Brief communication

Does carbonation of steel slag particles reduce their toxicity?
An in vitro approach

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ABSTRACT

Mineral carbonation can stabilize industrial residues and, in the steel industry, may contribute to simultaneously valorize CO2 emissions and slag. We hypothesized that, by restricting the leaching of metals of toxicological concern such as Cr and V, carbonation can suppress the toxicity of these materials. The cytotoxic activity (WST1 assay) of slag dusts collected from a stainless and a Linz-Donawitz (LD) steel plant, before and after carbonation, was examined in J774 macrophages. The release of Cr, V, Fe, Mn and Ni was measured after incubation in artificial lung fluids mimicking the extracellular and phagolysosomal milieu to which particles are confronted after inhalation.

LD slag had the higher Fe, Mn and V content, and was more cytotoxic than stainless steel slag. The cytotoxic activity of LD but not of stainless dusts was reduced after carbonation. The cytotoxic activity of the dusts toward J774 macrophages necessitated a direct contact with the cells and was reduced in the presence of inhibitors of phagocytosis (cytochalasin D) or phagolysosome acidification (bafilomycin), pointing to a key role of metallic constituents released in phagolysosomes. This in vitro study supports a limited reduction of the cytotoxic activity of LD, but not of stainless, steel dusts upon carbonation.

1. Introduction

The world production of steel has been constantly growing over the last years, from about 1 million in 2003 to 1.5 million in 2012 (Steel statistical yearbook, 2013), and an increasing demand is still expected to support economic developments in emerging countries (>3% in 2014 Worldsteel Association, 2013). A significant amount of waste is, however, generated by the production of steel, among which slag and greenhouse gas emissions (primarily CO2) represent major environmental challenges. The amount of slag produced is usually between 150 and 200 kg per ton of steel and only the coarse fractions can currently be used in economically valuable industrial applications, mainly in road construction. Fewer valorization opportunities exist for the fine fraction (<10 μm) which may represent up to 50% of the total slag produced. Limiting the generation of greenhouse gas such as CO2 also represents a major challenge in the management of global climatic changes. Following the Rio convention, the European Union aims by 2020 to reduce its emissions of greenhouse gas by 20% compared to 1990. Approximately 30 Gt of CO2 are emitted in the atmosphere worldwide and steel industry represents a significant proportion of it. Methods are being developed to sequester CO2 from industrial emissions but a valorization of the gas does usually not exist. There is, therefore, an increasing pressure to develop new approaches to recycle and valorize these wastes, and to mitigate their environmental impacts.

Mineral carbonation is a process whereby CO2 is chemically reacted with calcium- and/or magnesium-containing minerals to form stable carbonate materials. This process has been proposed to stabilize solid residue streams generated from diverse industrial settings, including coal fired power stations and other types of combustion residues such as municipal solid waste ashes (Lim et al., 2010; Olajire, 2013). In the steel industry, this process has the potential to simultaneously remediate CO2 emissions and valorize fine slag. In the CARMAT project financed by the Walloon region, a direct gas–solid process has been developed in which CO2 reacts mainly with alkaline Ca/Mg silicate minerals to produce carbonated products from fine steel slag. Different types of carbonated products can be produced, including pellets to replace sand in road construction applications, massive parts produced at low CO2 pressure for the construction sector (building blocks, paving stones), or massive parts produced at high CO2 pressure for pavement or terraces in substitution of natural stones.

The main constitutive elements of steel slags are calcium, silicon, iron and manganese but, depending on their origin, they
may also contain traces of Cr and V which represent potential sources of toxicological concern (Chaurand et al., 2007). Inhalation is the main route of exposure to these particles for humans in occupational settings, but possibly also consumers or the general population indirectly exposed via environmental contamination. Upon carbonation, the concomitant formation of carbonates and pH decrease (from pH 11–12 to 8–9) contribute to limit the leaching of these metallic ions from the material (Van Gerven et al., 2005; Navarro et al., 2010). It remains, however, to determine whether carbonation and reduced metal leachability suppress toxicity.

