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Toxicological profile of calcium carbonate nanoparticles for industrial applications



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ABSTRACT

Calcium carbonate nanoparticles (CaCO₃NPs) derived from CO₂ are promising materials for different industrial applications. It is imperative to understand their toxicological profile in biological systems as the human and environmental exposures to CaCO₃NPs increases with growing production. Here, we analyse the cytotoxicity of CaCO₃NPs synthesized from a CaO slurry on two cell lines, and *in vivo* on zebrafish (*Danio Rerio*). Our results demonstrate the CaCO₃NPs *in vitro* safety as they do not cause cell death or genotoxicity. Moreover, zebrafish treated with CaCO₃NPs develop without any abnormalities, confirming the safety and biocompatibility of this nanomaterial.

1. Introduction

Currently, the employment of several nanomaterials to improve the performances and the mechanical properties of the cement has gained considerable research interest [1,2]. Different nanoparticles, including titanium dioxide nanoparticles (TiO₂ NPs) [3], silica nanoparticles (SiO₂ NPs) [4], alumina nanoparticles (Al₂O₃ NPs) [5], and carbon nanotubes (CNTs) [6] have been included in cement-based materials, presenting potential benefits or limitations. In this framework, calcium carbonate nanoparticles (CaCO₃NPs) derived from CO₂ are currently being investigated as potential nanomaterials to be employed in these industrial applications [7,8], with the purpose of contributing to CO₂ capture and utilization directly in the industrial site in which CO₂ is available or produced [9,10]. In this regard, the cement industry is one of the major contributors to anthropogenic CO₂ emissions; therefore, the possibility to incorporate a CO₂-derived filler in the cement matrix is very attractive [10,11].

Calcium carbonate nanoparticles are believed to increase the strength of the cement, thanks to their unique properties, including a high surface area to volume ratio and high porosity. The CaCO₃ increases the kinetic of the C–S–H bonds formation since it acts as seeds for the hydration of the cement, which turns out to be accelerated by the CaCO₃. Thus the early age compressive and flexural strengths of the cement are increased [12–14]. Moreover, CaCO₃ increases the mechanical properties, because of their filling properties [13,14].

The extensive surge in the production and use of CaCO₃NPs,

exposure for industry workers and the impact of their release, gives rise to an urgent need for a careful toxicological evaluation of these nanoparticles on ecosystems and humans. To address this issue, we evaluated the toxicity of CaCO₃NPs on two different cell lines: a mouse embryonic fibroblast cell line (NIH 3T3) and a human breast adenocarcinoma cell line (MCF7). The cytotoxic assessment was performed by the evaluation and estimation of viability, reactive oxygen species (ROS) generation and DNA damage under *in vitro* conditions and after treatment with different concentrations of CaCO₃NPs. Our results reported that CaCO₃NPs did not lead to an increase of cell death and reactive oxygen species or oxidative DNA damage, demonstrating their no cytotoxicity in both NIH 3T3 and MCF7 cells.

We further analyse the behavior of CaCO₃NPs on embryonic/larval zebrafish. Zebrafish are increasingly employed as models in high-throughput toxicological studies, to speed up the nanotoxicity assessment [15–17]. The value of these organisms relies on their genetic similarity with humans, small size and transparency [18,19]. We examined the growth and survival of embryos treated with different suspensions of CaCO₃NPs from 4 to 120 h post fertilization (hpf). CaCO₃NPs exposure produced no significant changes in the zebrafish biological parameters.

Our results demonstrated that calcium carbonate nanoparticles had no *in vitro* and *in vivo* toxic effect, reporting their high biocompatibility. Our study provides insights into the safety and healthy working environments in industries that use CaCO₃NPs derived from CO₂.

