

Biocompatibility of Porous Spherical Calcium Carbonate Microparticles on Hela Cells

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ABSTRACT

Recently there has been a wide concern on inorganic nanoparticles as drug delivery carriers. CaCO₃ particles have shown promising potential for the development of carriers for drugs, but little research had been performed regarding their safe dosage for maximizing the therapeutic activity without harming biosystems. In this study, we assessed the biological safety of porous spherical CaCO₃ microparticles on Hela cells. The reactive oxygen species (ROS), glutathione (GSH), carbonyl content in proteins (CCP), DNA-protein crosslinks (DPC) and cell viability were measured. Results showed that with the exposure concentration increase, ROS and CCP in Hela cells presented a significant increase but GSH contents in Hela cells and cell viability showed a significant decrease respectively compared with the control. DPC coefficient ascended, but no statistically significant changes were observed. The results indicated that porous spherical CaCO₃ microparticles may induce oxidative damage to Hela cells. But compared with other nanomaterials, porous spherical CaCO₃ appeared to have good biocompatibility. The results implied that porous spherical calcium carbonate microparticles could be applied as relatively safe drug vehicles, but with the caveat that the effect of high dosages should not be ignored when attempting to maximize therapeutic activity by increasing the concentration.

Keywords: Calcium Carbonate Microparticles; Hela Cells; Biological Safety

1. Introduction

During the past decades, inorganic nanoparticles as drug delivery carriers have attracted much attention in modern pharmaceutical and medication area. Many inorganic materials, such as calcium phosphate [1], colloidal gold [2], carbon nanotubes [3], silicon [4], iron oxide [5] and layered double hydroxide (LDH) [6] have been studied.

In addition to the conventional applications in toothpastes and cosmetics, paper industry, and water treatment as filtering materials, CaCO₃ particles have also shown potentiality for the development of carriers for drugs [7-12]. Y. Ueno [10] *et al.* incorporated betamethasone phosphate (BP) and erythropoietin into nano-CaCO₃ particles, which were chemically stable and released very slowly. Chaoyang Wang [11] *et al.* loaded amorphous ibuprofen in the pores of the CaCO₃ microparticles which had a rapider release in the gastric fluid and a slower release in the intestinal fluid. Caiyu Peng [12] *et al.* prepared carboxymethyl cellulose (CMC)-doped CaCO₃ microparticles with an average diameter of 5 μm and coated them

by chitosan and alginate multilayers. These particles could spontaneously load positively charged doxorubicin (DOX) molecules whose releasing from the CaCO₃ microparticles could be sustained to more than 150 h. Moreover, the multilayer coating could lower down the release amount of the loaded DOX within the same incubation time.

With the development of new drug delivery systems, both the positive and negative sides of nanotechnology appeared. The need to maximize therapeutic activity may lead to negative side effects. They could exhibit unexpected toxicity to living organisms [13-15]. It had been mentioned in some reports that CaCO₃ microparticles was relatively safe drug carriers, but little research had been performed regarding its safe dosage for maximizing the therapeutic activity without harming biosystems.

Currently, oxidative stress is a well-defined paradigm to explain toxic effects induced by nanomaterials [16]. The generation of reactive oxygen species (ROS) and the formation of oxidative stress are the best developed model to explain the toxic effects of nanomaterials [17]. Oxidative stress refers to a state in which glutathione (GSH) is depleted while oxidized glutathione accumu-

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lates. In addition, DNA-protein crosslinks (DPC) can also be used as biomarkers for assessing group modifications on DNA and protein and can be regarded as a signal of cancer [18,19]. Thus, in the present study, the reactive oxygen species, DNA-protein crosslinks and cell viability were measured to access the biological safety of porous calcium carbonate microparticles on HeLa cells.

2. Experimental Detail

2.1. Cell Culture and Exposure to CaCO₃ Particles

HeLa cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% newborn bovine serum (NBS, GIBCO), penicillin and streptomycin (each at 100 mU/mL) at 37°C in a humidified atmosphere containing 5% CO₂. CaCO₃ were dispersed in a complete culture medium with 10% NBS at final concentrations of 50, 100, 200 and 400 µg/mL. Before exposure to cells, all suspensions were sonicated for 40 min. Cells were then inoculated into the medium containing different concentrations of nanomaterials and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 12 h.

