditions were done in triplicate.

218 The principle of the biphasic assay is based on the hypothesis that bioavailable ions would migrate into
219 the dynamic fluid phase, while particles or complexes formed with biological ligands would remain
220 trapped in the static phase. The validation of our assay was done using actinides (Griffiths et al., 2016;
221 Van der Meeren et al., 2019). To ensure that it was also applicable to cobalt, we compared the
222 transferability of different chemical forms of cobalt incorporated in the gel. Our results (shown in Figure
223 S1) demonstrate the low retention of CoCl₂ in the static phase (20% in 7 days) as opposed to the high
224 retention of particles (> 99% on 7 days). In addition, to make sure that only Co²⁺ ions were transferred
225 in the dynamic phase following the dissolution of particles, the dynamic phase was filtered through 100
226 nm diameter pore filters. Cobalt concentrations measured in the filtered samples did not differ from
227 those of the non-filtered samples (data not shown).

228

229 **Statistical Analysis**

230 Results are expressed as mean ± SD. One-way ANOVA followed by Dunnett's multiple comparisons
231 test were performed using GraphPad Prism version 8 (GraphPad Software; La Jolla, CA, USA).

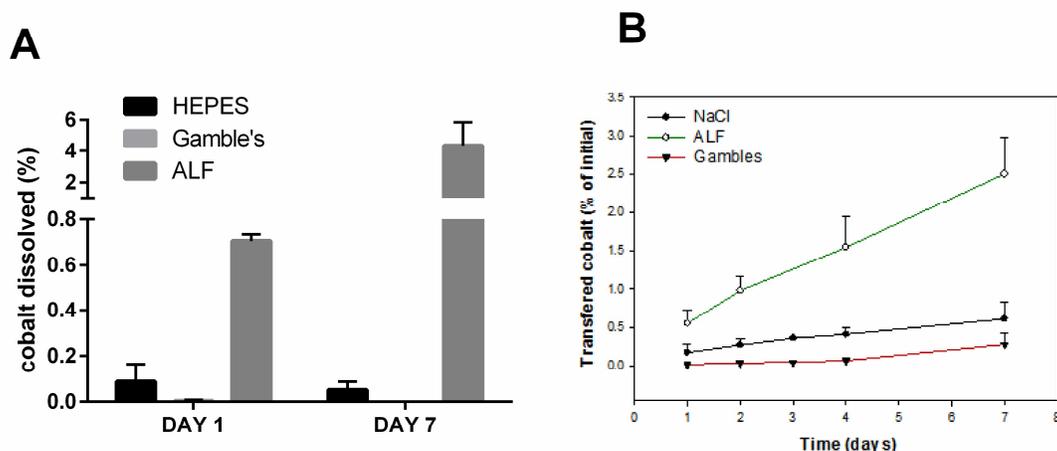
232

233 **Results**

234 **1/Intrinsic Co₃O₄P dissolution rate in NaCl, Hepes, ALF and Gamble's.**

235 We first evaluated the intrinsic dissolution properties of Co₃O₄P in HEPES and NaCl/KCl, for a 7-day
236 incubation period. Next, Gamble's solution and ALF were used, as the common surrogate fluids of the

237 main lung retention compartments. Gamble's solution mimics the interstitial and airway lining fluids,
 238 whereas ALF simulates intracellular conditions in phagocytes, such as macrophages (Marques et al.,
 239 2011).
 240 A low solubility of $\text{Co}_3\text{O}_4\text{P}$ was observed in the HEPES medium for which no interaction/complexation
 241 with ligands occurred. Less than 0.2 % of Co^{2+} was released over the 7-day incubation period (Figure
 242 1A). Dissolution was even lower in Gamble's, with less than 0.02% cobalt dissolution over 7 days. A
 243 significantly higher dissolution was observed in ALF, with 0.7 % dissociated cobalt after a one-day
 244 incubation period and up to 4.3% after 7 days (Figure. 1A). Similar results were obtained with the
 245 biphasic dynamic assay although a lower dissolution was measured in ALF (2.6% in 7 days) (Figure
 246 1B). Both the presence of citrate and low pH could explain the higher dissolution in ALF.



247

248 **Figure 1: Dissolution of $\text{Co}_3\text{O}_4\text{P}$.**

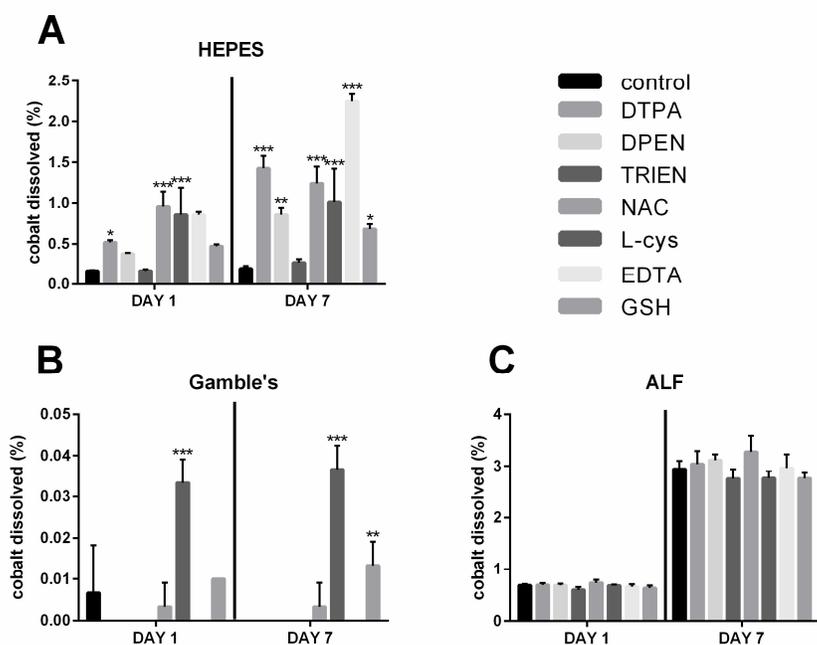
249 $\text{Co}_3\text{O}_4\text{P}$ dissolution was assessed in solution. (A), $\text{Co}_3\text{O}_4\text{P}$ particles were incubated 1 day and 7 days,
 250 in HEPES, Gamble's and ALF. Results are expressed as mean % \pm SD of solubilized Co versus total
 251 cobalt. In the two-phase dynamic assay, (B), $\text{Co}_3\text{O}_4\text{P}$ were included in gels prepared in NaCl/KCl, ALF
 252 or Gamble's. Results are expressed as mean % \pm SD of cumulative Co measured in the dynamic phase
 253 versus initial Co in the gel. Each experimental condition was done in triplicate and repeated 3 times.
 254

255 **2/ Molecules showing Co^{2+} -decorporating efficacy: effect on $\text{Co}_3\text{O}_4\text{P}$ dissolution**

256 Various molecules, all being Co^{2+} - ligands with different affinities (Table S1), have shown some
 257 efficacy for decorporation of soluble forms of cobalt in rats (Levitskaia et al., 2011; Levitskaia et al.,
 258 2010b; Llobet et al., 1986, 1988). However, none of these molecules have been tested for decorporation
 259 of poorly soluble $\text{Co}_3\text{O}_4\text{P}$. Therefore, using the dissolution assay in solution, we evaluated the ability of

260 these ligands to increase $\text{Co}_3\text{O}_4\text{P}$ dissolution in the lung fluids surrogates Gamble's and ALF, as well as
 261 in HEPES. The efficacy of the different drugs N-acetyl-L-cysteine (NAC), EDTA, L-cysteine (L-cys),
 262 glutathione (GSH), N,N-bis (2-aminoethyl) -1,2-ethanediamine dihydrochloride (Trien) and D-
 263 penicillamine (D-PEN) was assessed and compared to that of DTPA, as DTPA is currently the
 264 recommended treatment in case of accidental internal contamination with $\text{Co}_3\text{O}_4\text{P}$ (ASN, 2008;
 265 Giampreti et al., 2016).