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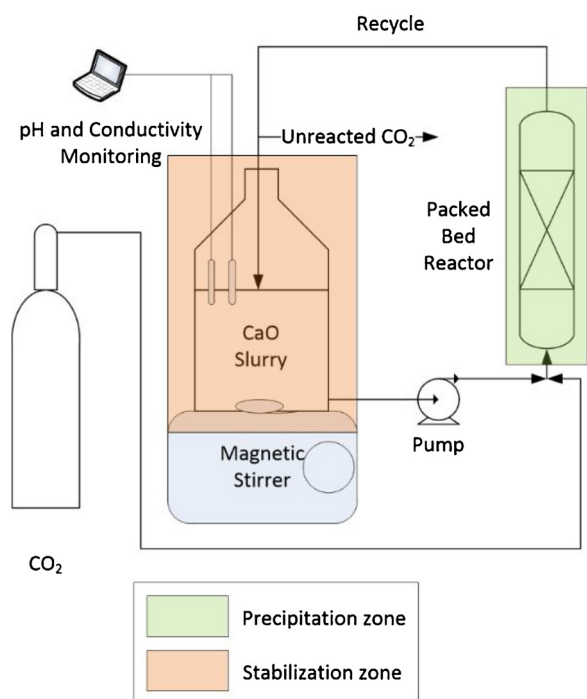


Fig. 1. Experimental setup for the synthesis of CaCO_3 NPs.

2. Materials and methods

2.1. Synthesis of CaCO_3 NPs

In the CaCO_3 synthesis, a slurry prepared with an analytical grade of CaO (Merck, purity $\geq 99\%$) and deionized water, and CO_2 (purity: 99.9%, supplied from SIAD, Italy) were employed. The CaCO_3 NPs were synthesized through carbonation with pure CO_2 of a CaO slurry. The experimental setup consisted in semi-batch process employing a Packed Bed Reactor (PBR) filled with Raschig rings as random packing, shown in Fig. 1. The slurry is conveyed through a peristaltic pump into the PBR, where it gets in contact with the CO_2 and the precipitation takes place. The precipitated particles were immediately recirculated to the vessel, which is maintained at a constant stirring velocity. In such a way, two zones are individuated: i) the crystallization one, inside the PBR, ii) the stabilization one, inside the feed tank, where the pH is maintained high enough to provide a stable environment for the CaCO_3 particles, since under alkaline conditions growth and agglomeration phenomena of the CCnPs are not favoured [20,21]. The CO_2 flow was stopped once the pH was less than 10.5, a value at which, according to the carbonate equilibria, the CO_3^{2-} formation is not favoured, thus reducing the CaCO_3 saturation (see Fig. 2). Then, once the process was finished, the synthesized particles were rapidly filtered by vacuum (pore size = 0.45 μm) and repeatedly washed with deionized water to eliminate ion excess. At last, the CaCO_3 powder was dried at 60 $^\circ\text{C}$ overnight and it was finally ready for characterization of their size, morphology and crystal phase. The parameters of the PBR are summarized in Table S1.

2.2. CaCO_3 NPs characterization

Different characterization analysis were performed on the CaCO_3 powder. The dried powder was dispersed in isopropanol by means of ultrasonic mixing in order to obtain a stable crystal suspension (1 g L^{-1}) [22] and about 1 mL of sample was put into a disposable polystyrene cuvette and size and size distribution were measured by dynamic light scattering (DLS) method using particle size analyzer (Malvern nano ZS model). A 1 mL was also introduced into a zeta cell, and

zeta potential values were measured by the Malvern Zetasizer.

The phase purity of the samples was examined by X-ray diffraction in the 2θ range of 20–70 $^\circ$ with a scanning step of 0.013 $^\circ$ and a radiation $\text{CuK}\alpha$, $k = 1.54056 \text{ \AA}$. The crystalline phase was identified by employing the Powder Diffraction File PDF-4/Minerals 2020 of JCPDS. Morphological characterization was obtained using Field Emission Scanning Electron microscopy (ZEISS MERLIN FE-SEM operated at 3 kV). The samples were prepared for electron microscopy observations by suspending a small amount of nanoparticles in isopropanol, through ultrasonic mixing for 30 min, subsequently by placing a drop of the dispersion on a copper grid coated with a layer of amorphous carbon, and finally the sample was dried at room temperature before FESEM analysis.