We assessed here the impact of carbonation on the toxicity of fine slag dusts collected from two different production lines representative of a stainless and a Linz-Donawitz (LD) steel plant, respectively. We assessed the in vitro cytotoxic activity of these materials, carbonated or not, toward macrophages (J774 cell line) which represent a critical toxicity component for inhaled dusts. We correlated the results of these cellular experiments with the release of metals of potential toxicological concern (Cr, V, Fe, Mn and Ni) measured after incubation in two artificial lung fluids (Stopford et al., 2003).

2. Materials and methods

2.1. Steel slag particles

Particle samples were obtained from the fine slag recovered in a stainless steel production (P1) or in a LD production plant (P5). Both materials were carbonated under 3 conditions: pellets, massive pieces in a low- or high-pressure autoclave (P1p, P1m_lp, P1m_hp, P5p, P5m_lp and P5m_hp, respectively). Pellets were generated on a pelletizing plate and carbonated in a fluidized bed, whereas the massive pieces were molded in a Controlab E0160S equipment (Saint-Ouen, France) and carbonated in a low- or high-pressure autoclave. The carbonated products were ground with a vibromill (Retsch, Haan, Germany) and mesh-sieved to obtain a number size distribution with at least 50% of the particles <10 \( \mu \text{m} \) in diameter, as measured by laser diffraction (CILAS, Orléans, France) after ultrasonic dispersion. The final samples were analyzed for their carbon content on a Shimadzu TOC-V Series SSM-5000 A equipment (Duisburg, Germany) and their elemental composition was determined by ICP-MS after microwave mineralization in \( \text{HNO}_3 / \text{HCl} \) (limits of detection < 0.5 \( \mu \text{g/L} \)). The hydrodynamic size distribution (intensity %) was determined in triplicates in culture medium (DMEM) by DLS (Zetasizer, Malvern). Before testing, dry particles were heated at 200 °C for 2 h for sterilization and destruction of any possible trace of endotoxin, and suspended in phosphate buffered saline (PBS) at a stock concentration of 3.33 mg/ml immediately before cell exposure. DQ12 crystalline silica particles were used as positive control in cytotoxicity assays (Rabolli et al., 2011).

2.2. Cell lines and culture conditions

Monocyte/macrophages (J774) were obtained from ATCC (LGC, Molsheim, France) and routinely cultured in complete Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS and 1% antibiotic–antimycotic. The cells were seeded in 96-well plates

<table>
<thead>
<tr>
<th>Metal content (( \mu \text{g/g} ))</th>
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<tr>
<td>Al</td>
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| Inorganic carbon content (% CO2) | 0.55 | 8.6 | 8.1 | 18.5 | 0.55 | 8.2 | 12.0 | 11.5 |
| Size distribution (number % < 10 \( \mu \text{m} \)) | 67 | 70 | 75 | 75 | 84 | 68 | 73 | 74 |
| Median hydrodynamic diameter in DMEM (\( \mu \text{m} \)) | 12 | 10.5 | 10 | 10 | 12 | 12.5 | 8 | 9 |

Table 1

Particle characteristics.

P1, inox steel slag particles; P5, LD steel slag particles.

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Fig. 1. Carbonation of steel slag particles does not suppress their cytotoxic activity to J774 macrophages. J774 cells were exposed for 24 h to increasing doses of the different particles suspended in DMEM in the absence of serum, and cell viability was assessed by the WST1 assay. P1, inox steel slag particles; P5, LD steel slag particles. p, carbonated in pellet form; m_lp, carbonated in massive form under low pressure; m_hp, carbonated in massive form high pressure.
during 24 h to the different particles suspended in serum-free DMEM (12.5–200 µg/cm² culture well surface area). In selected experiments, J774 cells were treated with cytochalasin-D (2.5 µM, Sigma–Aldrich, St Louis, MO) or bafilomycin A1 (250 nM, Sigma–Aldrich, St Louis, MO) 30 min before and during particle exposure. J774 cells were also exposed in 24-well plates to the different particles (200 µg/cm²) added directly to the culture medium or to the soluble fraction released from the particles through a polycarbonate membrane (0.4 µm pore size, Millipore, Merck Millipore, Overijssse, Belgium).