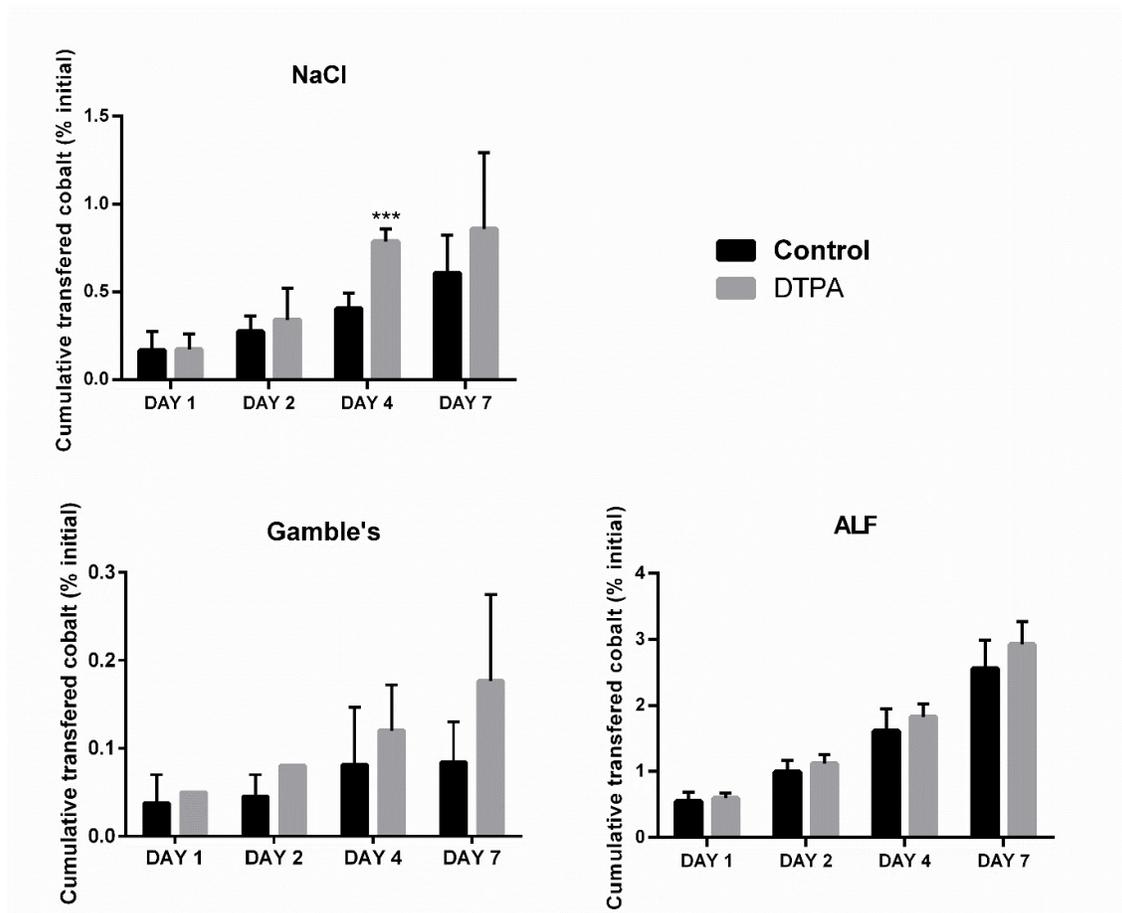
266 In HEPES, a statistically significant increase in $\text{Co}_3\text{O}_4\text{P}$ dissolution was observed at day 7 with all the
 267 tested molecules except TRIEN (Figure 2A). The highest efficacy was obtained with EDTA leading to
 268 2.2% dissolution ($p < 0.001$) at day 7. In contrast, none of the tested molecules induced a statistically
 269 significant increase in $\text{Co}_3\text{O}_4\text{P}$ dissolution in Gamble's except the two sulphur-containing molecules, L-
 270 cys and to a lower extent GSH (Figure 2B). However, the percentage of $\text{Co}_3\text{O}_4\text{P}$ dissolution remained
 271 very low ($< 0.05\%$). In ALF, no increase in particle dissociation was observed whatever the drug tested
 272 (Figure 2C) as compared to the untreated controls.



273

274 **Figure 2: Effect of Co^{2+} -decorporating agents on $\text{Co}_3\text{O}_4\text{P}$ dissolution.**
 275 $\text{Co}_3\text{O}_4\text{P}$ were incubated for 1 or 7 days in HEPES, Gamble's and ALF. At the end of the incubation, Co
 276 concentration was measured in the supernatant. Results are expressed as mean % \pm SD of dissolved Co/
 277 total cobalt. Each experimental condition was done in triplicate. Statistically significant differences
 278 from control were determined by one-way ANOVA followed by Dunnett's multiple comparisons test: * p
 279 < 0.05 , ** $p < 0.01$, *** $p < 0.001$.

280 Experiments using the dynamic biphasic assay (Figure 3) confirmed the absence of significant increase
281 in $\text{Co}_3\text{O}_4\text{P}$ dissolution with DTPA in the lung fluid surrogates Gamble's and ALF. A slight and transient
282 enhanced dissolution was seen in the presence of DTPA as compared to non-treated controls in NaCl
283 ($p < 0.001$) at day 4.



284

285 **Figure 3: Effect of DTPA on the dissolution of $\text{Co}_3\text{O}_4\text{P}$ assessed in the two-phase dynamic assay.**
286 $\text{Co}_3\text{O}_4\text{P}$ were included in the static retention phase (agarose gels) prepared in NaCl, Gamble's or ALF.
287 DTPA 500 μM was added in the dynamic phase. Results are expressed as mean % \pm SD of cumulative
288 cobalt transferred in the dynamic phase/initial cobalt in the gel. Each experimental condition was done
289 in triplicate and repeated 1 to 3 times. *** $p < 0.001$.
290

291 The contrasting results obtained using both assays in the non-physiologic buffers and in the lung
292 surrogates (Gamble's and ALF) highlight the importance of using biologically-relevant media for
293 dissolution studies, as also previously reported by others (Kastury et al., 2017).

294

295

296 **3/ Molecules with reported properties for cobalt chelation or for metal oxide particles dissolution**

297 The absence of effect of the above tested molecules on $\text{Co}_3\text{O}_4\text{P}$ dissolution in lung fluid surrogates is
 298 likely to predict a low decorporation efficacy of those drugs following pulmonary contamination with
 299 $\text{Co}_3\text{O}_4\text{P}$.

300 Therefore, from an exhaustive review of the literature summarized in Table 1, we selected various
 301 molecules with metal chelating properties as well as molecules enhancing other metal oxide particle
 302 dissolution.