2.3. Cell culture

Mouse embryonic fibroblast cells (NIH 3T3) and human breast adenocarcinoma cells (MCF7) were grown in Dulbecco's modified Eagle's medium without phenol red (DMEM) (Life Technologies), supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 100 IU mL^{-1} penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin (Life Technologies) at 37 $^\circ\text{C}$ in a humidified atmosphere containing 5% CO_2 .

2.4. Cell viability

NIH 3T3 and MCF7 cells were seeded at 1×10^4 cells/well in a 96 well plate and incubated overnight (o.n) in DMEM (37 $^\circ\text{C}$, 5% CO_2). On the next day, the medium was removed and the cells were treated with CaCO_3 NPs at different concentrations (1, 5, 10, 25 and 50 $\mu\text{g/mL}$) in DMEM for 72 h. Cell viability was assessed using WST-1 assay (Roche Applied Science) as previously described [23,24].

2.5. Oxidative reactivity

Intracellular reactive oxygen species (ROS) generated by CaCO_3 NPs were measured by using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay. A methanol DCFH-DA stock solution was diluted in DMEM without phenol red to obtain a 100 μM working solution. Cells were seeded in a 96 well plate and exposed to different concentrations of CaCO_3 NPs for 2, 6 and 24 h. After the different time points, cells were rinsed three times with phosphate buffered saline (PBS, pH 7.4). 75 μL /well of DCFH-DA working solution was added to each well and plates were incubated at 37 $^\circ\text{C}$ for 30 min. PBS was used as a negative control, while hydrogen peroxide (H_2O_2) as a positive one. Subsequently, cells were rinsed with PBS and the fluorescent intensity was measured at 485/520 nm measured every 5 min over 30 min using a plate reader. ROS increase was calculated as the mean slope per min and normalized to the unexposed control. Each experiment was performed three times and the data are presented as mean values \pm standard error of the mean of the replicates.

2.6. Comet assay

The levels of DNA damage in NIH 3T3 and MCF7 cells induced by CaCO_3 NPs were measured by using the alkaline single-cell comet assay. Cells were seeded in 24 well and treated for 24 h with 1, 5, 10, 25 and 50 $\mu\text{g mL}^{-1}$ of CaCO_3 NPs for 6 and 24. PBS was used as a negative control, while H_2O_2 as a positive one. Cells were harvested, were rinsed twice in PBS and were trypsinized. The trypsin was inactivated by adding 1 mL of DMEM and the final suspension was by centrifugation (4 min at $18.000 \times g$, 4 $^\circ\text{C}$). The cell suspension was mixed with low-melting agarose. 30 μL of aliquots were added on agarose-coated slides. Cells were lysed for 1 h on ice with lysis buffer. Next, they were placed in alkaline solution for 40 min followed by electrophoresis in the same buffer at 29 V for 30 min. Slides were neutralized, dried o.n. and fixed in methanol (5 min). The samples were stained with SYBR-green