2.3. Cytotoxicity assays

The cytotoxic activity of the different particles was assessed after 24 h exposure by monitoring the cell metabolic activity (WST1 assay, Roche, Vilvoorde, Belgium) (Rabolli et al., 2010). The culture medium was removed after particle exposure and replaced with 200 µl of serum-free DMEM containing 10% WST1. The cells were then incubated for up to 2 h and measured every 30 min at the appropriate wavelengths. Results are expressed as relative cell activity (vs sham-exposed cell activity). Positive control cells were exposed to a cytotoxic dose of DQ12 silica (300 µg/cm²). We verified in parallel experiments conducted in the absence of CO₂ in a carbonate-free DMEM buffered with HEPES that the experiments were not confounded by a potential carbonation of the particles by the culture medium.

2.4. Metal solubilization assays

The capacity of the particle samples to release soluble metallic ions was measured in 2 artificial fluids mimicking the extracellular (pH 7.3) and the phagolysosomal (pH 4.2) compartments. The composition of these artificial fluids is given in Table S1. The neutral fluid mimics the extracellular compartment to which pulmonary cells might be exposed, whereas the acidic fluid reflects the intracellular phagolysosome milieu to which particles are confronted after phagocytosis by lung macrophages. Particles were suspended (8 mg/ml) in 50 ml tubes containing the respective fluids and incubated at 37°C during 6 or 24 h under rotating agitation. At the end of the incubation period, the tubes were centrifuged and the supernatant was filtered (0.22 µm) and analyzed directly by ICP-MS for selected metallic elements (Cr, Fe, Mn, Ni and V). The results are expressed in % of the total elemental content in each particle sample tested (Table 1).

2.5. Statistics

Results are expressed as means ± SEM. The data were analyzed with GraphPad Prism (GraphPad software, La Jolla, CA). Differences between means were assessed by a Student t-test or ANOVA followed by a Student–Neuman–Keuls test for multiple comparisons as appropriate. Statistical significance was set at p < 0.05 (two-tailed).

3. Results and discussion

3.1. Particle characteristics

The characteristics of the test samples are summarized in Table 1. As expected, the elemental composition of P1 and P5 samples was different; slag particles obtained from the stainless steel plant (P1) contained more Ni, Cr and Mo than the LD plant particles (P5). P5 contained, in contrast, more Fe, Mn and V than P1. The size distribution of the dry particles was largely compatible with a respirable fraction, >50% of the particles being smaller than 10 µm. In

(Greiner Bio One, Wemmel, Belgium) at a density of 50,000 cells/well and kept overnight in complete medium at 37°C in a 5% CO₂ atmosphere. Cells were then rinsed with PBS and exposed.
culture medium, the particles did not significantly aggregate and all showed a hydrodynamic diameter of about 10 \( \mu m \) (Table 1).

### 3.2. Cytotoxicity assays

In preliminary experiments, we compared the response of J774 cells exposed to the pristine particles (P1 or P5) using the WST1, alamar blue and LDH release assays. We selected WST1 for subsequent experiments because this assay yielded the most consistent results and was also the most sensitive. When J774 cells were exposed to P1 (Fig. 1, upper panel), we observed a dose-dependent reduction of the WST1 cell activity from 50 \( \mu g/cm^2 \) on \((p < 0.01, \) not shown) and no significant difference between the pristine and carbonated samples (P1p, P1m_lp or P1m_hp). P5 appeared more cytotoxic than P1 (Fig. 1, lower panel), a significant reduction of cell viability being already detected from 12.5 \( \mu g/cm^2 \) (not shown). Carbonated P5 particles were slightly but statistically less cytotoxic than pristine P5 at high doses.