303 **Table 1:** Literature review of molecules involved in metal oxide dissolution

Acting molecule	Metal (form)	Chemical effect	Reference
Ascorbate	Fe (Fe_2O_3 , Fe_3O_4) Mn oxides	Redox, chelation Reductive dissolution	Huang et al. 2017 Joseph et al. 1996 Afonso et al. 1990 Suter et al. 1991 Panas et al. 1996 Stone et al. 1984
Ascorbate + EDTA	Fe (Fe_2O_3 , Fe_3O_4)	Auto accelerated reductive dissolution	Afonso et al. 1990
Ascorbate + oxalate	Fe (Fe_2O_3 , Fe_3O_4)	Reductive dissolution and chelation	Afonso et al. 1990
Catechols	Mn oxides	Reduction and chelation	Stone et al. 1984
Citrate	Fe (Fe_2O_3)	pH	Joseph et al. 1996
Combination CEA (citrate, ascorbate, EDTA)	Fe ($\text{Fe(III)}_2\text{O}_3$)	Ligand assisted reductive dissolution	Joseph et al. 1996 Anathan et al. 2003
DFOB	Co (CoOOH) Fe (oxides) Fe (Cr(III)-Fe(III)- (oxy)hydroxides)	Redox Chelation	Bi et al. 2010 Kraemer. 2004 Saad et al. 2017
DFOB + oxalate	Fe (Cr(III)-Fe(III)- (oxy)hydroxides) Al (Fe_3O_4)	Redox, chelation	Saad et al. 2017 Cervini-Silva et al. 2002
DIPIC (2,6- pyridinedicarboxylate)	Co (Co(II) , Co(III))	Chelation	Yang et al. 2002
enterobactin	Fe (Fe-OOH)	Ligand metal affinity and adsorbtion	Dubbin et al. 2017
Ferrichrome	Fe (Fe-OOH)	Ligand metal affinity and adsorbtion	Dubbin et al. 2017
HEDP	Co (II)	Ligand	Jurisson et al. 1983
Histidine	Co (II)	Ligand	Bornhost et al. 2000
Oxalate	Fe (oxides)	Redox, chelation	Kraemer. 2004 Panas et al. 1996 Zinder et al. 1986
phenolic reductants (catechol)	Fe (FeOOH , Fe_2O_3)	redox	Lakind et al. 1989
Tannic acid	Cu (CuO NPs)	chelation	Zhao et al. 2017

304

305 A total of 32 assays were carried out using the dissolution assay in solution for efficacy testing of a total
306 of 21 molecules and 11 different combinations. This screening was done in Gamble's and ALF given
307 the lack of consistency between results obtained in HEPES saline solution and lung fluid surrogates.
308 Co^{2+} was measured at days 1 and 7 (Table 2). For ALF, in the absence of added molecules, the percentage
309 of dissolution differed between experiments (from 0.57 to 0.85% at day 1 and from 2.9 to 4.9% at day
310 7). Therefore, to allow a comparative evaluation of dissolution efficacy in ALF, we used the percentage
311 of dissolution measured in the presence of the molecule tested divided by the percentage of dissolution
312 observed in the related control, to calculate a ratio. This calculation mode was not necessary for results
313 obtained in Gamble's, because of lower day variability. Thus, results obtained in Gamble's were
314 expressed as % of solubilized cobalt/ total cobalt content.

315 The tested molecules were classified in different categories: Co^{2+} - ligands, high SH-containing ligands
316 and molecules enhancing metal oxide dissolution. Association of molecules from these categories were
317 also tested (Table 2). Our results will be described in the following paragraphs, according to the category
318 of the tested molecules.

319
320
321

Table 2: Dissolution of Co_3O_4P in the presence of various molecules assessed in Gamble's and ALF using the dissolution assay in solution.

Ligand	GAMBLE'S		ALF	
	% dissolved Co/total Co		fold increase dissolution treated/untreated	
	(mean % \pm SD)		(mean ratio \pm SD)	
	DAY 1	DAY 7	DAY 1	DAY 7
0	$\leq 0.1\%$	$\leq 0.1\%$	1.00	1.00
DTPA	$\leq 0.1\%$	$\leq 0.1\%$	1.03 \pm 0.02	1.00 \pm 0.05
COBALT LIGANDS				
DIPIC	$\leq 0.1\%$	$\leq 0.1\%$	1.06 \pm 0.03	1.07 \pm 0.13
HEDP	$\leq 0.1\%$	$\leq 0.1\%$	0.91 \pm 0.01	1.01 \pm 0.04
Histidine	$\leq 0.1\%$	$\leq 0.1\%$	0.82 \pm 0.09	1.00 \pm 0.13
HIGH -SH LIGANDS				
DOTA(CysNH ₂) ₄	$\leq 0.1\%$	$\leq 0.1\%$	1.14 \pm 0.01	0.90 \pm 0.02
EDTA(CysNH ₂) ₄	$\leq 0.1\%$	$\leq 0.1\%$	1.11 \pm 0.03	0.89 \pm 0.01
NTA(CysNH ₂) ₃	$\leq 0.1\%$	$\leq 0.1\%$	1.13 \pm 0.07	1.06 \pm 0.11
MOLECULES ENHANCING METAL OXIDE DISSOLUTION				
Asc	0.11% \pm 0.01	$\leq 0.1\%$	1.95 \pm 0.09	1.41 \pm 0.03
Asc + EDTA	0.88% \pm 0.13	0.91% \pm 0.12	2.21 \pm 0.16	1.38 \pm 0.09
Asc + oxalate	$\leq 0.1\%$	$\leq 0.1\%$	2.41 \pm 0.10	1.65 \pm 0.13
DFOB	$\leq 0.1\%$	$\leq 0.1\%$	1.24 \pm 0.04	1.38 \pm 0.04
DFOB + oxalate	$\leq 0.1\%$	$\leq 0.1\%$	1.21 \pm 0.06	1.50 \pm 0.10
DFP	$\leq 0.1\%$	$\leq 0.1\%$	1.28 \pm 0.04	1.11 \pm 0.04
DFT	ND	ND	1.03 \pm 0.01	1.32 \pm 0.20
Enterobactin	0.34% \pm 0.03	0.47% \pm 0.06	1.32 \pm 0.09	1.16 \pm 0.06
Ferrichrome	$\leq 0.1\%$	$\leq 0.1\%$	0.97 \pm 0.01	1.16 \pm 0.40
Oxalate	$\leq 0.1\%$	$\leq 0.1\%$	0.95 \pm 0.03	1.25 \pm 0.07
Pyrocatechol	$\leq 0.1\%$	$\leq 0.1\%$	1.39 \pm 0.05	1.26 \pm 0.05
Tannic acid	ND	ND	1.78 \pm 0.01	1.27 \pm 0.10
NEW TESTS OR COMBINATIONS				
Asc + DFOB	0.16% \pm 0.03	0.16% \pm 0.01	1.87 \pm 0.13	1.59 \pm 0.16
Asc + DOTA(CysNH ₂) ₄	0.55% \pm 0.04	0.61% \pm 0.01	1.97 \pm 0.06	1.27 \pm 0.06
Asc + DTPA	0.55% \pm 0.39	0.54% \pm 0.36	2.07 \pm 0.26	1.45 \pm 0.14
Asc + EDTA(CysNH ₂) ₄	0.28% \pm 0.04	0.33% \pm 0.01	1.81 \pm 0.32	1.08 \pm 0.09
Asc + EDTA Na + OPD	0.94% \pm 0.08	0.99% \pm 0.17	2.11 \pm 0.07	1.30 \pm 0.01
DIPIC + Hist	$\leq 0.1\%$	$\leq 0.1\%$	0.84 \pm 0.04	1.02 \pm 0.01
DIPIC + L-ornithine	$\leq 0.1\%$	$\leq 0.1\%$	0.90 \pm 0.01	1.14 \pm 0.05
DIPIC + OPD	$\leq 0.1\%$	$\leq 0.1\%$	1.95 \pm 0.09	1.18 \pm 0.05
DIPIC + Thiamine	$\leq 0.1\%$	$\leq 0.1\%$	1.06 \pm 0.04	1.27 \pm 0.02
DTT	ND	ND	1.14 \pm 0.03	1.22 \pm 0.13
Imidazole	$\leq 0.1\%$	$\leq 0.1\%$	0.91 \pm 0.03	1.10 \pm 0.13
L-cysteine + EDTA	$\leq 0.1\%$	$\leq 0.1\%$	0.91 \pm 0.05	0.85 \pm 0.09
L-ornithine	$\leq 0.1\%$	$\leq 0.1\%$	0.88 \pm 0.00	1.10 \pm 0.11
OPD	$\leq 0.1\%$	$\leq 0.1\%$	1.96 \pm 0.09	1.14 \pm 0.10
Thiamine	$\leq 0.1\%$	$\leq 0.1\%$	0.94 \pm 0.01	1.14 \pm 0.13