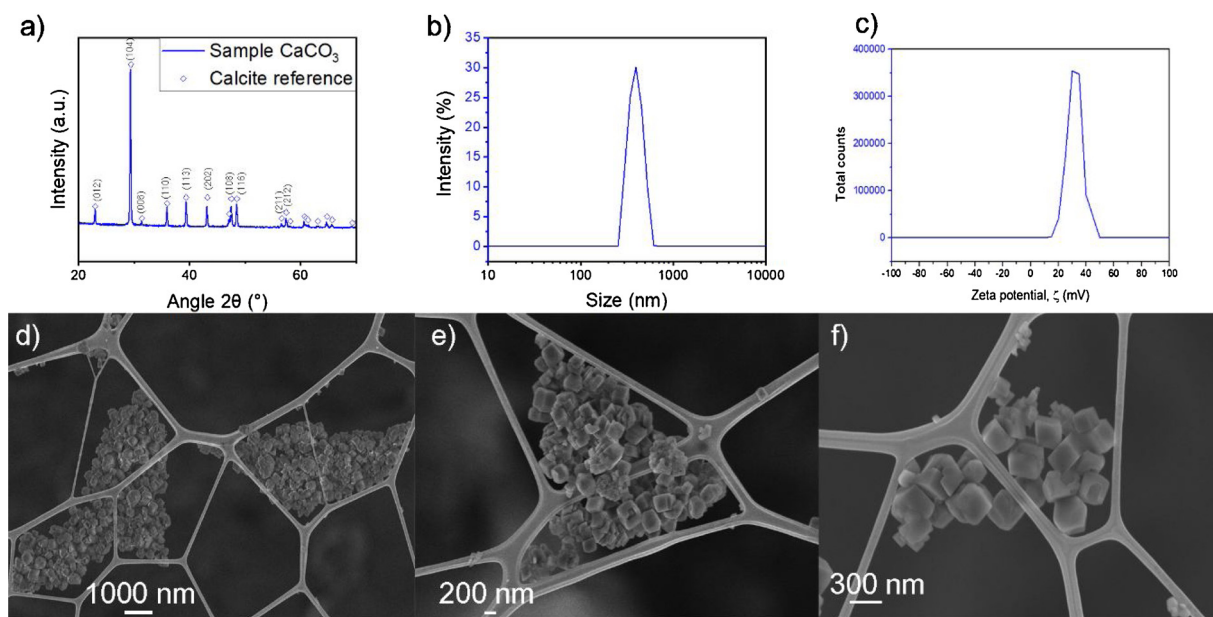


Fig. 2. CaCO_3NPs characterization. a) XRD spectra, b) CaCO_3NPs size distribution, c) Zeta potential measurement, d-f) FE-SEM micrographs.

(Thermo Fisher Scientific, Waltham, MA) and scored by means of confocal microscopy (Nikon A1, Tokyo, Japan) with an automated image analysis system. At least 100 cells were analyzed per sample. Each experiment was performed three times. The extent of DNA damage was measured as the % DNA in the tail, which represents the fraction of the total DNA that is contained in the comet tail.

2.7. *In vivo* toxicity

Adult zebrafish (*Danio Rerio*) were kept and were spawned as previously described [25]. Embryos aged 4–5 hpf were collected, rinsed with E3 medium [26] and treated with 10, 50, 100 or 200 $\mu\text{g}/\text{mL}$ of CaCO_3NPs until 120 hpf. Survival rate and hatching success were monitored every 24 h. The swimming and cardiac activities were noted at 72 hpf and the morphological changes at 96 hpf. Each experiment was performed three times. All animal experiments were performed in full compliance with the revised directive 2010/63/EU.

2.8. Statistical analysis

Results were expressed as mean \pm standard deviations of three determinations. In the *in vitro* experiments, they were tested by one-way ANOVA or Student's t-test. Statistically, a significant difference was considered at $p < 0.05$. In the *in vivo* experiments, data were tested by one-way analysis of variance (ANOVA) in combination with Holm-Sidak post hoc to compare each treatment group with the negative controls. Statistically, a significant difference was considered at $p < 0.01$.

3. Results and discussion

3.1. Characterization of CaCO_3NPs

The synthesized CaCO_3 phase purity was characterized by XRD and compared to reference data and literature [27]. Fig. 2a shows the results from this analysis. The diffraction peaks are well consistent with pure calcite pattern, which is the most stable CaCO_3 crystalline phase [28–30]. No presence of other CaCO_3 crystalline phases, such as aragonite and vaterite, was determined. In fact, vaterite and aragonite phases could present some issues when employed as fillers, since they can solubilize and re-precipitate as calcite because of their greater

instability. The sharpness of these peaks indicates the highly crystalline nature of the material. Fig. 2b shows a narrow size distribution (PSD), which is in a good agreement with the FESEM micrographs (Fig. 2d–f), where nanosized cubic calcite primary particles are seen. Zeta potential of -30 mV indicates that the particles are stable and do not agglomerate [20,21]. These particles have a cubic shape as seen in the FESEM micrographs (Fig. 2d–f), which is the classical morphology of calcite crystals [28,31]. Furthermore, this experimental setup provides CO_2 conversions equal to 18 %, which could mean a significant decrease in carbon footprint of the cement industry.