The higher cytotoxic activity of the dust obtained from the LD steel mill (P5) could be explained by its higher Fe, Mn and/or V content. A reduced bioaccessibility of (one of) these elements upon carbonation could possibly explain the limited reduction in cytotoxic activity of carbonated compared to pristine P5 particles.

We then examined whether the cytotoxic activity of the particles was mediated by metallic ions possibly released extracellularly in the culture medium. J774 cells were, therefore, incubated with the different particles (200 \( \mu g/cm^2 \)) directly in contact with the cells or through a 0.4 \( \mu m \) polycarbonate membrane (Fig. 2A). The particles did practically not reduce cell viability when incubated in the insert, suggesting that extracellularly released metallic ions did not markedly contribute to their cytotoxic activity. We then hypothesized that the cytotoxic activity could be mediated by metallic ions released intracellularly after phagocytosis by J774 cells. This possibility was supported by the results obtained in the presence of cytochalasin D, an inhibitor of phagocytosis, or bafilomycin A1, a potent inhibitor of phagolysosome acidification. Pristine and carbonated particles induced less cytotoxicity in the presence of these inhibitors (Fig. 2B and C). The cytotoxic activity of steel slag particles toward J774 cells appeared, therefore, mediated by phagocytosis and, possibly, the subsequent intracellular release of (metallic) species in the phagolysosomes. A similar mechanism has been reported recently for indium-containing

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**Fig. 3.** Metal release from steel slag particles after 24 h incubation in neutral or acidic artificial lung fluid. The particles were incubated at 37 °C in a shaking bath containing either neutral or an acidic fluid. After 24 h, suspensions were filtered (0.22 \( \mu m \)) and the filtrate was analyzed by ICP-MS for the determination of metal content. P1, inox steel slag particles; P5, LD steel slag particles. p, carbonated in pellet form; m_lp, carbonated in massive form at low pressure; m_hp, carbonated in massive form at high pressure. Results are expressed as \( \mu g \) metal/g dust.
particles which released indium ions in the phagolysosomes of RAW macrophages (Gwinn et al., 2013). These investigators observed that indium-containing particles were significantly more cytotoxic to RAW macrophages than to a lung epithelial cell line (LA-4), consistent with a larger uptake and indium release by RAW than LA-4 cells. We also noticed a lower sensitivity of LA-4 cells to the slag particles, compared to J774 cells (not shown).

3.3. Solubilization experiments

We then examined the release of metallic ions from the different particles (Fig. 3). At neutral pH, a slight release of Cr (<2% of the particle content) was found with P1, less with P5 particles. P5 released twice more vanadium than P1. No clear modification of metal release was evidenced when comparing pristine and carbonated particles incubated under neutral conditions. At acidic pH, a more extensive release of all the metals examined was observed. Higher Cr and Ni concentrations were generated from P1 compared to P5. Conversely, higher Fe, Mn and V concentrations were released from P5 compared to P1. No clear modification of metal release was evidenced when comparing pristine and carbonated particles incubated under acidic conditions. These results, together with the cellular experiments summarized in Fig. 2, indicate that the cytotoxic activity of the slag particles might be mediated by metallic ions released in the phagolysosomes after particle uptake. Carbonates are unstable under such acidic conditions. We could not confirm our initial hypothesis of a reduced bioaccessibility of Fe, Mn or V from carbonated P5 particles under acidic conditions.

In conclusion, this in vitro study supports a limited reduction of the cytotoxic activity of LD, but not of stainless, steel dusts upon carbonation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

The authors thank Gauthier Hermant and Hervé Brecquel (CTP, Tournai, Belgium) for the preparation and characterization of the dusts used in the present study. This work was conducted under the CARMAT project (Développement de nouveaux types de matériaux à usage du BTP obtenus par carbonatation, au moyen de fumées industrielles et de fractions de scories d’aciéries difficillement valorisables) coordinated by RECOVAL and funded by the DG06 of the Walloon region (convention No. 6380).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2015.02.013.

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