322
323

In Gamble's, results are expressed as mean % \pm sd (Co^{2+} / total cobalt content). Each experimental condition was done in triplicate and repeated 1 to 11 times. Percentages above 0.1 are shown in red and bold characters. In

324 ALF, to allow a comparative evaluation of dissolution efficacy, we used the percentage of dissolution measured
325 in the presence of the molecule tested divided by the percentage of dissolution observed in the related control, to
326 calculate a ratio. Results are expressed as mean \pm sd. Ratios above 1.3 are shown in red and bold characters. ND:
327 not determined
328

329 **3.1/ Effect of cobalt ligands and SH-rich ligands on Co₃O₄P dissolution**

330 The first category of selected molecules included cobalt ligands listed in Table 1. Both histidine, DIPIC
331 (2,6-pyridinedicarboxylate) and HEDP (1-Hydroxyethylidenediphosphonic acid) are known Co²⁺-
332 ligands (Bornhorst and Falke, 2000; Jurisson et al., 1983; Yang et al., 2002). In our hands, these
333 molecules had no influence on Co₃O₄P dissolution neither in Gamble's nor in ALF (Table 2).

334

335 The second category of chosen molecules were SH-rich ligands, since sulfur-containing molecules, i.e
336 L-cys and NAC induced a low but significant increase in Co₃O₄P dissolution in Gamble's (Figure 2).

337 We synthesized sulphur pseudopeptides bearing a larger number of sulphur atoms in their chelating
338 scaffolds, in an attempt to enhance the dissolution effect observed in Gamble's with the monothiol

339 molecules. These sulfur-containing pseudopeptides are polydentate ligands built on NTA, EDTA and
340 DOTA, grafted with three to four cysteines. The structures of the three pseudopeptides are reported

341 in Scheme S1 together with their affinity for Co²⁺ measured at physiological pH. NTA(CysNH₂)₃ is a
342 trithiol ligand previously designed for metal chelation, demonstrating a large affinity for Cu⁺ and to a

343 lesser extent for Zn²⁺ (Pujol et al., 2009, 2011). It forms a CoL complex with a micromolar affinity at
344 pH 7.4, which is significantly larger than the affinities reported for Co²⁺ with L-cys and GSH (Table

345 S1). The two other pseudopeptides derived from EDTA and DOTA. EDTA(CysNH₂)₄ and

346 DOTA(CysNH₂)₄, that were specifically designed for this study, have four thiolates in their structure.

347 As expected, their affinity for Co²⁺ is larger than with NTA(CysNH₂)₃ since Co²⁺ is known to be

348 efficiently coordinated in a tetrahedral tetrathiolate coordination environment. Their CoL complexes

349 exhibit stabilities in the nanomolar range at pH 7.4, the cyclic DOTA derivative demonstrating a

350 slightly larger affinity. It has to be noted that the two latter sulphur-rich ligands are able to complex a

351 second Co²⁺ ion probably through the N/O atoms of the chemical scaffold in a Co₂L complex.

352 However, despite their enhanced affinity for Co²⁺ with respect to monothiol molecules, none of these

353 sulfur pseudopeptides led to an increased Co₃O₄P dissolution whatever the medium used (Table 2).

354 **3.2/ Effect of molecules enhancing metal oxide dissolution on Co₃O₄P dissolution.**

355 Since none of the molecules tested above were able to induce significant increase in the dissolution of
356 Co₃O₄P, we selected other molecules from literature (Table 1) which, alone or in combination, have
357 been shown to induce a strong dissolution of metallic oxides particles of iron, copper, or manganese.
358 We also tested the effect of ligands susceptible of releasing protons, since protons (or low pH) have a
359 strong effect on metallic oxide particle dissolution.

360 The role of metal ion reduction on particle dissolution has been demonstrated with ascorbate (Afonso et
361 al., 1990; Huang et al., 2017; Joseph et al., 1996; Panias et al., 1996; Stone and Morgan, 1984; Suter et
362 al., 1991), oxalate (Kraemer, 2004; Panias et al., 1996; Zinder et al., 1986) and with phenolic reductants
363 as catechol (Lakind and Stone, 1989) or tannic acid (Zhao et al., 2017). These compounds have been
364 shown to increase the dissolution of metal oxides, such as Fe and Mn by reduction. Some of these
365 molecules seem to act through combined reductive and metal-binding effects. Moreover, association of
366 different kinds of molecules was shown to induce efficient particle dissolution, such as ascorbate with
367 oxalate or EDTA as well as with a mixture of citric acid and EDTA (Afonso et al., 1990; Joseph et al.,
368 1996). It should be mentioned that all these studies were performed in non-physiologic buffers and
369 mainly at acidic pH (≤ 4). In the present study, tannic acid, pyrocatechol and ascorbate alone or in
370 association with oxalate or with EDTA significantly enhanced Co₃O₄P dissolution in ALF. The highest
371 efficacy was obtained with the association of ascorbate and oxalate (2.41 fold increase as compared to
372 controls at day 1 and 1.65 fold at day 7, these ratios corresponding respectively to 1.6 and 5.2% of
373 dissolution). However, this association had no effect in Gamble's. The association of ascorbate and
374 EDTA increased significantly the dissolution of Co₃O₄P in both lung fluid surrogates, reaching 0.91%
375 dissolution in Gamble's and a ratio to a 1.38 fold increase as compared to controls in ALF at day 7 (Table
376 2). It is interesting to keep in mind that citrate is one of the components of ALF. Except for the
377 combination of ascorbate and EDTA, no other significant increase in Co₃O₄P dissolution was observed
378 in Gamble's (Table 2).