3.2. Toxicity evaluation

3.2.1. *In vitro* studies

3.2.1.1. Cellular viability. The cytotoxicity profile of CaCO_3NPs was determined in NIH 3T3 and MCF7 cells through WST1 cell metabolism-based assay to evaluate the mitochondrial activity for estimating cell proliferation and viability. The WST1 test estimates the cell metabolic activity via the reduction of salt to formazan and the amount of formazan is directly correlated to the number of metabolically active cells.

Different concentrations of CaCO_3NPs ($1\text{--}50$ $\mu\text{g mL}^{-1}$) were exposed to the two cell lines for 12, 24, 48, and 72 h. As shown in Fig. 3a, CaCO_3NPs did not enhance the WST1 reduction in the NIH 3T3 cells at different time points (Fig. 3a). Besides, the WST-1 did not form crystals with CaCO_3NPs in MCF7 cells was not affected by the presence of CaCO_3NPs (Fig. 3b). The evaluation of the WST-1 assays showed no cytotoxic effects of CaCO_3NPs at high concentrations (50 $\mu\text{g mL}^{-1}$) and long incubation time (72 h) in normal and cancer cells. Both cell lines were not susceptible to, CaCO_3NPs showing a significant cell viability increase for all the analyzed concentrations after 72 h and in some cases also after 48 (asterisks in Fig. 3a and b).

3.2.1.2. Oxidative reactivity. After a preliminary assessment of the potential harmful effects of CaCO_3NPs by cell viability on two different cell lines, we next evaluated their possible induction of intracellular oxidative stress and DNA damage. The ability of CaCO_3NPs to produce reactive oxygen species (ROS) was measured with the DCFH-DA assay. The experimental conditions were similar to the ones of cell viability studies. We exposed NIH 3T3 and MCF7 cells to different concentrations of CaCO_3NPs ($1, 5, 10, 25$ and 50 $\mu\text{g mL}^{-1}$) for

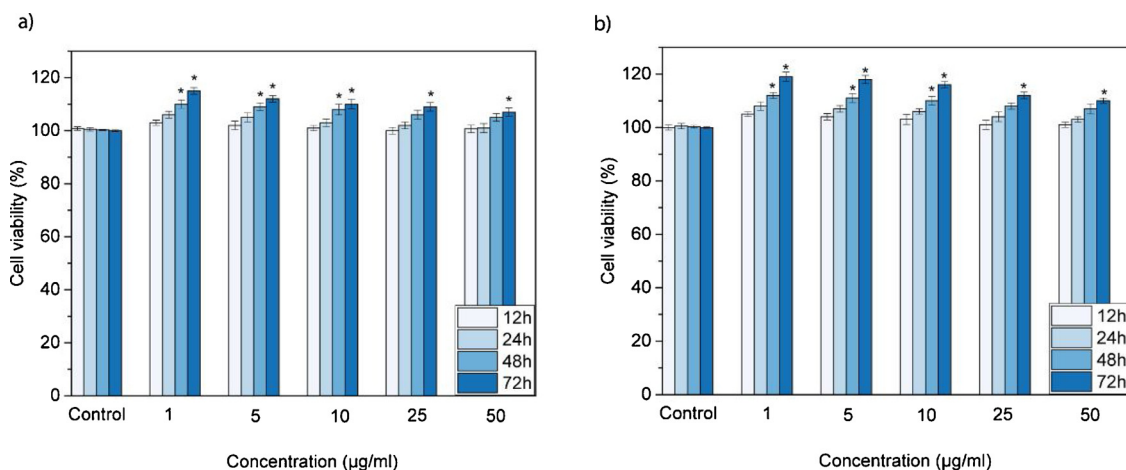


Fig. 3. (a) Cell viability of NIH 3T3 and (b) MCF7 cells treated with different concentrations (1, 5, 10, 25 and 50 µg mL⁻¹) of CaCO₃NPs for different times. Data are presented as the mean ± SD of three independent experiments (*p ≤ 0.05 in comparison to the control).

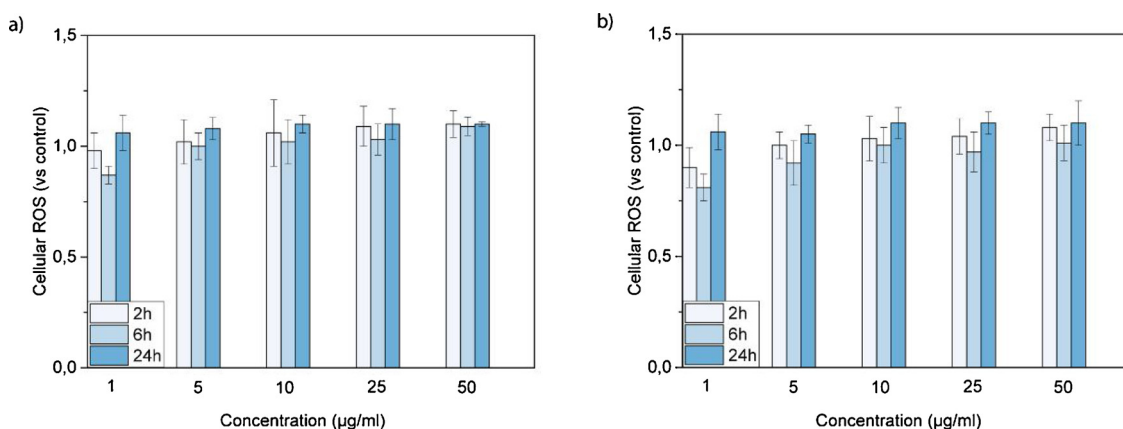


Fig. 4. (a) Intracellular ROS generation in (a) NIH 3T3 and (b) MCF7 cells treated with CaCO₃NPs revealed using the DCFH-DA assay. The ROS increase was calculated as the mean slope per min and normalized to the unexposed control. Data are represented as the mean ± SD of three independent experiments.

2, 6 and 24 h. None of the CaCO₃NPs doses caused any significant increase in the reactive oxygen species after the three different time points of exposure in NIH 3T3 (Fig. 4a) and MCF7 cells (Fig. 4b). These results indicated that CaCO₃NPs did not induce oxidative stress in both cell lines, confirming the non-cytotoxicity of this nanomaterial.

3.2.1.3. Genotoxicity. The level of DNA damage in NIH 3T3 and MCF7 cells induced by CaCO₃NPs was evaluated by using the comet assay. NIH 3T3 and MCF7 cells were treated with 1, 5, 10, 25 and 50 µg mL⁻¹ of CaCO₃NPs for 6 and 24 h. As shown in Fig. 5, no DNA damage was induced at any concentration and time point in both cell lines. The tail intensities obtained for the different concentrations of CaCO₃NPs at 6 and 24 h were comparable with the values of the negative control samples. However, a significant difference with the controls was observed at the higher concentration of CaCO₃NPs. Cells showed again no toxic effects induced by CaCO₃NPs treatment.

3.2.2. In vivo studies

All the *in vitro* studies performed to assess the impact of CaCO₃NPs on cells have demonstrated the high cyto-biocompatibility of these particles. However, since the use of CaCO₃NPs will lead to human exposure, we complete their risk assessment in zebrafish during the development.

To evaluate the developmental toxicity of CaCO₃NPs, zebrafish embryos were treated with different concentrations of CaCO₃NPs (10, 50, 100 and 200 µg mL⁻¹). The survival rate, hatching success, swimming, and cardiac activities and morphological changes were

analyzed during the early embryonic growth. CaCO₃NPs did not affect the survival rate (Fig. 6a), even if a significant difference (p ≤ 0.01) was observed at 120 hpf, at higher concentration CaCO₃NPs (200 µg mL⁻¹). Correspondingly, the hatching success was not delayed or inhibited from the treatment with the different concentrations of CaCO₃NPs (Fig. 6b), presenting a significant difference only at 48 and 120 hpf at high concentration. Embryos hatched in their normal temporal window, between 48 and 72 hpf. The high values of survival and hatching rates revealed no significant toxic effects of CaCO₃NPs on these two biological parameters.