379 Siderophore-promoted dissolution has also been described for iron oxides. Dubbin et al., described an
380 increase in iron release from synthetic goethite in the presence of ferrichrome and enterobactin at pH
381 6.5 (Dubbin and Bullough, 2017). Desferrioxamine B (DFOB) has been shown to increase Fe release

382 from Cr(III)-Fe(III)-(oxy)hydroxides in an HEPES buffer at pH 7, and oxalate acted synergistically with
383 DFOB to increase the Fe release (Saad et al., 2017). Bi et al demonstrated the solubilizing effect of
384 DFOB on heterogenite in a pH dependent manner (Bi et al., 2010). We thus tested four siderophores:
385 DFOB, enterobactin, desferrithiocin (DFT) and deferiprone (DFP). In Gamble's, only enterobactin
386 induced an increase in $\text{Co}_3\text{O}_4\text{P}$ dissolution (0.47% dissolution at day 7). In ALF, both enterobactin and
387 DFOB slightly enhanced dissolution. DFOB induced a 1.38 fold higher dissolution than that of controls
388 at day 7, which is consistent with published data obtained for heterogenite particles (Bi et al., 2010).
389 The two other siderophores, DFT and DFP also increased the dissolution of $\text{Co}_3\text{O}_4\text{P}$ in ALF but to a
390 lesser extent than DFOB (Table 2).
391 Saad et al. demonstrated that siderophore and oxalate promoted dissolution of Cr(III)-Fe(III)-
392 (oxy)hydroxides (Saad et al., 2017). By using this combination, we reached a 1.5 increase in $\text{Co}_3\text{O}_4\text{P}$
393 dissolution in ALF at day 7 as compared to the controls, which makes this combination one of the best
394 candidates (Table 2).

395

396 **3.3/ Effect of novel molecule associations on $\text{Co}_3\text{O}_4\text{P}$ dissolution**

397 Altogether, the above reported results highlight the improved efficacy of associations of molecules with
398 complementary properties such as metal reduction and strong Co^{2+} -binding properties as compared to
399 each molecule alone. We thus tested molecules not reported in the literature for metal oxides dissolution
400 but for which intrinsic properties might enhance the dissolution of cobalt particles.

401 We first tested dithiothreitol (DTT) for its strong reducing properties. However, DTT showed a lower
402 efficacy to dissociate Co_3O_4 particles than ascorbate in ALF (Table 2). In contrast, L-Cys, which is also
403 a reductant, was shown to slightly increase $\text{Co}_3\text{O}_4\text{P}$ dissolution in Gamble's (Figure 2B). Thus, we
404 evaluated the efficacy of a combination of ascorbate or L-Cys and high affinity Co^{2+} ligands (Table 2).
405 The combination of L-cys with EDTA did not improve $\text{Co}_3\text{O}_4\text{P}$ dissolution. Therefore, we focused on
406 combinations involving ascorbate and cobalt ligands with different coordinating atoms and affinities
407 (Table S1).

408 We did not observe any significant synergistic effect between ascorbate and DFOB. In contrast, the
409 addition of ascorbate to the SH-rich pseudopeptides $\text{DOTA}(\text{Cys})_4$ and $\text{EDTA}(\text{Cys})_4$ led to 0.61% and

410 0.33% dissolution respectively at day 7 in Gamble's, showing a good synergistic effect as compared to
411 each molecule used alone. This effect was not observed in ALF. In Gamble's we observed a strong
412 synergistic effect with ascorbate and DTPA as compared to DTPA alone with 0.54% of dissolution at
413 day 7 versus <0.1% for DTPA alone. This was not the case in ALF.

414 The combination of ascorbate with EDTA was one of the most effective in Gamble's, with 0.91% of
415 dissolution at day 7. Since the affinity of DTPA for Co^{2+} is larger than that of EDTA (Table 1. Sup mat),
416 the affinity for Co^{2+} does not seem to be the determining factor for an effective dissolution of $\text{Co}_3\text{O}_4\text{P}$
417 in the lung fluid surrogates.

418 pH or the ability to deliver protons at the particle surface has also been reported as important factors in
419 particle dissolution. A low pH however may prevent efficient chelation of Co^{2+} by chelating groups with
420 mid or high pKA values. Dipicolinic acid (DIPIC) was shown to be of interest, as the $[\text{Co}(\text{dipic})_2]^{2-}$
421 complex is stable even at low pH due to the low basicity of the nitrogen atom found in the aromatic
422 heterocycle (Yang et al., 2002). In addition, combination of DIPIC with proton acceptors (histidine,
423 thiamine, ornithine and o-phenylenediamine, OPD) were described to enhance and stabilize the
424 formation of the $[\text{Co}(\text{dipic})_2]^{2-}$ complexes at low pH (Ghasemi et al., 2014). However, no significant
425 effect of these combinations was observed on the dissolution of $\text{Co}_3\text{O}_4\text{P}$ in Gamble's or in ALF (Table
426 2). In contrast, OPD alone had a slight effect in ALF. Therefore, we also tested OPD in combination
427 with ascorbate and EDTA. OPD addition significantly increased $\text{Co}_3\text{O}_4\text{P}$ dissolution efficacy in
428 Gamble's, with a dissolution ratio of 0.94% and 0.99% at day 1 and day 7, respectively, but not in ALF.
429 Whereas it should be noted that addition of OPD markedly increased the efficacy of ascorbate and
430 EDTA, those combinations were not retained for the rest of the study due to the toxicity of OPD
431 (Matsumoto et al., 2012).

432 In summary, the best combinations of molecules are: ascorbate alone, DFOB alone and the combination
433 of ascorbate with EDTA, ascorbate with oxalate, ascorbate with DTPA, ascorbate with DFOB and
434 DFOB with oxalate.

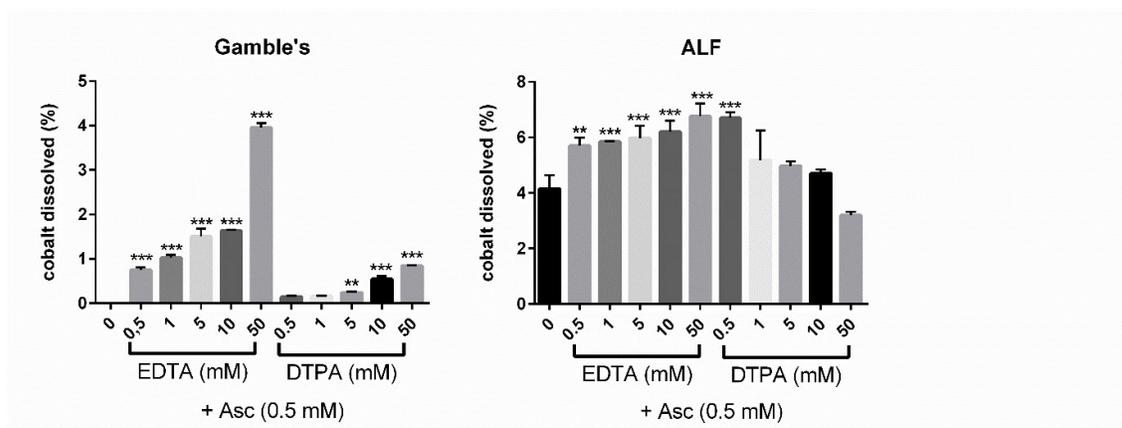
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437

438 **3.4/ Influence of ligand concentration on Co₃O₄P dissolution.**

439 We studied then the impact of DTPA or EDTA concentration in both lung fluids when used in
440 association with ascorbate. Indeed, a strong effect of EDTA concentration on the dissolution of hematite
441 in citric acid-EDTA-ascorbic acid mixtures has been previously observed (Joseph et al., 1996). The
442 concentration of EDTA and DTPA was increased up to 50 mM. Whereas this high concentration has no
443 physiological relevance, this study was made to identify the importance of Co²⁺-chelation in the
444 dissolution process. By increasing 100 fold the EDTA concentration (50 mM), the percentage of
445 dissolution increased by a factor of 5 (from 0.8% to 4%) in 7 days in Gamble's (Figure 4). In ALF, only
446 a slight but significant increase was observed. These results are in agreement with what was described
447 with hematite in citric acid-EDTA-ascorbic acid mixtures (Joseph et al., 1996). Noticeably, raising
448 DTPA concentration led to only a moderate increase in Co₃O₄P dissolution in Gamble's. The decrease
449 in dissolution observed in ALF as compared to the lower DTPA concentration of 0.5 mM, suggests
450 different mechanisms of action for the two ligands.