Moreover, the swimming and cardiac activities of larvae of 72 hpf were monitored. Exposure to CaCO₃NPs induced no significant effects on heartbeats. Spontaneous movements of treated larvae remained unchanged in comparison with the control group (see Fig. S1a and b in the Supplementary Material). These results confirmed that CaCO₃NPs did not cause zebrafish toxicity in the temporal window of investigation.

Other reports on nanomaterials that can be employed to improve the mechanical properties of the cement showed a perturbation in all these toxicological endpoints. In particular, silica NPs [32] and CNTs [33,34] induced a decrease in the survival rate or an increase in the mortality and a delay and strong inhibition in the hatching rate. Moreover, larvae treated with silica NPs presented an altered larval locomotor activity and bradycardia [35], while larvae exposed to CNTs had a significant reduction of the number of heartbeats [33].

Besides, the morphological changes in zebrafish groups treated with CaCO₃NPs were monitored at 96 hpf. The deformities produce by

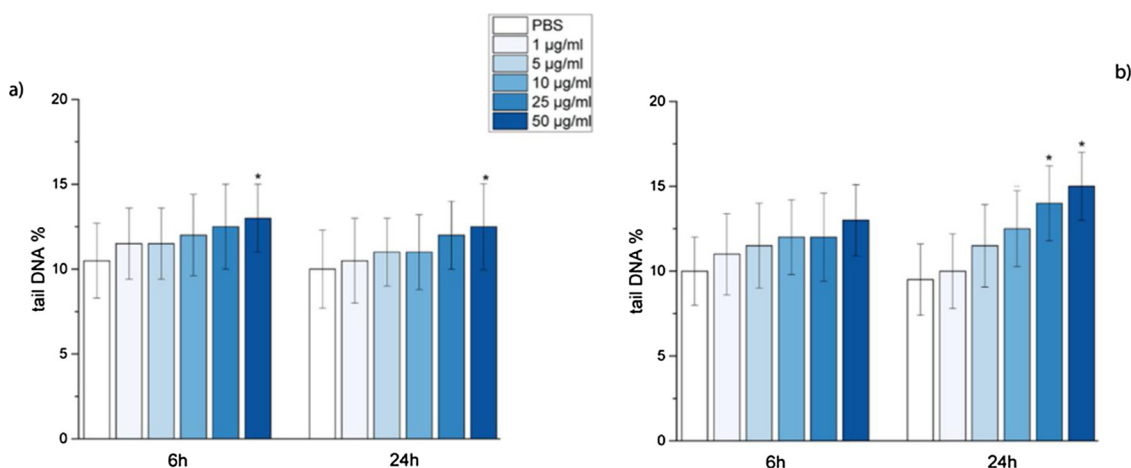


Fig. 5. Percent of DNA in the tail (tail DNA %) in (a) NIH 3T3 and (b) MCF7 cells. Data are represented as the mean \pm SD of three independent experiments (* $p \leq 0.05$ in comparison to the control).