451

452 **Figure 4: Impact of ligand concentration on particle dissolution at day 7 in Gamble's and ALF.**
453 Cobalt concentration remained constant while the ligand concentrations were increased from 0.5 to 50
454 mM, leading to ligand:cobalt ratios varying from 0.5 to 50 respectively. Results are expressed as mean
455 % ± SD of dissolved cobalt/ total cobalt content. n=3. Statistically significant differences from control
456 were determined by one-way ANOVA followed by Dunnett's multiple comparisons test: *p < 0.05, **p
457 < 0.01, ***p < 0.001.

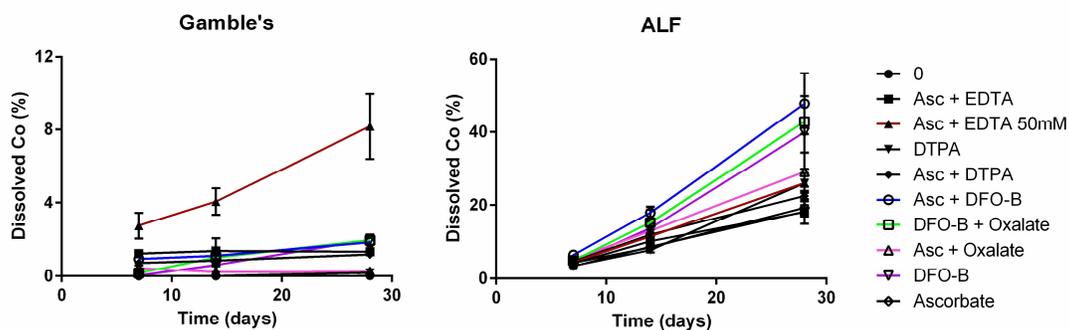
458

459 **4/ Long-term effect of various combinations on Co₃O₄P dissolution.**

460 To evaluate the long-term effect, the dissolution assay with the best combination of molecules was
461 conducted in solution over 28 days,. This could confirm the interest of chronic decorporation treatment

462 as it has been observed with DTPA after actinide pulmonary contamination (Guilmette and
463 Muggenburg, 1993).

464 Our results show that increasing the incubation time further increased $\text{Co}_3\text{O}_4\text{P}$ dissolution for most of
465 the conditions in Gamble's and for all in ALF. Ascorbate with EDTA 50 mM, in Gamble's, led to 8.2
466 +/- 1.8% dissolution although in the controls, soluble cobalt remained barely detectable (Figure 5). In
467 ALF, although the $\text{Co}_3\text{O}_4\text{P}$ dissolution in the controls was already of 18 +/- 3% in 28 days, the dissolved
468 fraction was much larger and reached 48 +/- 8%, 43 +/- 1% and 40 +/- 10% with the ascorbate and
469 DFOB, oxalate and DFOB and DFOB alone, respectively (Figure 5). A progressive and constant
470 dissolution occurred with time whatever the fluid surrogate in which particles were incubated,
471 particularly in presence of DFOB.



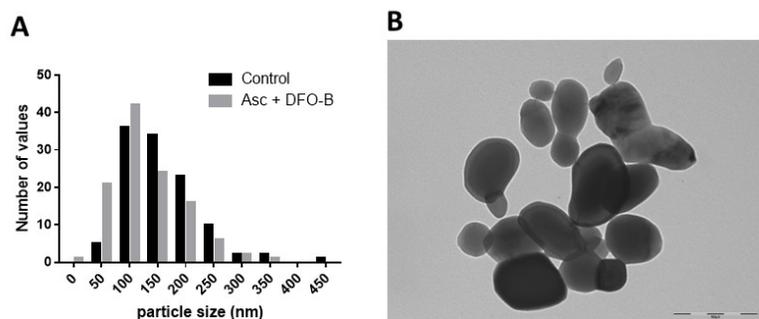
472

473 **Figure 5: Dissolution of $\text{Co}_3\text{O}_4\text{P}$ up to 28 days incubation with selected ligand combinations.**
474 Results are expressed as mean % \pm SD of dissolved cobalt/ total cobalt content, $n=6$.
475

476 Since 48% of the cobalt was released from particulate matter after an incubation period of 28 days by
477 ascorbate and DFOB in ALF, a decrease in particle size was expected to occur. Indeed, TEM analysis
478 showed that the mean size of particles was significantly smaller when incubated in ALF for 28 days in
479 the presence of ascorbate and DFOB as compared to controls in ALF alone (mean size 129 +/- 59 nm
480 vs 157 \pm 69 nm, $p<0.01$), (Figure 6).

481

482



483

484 **Figure 6: $\text{Co}_3\text{O}_4\text{P}$ size after a 28 day incubation with ascorbate and DFOB.**

485 *A: TEM pictures were analyzed with Image J software to determine the diameter of particles in each*
 486 *sample (n=113). B: representative TEM picture of $\text{Co}_3\text{O}_4\text{P}$ after a 28-day incubation with ascorbate*
 487 *and DFOB.*

488

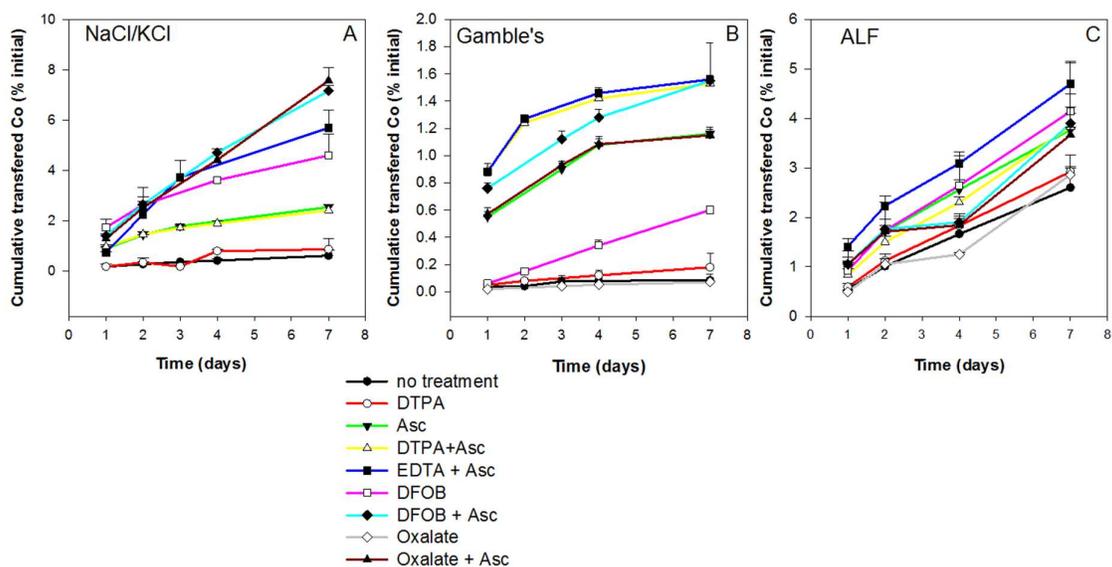
489

490 **5/ Validation of the results using the two-phase model**

491 To go further in the determination of efficacy of various drugs to enhance particle dissolution, we used
 492 the dynamic two-phase model assay to assess the efficacy of molecules selected from the dissolution
 493 assay in solution. For this assay, the static phase representing the retention compartments was prepared
 494 in NaCl, Gamble's or ALF. The molecules to be tested were added to the dynamic phase representing
 495 the transfer compartment.

496 Efficacy was assessed by measurement of Co in the dynamic phase from 1 to 7 days after incubation in
 497 saline or lung surrogates. $\text{Co}_3\text{O}_4\text{P}$ dissolution was increased in the presence of ascorbate whether or not
 498 associated with other molecules (Figure 7). Oxalate did not increase dissolution of $\text{Co}_3\text{O}_4\text{P}$ in ALF and
 499 Gamble's when used alone, nor improved the efficacy of ascorbate when used in association. DFOB
 500 used alone was able to increase the dissolution of $\text{Co}_3\text{O}_4\text{P}$ whatever the composition of the agarose gels.
 501 Remarkably, the higher efficacy, observed both in Gamble's and ALF, was obtained with the association
 502 of the reductant ascorbate with the chelating agents DTPA or EDTA. These results confirm those
 503 obtained with the dissolution assay in solution.

504



505

506 **Figure 7: Effect of various molecules on dissolution of Co_3O_4P assessed in the two-phase dynamic**
 507 **assay.**

508 Co_3O_4P were included in the static retention phase (agarose gels) prepared in NaCl/KCl (7A), Gamble's
 509 (7B) or ALF (7C). Treatments (500 μM) were added in the dynamic phase. Results are expressed as %
 510 cumulative cobalt measured in the dynamic phase/initial cobalt in the gel. Each experimental condition
 511 was done in triplicate and repeated 1 to 3 times.

512

513 As a summary, a schematic classification of the combinations with the highest efficacy at day 7 using
 514 both dissolution methods is presented in Table 3. Altogether, ascorbate associated with DTPA, EDTA
 515 and DFOB showed the highest efficacy on Co_3O_4P dissolution in both media.

516

517 **Table 3: Schematic overview of the dissolution of Co_3O_4P in the presence of various molecules assessed**
 518 **in Gamble's and ALF at day 7.**

519

	DTPA	Asc	DFOB	Ox	Asc/DTPA	Asc/EDTA	Asc/DFOB	Asc/ox
Gamble's	=	+	+	=	+++	+++	++	+
ALF	=	+	++	=	++	++	++	++

520 *The combinations with highest efficacies in both media are shown in red.*

521

522

523

524 **Discussion**

525 Understanding the fate of poorly soluble inhaled compounds and the mechanisms controlling particle
526 retention and clearance from the lungs is essential to evaluate the risk of long-term exposure and to
527 develop tailored decorporation strategies. Following inhalation of poorly soluble compounds, such as
528 $\text{Co}_3\text{O}_4\text{P}$, particle dissolution, occurring mainly in macrophages, represents the first step in lung
529 clearance. In case of inhalation of strong gamma emitters ^{58}Co or ^{60}Co , the reduction of the retention
530 time is of crucial importance to avoid chronic irradiation of target cells. Once the ionic form Co^{2+} is
531 released by particle dissolution, it is expected to be rapidly eliminated from the body, mainly in urine.
532 Indeed clearance of almost 100% after injection of CoCl_2 occurs in 45 h (Weber et al., 2012). To
533 accelerate urinary excretion, the use of chelating drugs is recommended. It is generally assumed that
534 ionic forms are more prompt to form complexes with biological ligands or chelating agents than
535 particulate forms, highlighting further the importance of increasing particle dissolution.

536 In the present study, our goal was to propose innovative strategies for $\text{Co}_3\text{O}_4\text{P}$ dissolution, as a first step
537 of decorporating drug development. The novelty of our work is four fold: 1- the use of particles with
538 similar properties than those involved in a real accidental exposure at the work place, 2- the use of two
539 complementary *in vitro* dissolution assay, the second one showing good correlation with *in vivo*
540 excretion data following contamination with actinides, 3- the evaluation of efficacy of molecules
541 differing in their mechanisms of action and 4- the evaluation of efficacy on dissolution $\text{Co}_3\text{O}_4\text{P}$, in
542 biologically-relevant conditions, representative of an inhalation scenario.

543 The dissolution assessment consisting in measuring the ionic fraction of cobalt in solution at different
544 times after incubation allowed the screening of a large number of molecules, used alone or in
545 combination in simple buffer as well as in lung fluid surrogates. On the other hand, the dissolution
546 assessment in the dynamic bi-compartmental assay, mimicking a retention phase and a transfer
547 compartment is expected to give a good correlation with *in vivo* data (Griffiths et al., 2016). Using these
548 two assays, we confirmed the low dissolution potential of $\text{Co}_3\text{O}_4\text{P}$ in simple buffers, in the absence of
549 interactions with chemical or biological entities (Collier et al., 1992). Using lung fluids surrogates, we
550 showed that dissolution of $\text{Co}_3\text{O}_4\text{P}$ was influenced by various parameters such as pH or the presence of

551 compounds as citrate, carbonate, proteins, etc..., in line with results obtained for a number of metal
552 oxide particles (Kastury et al., 2017; Pelfrene et al., 2017). The higher $\text{Co}_3\text{O}_4\text{P}$ dissolution in ALF,
553 representing the intracellular compartment of macrophages, likely results from the lower pH of this
554 medium (pH 4.5 vs pH 7.4 in HEPES and Gamble's) and the presence of citrate, since both parameters
555 have been shown to facilitate dissolution of cobalt oxides (Collier et al., 1992; Lundborg et al., 1992).
556 $\text{Co}_3\text{O}_4\text{P}$ dissolution measured in the present study in ALF was of the same order of magnitude than
557 previously reported (Cho et al., 2012; Collier et al., 1992; Ortega et al., 2014). The contrasting results
558 obtained in buffers as compared to lung fluid surrogates, (Figures 2 and 3) underlined the need for using
559 surrogates of relevant biological fluids for such studies.