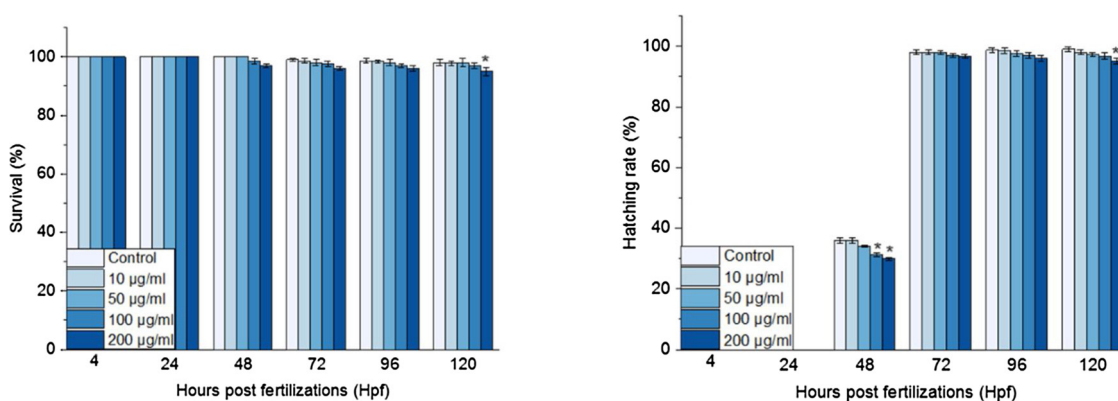


Fig. 6. Survival (a) and hatching rates (b) of zebrafish exposed to CaCO_3NPs . Data are presented as the mean \pm SD of three independent experiments $n = 80$ (* $p \leq 0.01$ in comparison to the control).

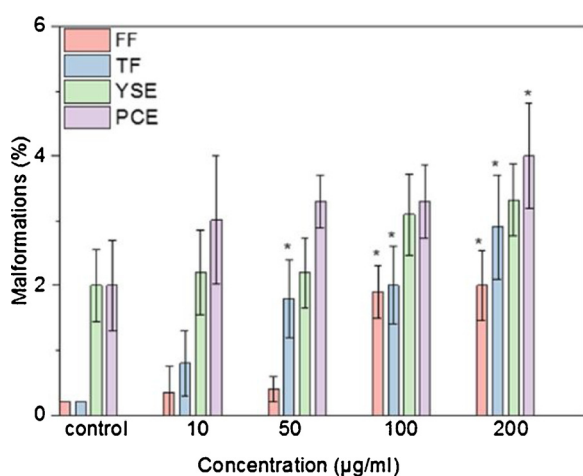


Fig. 7. Malformations of zebrafish induced by CaCO_3NPs . Data are presented as the mean \pm SD of three independent experiments $n = 80$ (* $p \leq 0.01$ in comparison to the control).

CaCO_3NPs , such as pericardial edema (PCE) and yolk sac edema (YSE), fin fold flexure (FF) and tail flexure (TF), were the most common induced by other classes of nanoparticles used as additives to the cement-based materials or other applications [32,36] (Fig. 7). As the concentration increases, the malformations percentages became higher. Even if significant differences between the phenotypes of samples of control and treated one was detected for some abnormalities and

endpoints (asterisks in Fig. 7), the total percentage of malformations was still $< 4\%$, indicating the absence of toxic effects.

In conclusion, the zebrafish embryo/larvae screening showed that CaCO_3NPs are inert. These *in vivo* findings agreed with the previous *in vitro* results, confirming the complete safety of CaCO_3NPs produced from CO_2 .

4. Conclusions

In the present study, we have reported on the fabrication of calcium carbonate nanoparticles from CO_2 and their toxicity assessments in cultured cells and more complex biological systems. We have shown that CaCO_3NPs can be easily and rapidly synthesized from a CaO slurry. Moreover, we have demonstrated that our CaCO_3NPs possess a high cyto-biocompatibility in normal and cancer cell lines. The cell viability presented high values and no cell death or increase in the levels of reactive oxygen species or DNA damage were detected on the two different cell lines. To gauge the safety of CaCO_3NPs with respect to human exposure, we investigated the specific interaction of the nanoparticles with zebrafish, as vertebrate models. We demonstrated the excellent biocompatibility of CaCO_3NPs in zebrafish during early development, reporting no mortality, inhibition of hatching, cardiac effects and no perturbation in the locomotor activity in embryos and larvae treated even at the higher concentration of nanoparticles evaluated. In conclusion, our work reported for the first time the complete biosafety of CaCO_3NPs derived from CO_2 , suggesting that they can be employed as additives in the materials cement-based without any exposure risks on the environments and human health.

Author contributions section

M.d.A and S.G. conceived the idea and designed the experiments. F.L. performed the synthesis and characterization of CaCO₃NPs. M.d.A. performed all the biological experiments. F.A.D and S.B. supervised the synthesis and characterization of CaCO₃NPs. S.G. supervised the biological experiments. M.d.A. wrote the paper. S.G and S.B. contributed to revise the paper. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.colsurfb.2020.110947>.

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