560 We evaluated the efficacy of various molecules to increase $\text{Co}_3\text{O}_4\text{P}$ dissolution, including DTPA as a
561 gold standard, since this chelating agent is the recommended treatment following internal contamination
562 with cobalt. In lung fluids, no dissolution enhancement was observed on $\text{Co}_3\text{O}_4\text{P}$ dissolution with DTPA,
563 nor in the presence of molecules previously described by others for their efficacy to decorporate soluble
564 Co^{2+} (Figures 2 and 3). Further testing with specifically designed molecules with high-affinity for Co^{2+}
565 or known Co^{2+} binding molecules (listed in Table 1) did not improve $\text{Co}_3\text{O}_4\text{P}$ dissolution either. In
566 addition, since the affinity of DTPA for Co^{2+} is larger than that of EDTA (Table 1. Sup mat), and the
567 $\text{Co}_3\text{O}_4\text{P}$ dissolution less efficient with DTPA than EDTA, the affinity for Co^{2+} does not seem to be the
568 determining factor for an effective dissolution of $\text{Co}_3\text{O}_4\text{P}$ in the lung fluid surrogates. These result
569 strongly suggest that chelation of cobalt at the surface of the $\text{Co}_3\text{O}_4\text{P}$ or destabilization of the particle
570 surfaces are determining factors, and that molecules efficient for Co^{2+} -binding in solution, may not be
571 efficient for particles dissolution.

572 The $\text{Co}_3\text{O}_4\text{P}$ are mixed-valence cobalt oxide particles, with Co(II) and Co(III). Therefore, destabilization
573 of the particle surface by modifying the Co(II)/Co(III) equilibrium is probably an important step of
574 $\text{Co}_3\text{O}_4\text{P}$ dissolution, as it was described for mixed-valence iron oxides.

575

576 This prompted us to review the literature (Table 1), and to develop a strategy based on the hypotheses
577 previously established for other metallic oxide particles dissolution. Particle dissolution depends mainly
578 on three mechanisms: 1) protonation, in which protons are competing with the positively charged cations

579 at the surface of the particle, 2) reduction, which leads to the destabilization of the particle surface by
580 reducing the lattice metal cations thus modifying their preferred coordination and leading to the release
581 of the dissolved cation and 3) chelation, acting directly at the particle surface or in solution. It is also of
582 importance to note that a same molecule can act both as a chelator and as a redox agent, as it has been
583 reported for ascorbate (Huang et al., 2017; Suter et al., 1991) and siderophores (Bi et al., 2010; Kraemer,
584 2004; Saad et al., 2017). For siderophores, literature data also propose a proton-promoted dissolution
585 mechanisms (Kraemer, 2004).

586 We also combined molecules with different properties to reach a higher efficacy. Indeed, Joseph et al.
587 demonstrated the positive effect of the combination of citric acid, EDTA and ascorbic acid for dissolving
588 hematite at pH 2.8 (Joseph et al. 1996). Ascorbic acid initiated the dissolution by Fe(III) reduction into
589 Fe(II) at the particle surface. In addition ascorbate can reduce Fe(III)-EDTA complexes formed in
590 solution, and the resulting Fe(II)-EDTA complex is proposed to accelerate the reductive particle
591 dissolution. Actually, the citric acid–EDTA–ascorbic acid was shown to completely dissolve hematite
592 at pH 3 (Ananthan et al. 2003).

593 Whatever the tested molecule, the overall dissolution in ALF was always higher than in Gamble's. In
594 contrast, the dissolution enhancement observed with given molecules as compared to untreated controls
595 was higher in Gamble's than in ALF. As an example in the biphasic model, the dissolution increased
596 18.5 fold in the presence of ascorbate and DTPA as compared to controls in 7 days, and 2.1 fold only in
597 ALF. The higher dissolution obtained in ALF suggests that the effect of pH is dominating over the effect
598 of (reductive) complexation in the process of $\text{Co}_3\text{O}_4\text{P}$ dissolution, at least at short incubation times. This
599 is not true anymore at longer incubation times, as demonstrated by the significantly higher dissolution
600 rate observed for $\text{Co}_3\text{O}_4\text{P}$ dissolution at day 28 in presence of DFOB with or without ascorbate or
601 oxalate, and with ascorbate and 50 mM EDTA (Figure 5) as compared to the control. On the other hand,
602 the very low level of $\text{Co}_3\text{O}_4\text{P}$ dissolution in Gamble's might be explained by a competition for
603 complexation between the Ca and Mg cations present at high concentration in the medium and Co
604 towards the added chelating molecules. We tested this hypothesis by incubating $\text{Co}_3\text{O}_4\text{P}$ in HEPES and
605 EDTA in presence of Ca^{2+} and Mg^{2+} at concentrations found in Gamble's, and the 7-day dissolution was
606 reduced 32 fold in the presence of these two cations (data not shown). The low but significant $\text{Co}_3\text{O}_4\text{P}$

607 dissolution increase obtained with L-Cys and GSH in Gamble's (Figure 2) also supports this hypothesis,
608 since these compounds are poor Ca^{2+} or Mg^{2+} ligands. We cannot exclude however, that these
609 compounds contribute to $\text{Co}_3\text{O}_4\text{P}$ dissolution by other mechanisms, such as a redox effect. In Gamble's
610 medium, the dissolution process could also be inhibited by possible "passivation" agents such as proteins
611 or small molecules as phosphate, which could irreversibly bind to the particles and protect their surface
612 against degradation.

613

614 Overall, our results strongly suggest that the combination of two molecules with different mechanisms
615 of action on cobalt oxide particles provides an effective strategy to increase their dissolution in the two
616 lung fluid surrogates (Tables 2 and 3). Of particular interest is the important long-term effect observed
617 in the dynamic assay in ALF, since these conditions correspond to the main retention compartment for
618 $\text{Co}_3\text{O}_4\text{P}$ in the lungs, following macrophage uptake. Ascorbate associated with chelating molecules as
619 EDTA, DTPA or DFOB showed a higher efficacy than when used alone. This suggests that, *in vivo*,
620 such combinations would be of great interest by promoting the particles dissolution using a redox
621 mechanisms, coupled by the chelation of the dissolved Co^{2+} ions. Translocation from lungs to blood
622 would consequently occur, accelerating lung clearance and decreasing the time of irradiation. The
623 subsequent committed effective dose in case of inhalation of radioactive cobalt compounds would thus
624 be limited. An important issue in the identification of new decorporating drugs, is their potential toxicity.
625 Indeed, a crucial point in the development of a treatment for use in humans is its absence of toxicity. In
626 this respect, it is important to note that among the molecules used in combination that allow the highest
627 dissolution rate of $\text{Co}_3\text{O}_4\text{P}$, DTPA, EDTA, ascorbate and DFOB are FDA approved molecules. Although
628 the unspecific chelation of other metallic ions cannot be excluded, no major side effects have been
629 reported for these molecules. Ca-DTPA has been used in workers contaminated with actinides for few
630 decades. As reported by the FDA administration and in a case study (Grappin et al. 2007), no evidence
631 for side effects in 95% of the treated workers was reported, even after long term administration.

632

633 **Conclusions**

634 In the present study, we evaluated the ability of various molecules to increase the dissolution of $\text{Co}_3\text{O}_4\text{P}$
635 in lung fluid surrogates. Dissolution was assessed using two complementary acellular *in vitro* models,
636 representative of retention compartments following pulmonary contamination with poorly soluble cobalt
637 particles. We first demonstrated that DTPA, the currently recommended decorporating treatment, had
638 no significant effect on the dissolution of $\text{Co}_3\text{O}_4\text{P}$. We then showed that combining redox active
639 molecules with chelating agents led to a better efficacy for $\text{Co}_3\text{O}_4\text{P}$ dissolution than each molecule used
640 alone as already reported for the dissolution of mixed-valence iron oxide particles. From the present
641 work, a small set of combinations involving FDA- approved molecules, very promising for new
642 decorporation strategies, were identified and can be now tested on main cellular targets, such as
643 pulmonary macrophages.

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