2.2. Intracellular ROS Measurement

The formation of intracellular ROS was measured by monitoring the changes in 2',7'-dichlorofluorescein-diacetate (DCFH-DA, Calbiochem) fluorescence. This membrane permeable dye enters the cell where intracellular esterases cleave off the diacetate group and the resulting DCFH retained in the cytoplasm and oxidized to DCF by ROS. A 10 mM DCFH-DA stock solution (in dimethyl sulfoxide) was diluted 1000-fold in PBS to yield a 10 µM working solution. After 12 h exposure to CaCO₃ suspension, cells in 96-well plate were washed twice with phosphate buffered saline (PBS, pH 7.4) to remove the CaCO₃ microparticles and then incubated with 150 µL DCFH-DA working solution at 37°C for 30 min. Fluorescence was determined using a fluorescence reader (FLx 800, Multi-Detection Microplate Reader, Bio-Tek Instrument Inc., USA) with excitation and emission at 485 nm and 528 nm, respectively [20].

2.3. Intracellular GSH Measurement

The concentration of GSH in cells was measured by 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, Sigma) method. After 12 h exposure to CaCO₃ suspension, cells in 6-well plate were suspended in 1 mL of PBS, frozen at -20°C and thawed at 37°C for three circles. Centrifuged at 10,000 rpm for 10 min and collected the supernatant. 200 µL of supernatant were mixed with 50 µL of 10% trichloroacetic acid (TCA). Centrifuged at 10,000 rpm for 5 min and collected the supernatant. 50 µL of the supernatant was obtained and placed into 96-well plate,

which was reacted with 150 µL of DTNB (60 µg/mL) for 5 min in dark place. The intracellular GSH reacted with DTNB to give TNB. The total TNB formed was determined by measuring the absorption at 412 nm with a microplate spectro-photometer (Power Wave XS, Bio-Tek Instrument Inc., USA).

2.4. DNA and Protein Crosslink (DPC) Assay

KCl-SDS method was used to detect DNA damage and the DPC content induced by CaCO₃ in the HeLa cell line. After 12 h exposure to CaCO₃ suspension, cells were harvested by centrifugation at 6000 rpm for 3 min, resuspended in 0.5 mL of PBS, and lysed using 0.5 mL of 2% SDS solution and gentle vortexing. Proteins were precipitated by KCl solution and digested by proteinase K (Amresco). The methods used here were based on Liu and Collins's report [21,22] with minor modification. Fluorescence was measured using a fluorescence spectrofluorimeter (F-4500 Microplate Scanning Spectrophotometer Operator, Bio-Tek Instrument Inc., USA) with excitation and emission at 350 nm and 450 nm, respectively, for measuring DNA and DPC. The sample DNA contents were determined quantitatively via a DNA standard curve generated from calf thymus DNA. The DPC coefficient was calculated as a ratio of the percentage of the DNA involved in DPC over the percentage of the DNA involved in DPC plus the unbound DNA fraction.

2.5. Carbonyl Content in Proteins (CCP) Assay

2,4-dinitrophenylhydrazine (2, 4-DNPH, Sigma) colorimetry was employed to detect CCP in HeLa cells. After 12 h exposure to CaCO₃ suspensions, cell proteins were collected by freezing cells at -20°C for 1 h and then melted at 37°C for three cycles, dispersed into suspension by minishaker, and then centrifugated at 3000 rpm for 5 min. The method followed Shiou-sheng Chen's report [23]. The absorbance of the samples at 370 nm was measured and the CCP was calculated by using Beer's Law.

2.6. MTT Assay

The MTT assay was applied to investigate the cytotoxicity of CaCO₃ particles. Cells were seeded in a 96-well plate at an initial density of 1×10^4 cells/well, incubated for 24 h, exposed to different concentrations of materials for 36 h, and then 20 µL of MTT solution (5 mg/mL) was added to each well. After incubation at 37°C in the dark for 4 h, the medium was removed, 150 µL of DMSO was added, and the absorbance at 570 nm was measured with a microplate spectrophotometer (Power Wave XS, Bio-Tek Instrument Inc., USA).

Cell viability was calculated using the Equation (1):

$$\text{Cell viability (\%)} = [A]_{\text{test}} / [A]_{\text{control}} \times 100\% \quad (1)$$

2.7. Data Analysis

Averages and standard deviations were based on 5 samples and all tests performed in triplicate. Test data for statistical treatment were performed by software Origin version 6.0. Results were evaluated statistically using the analysis of variance (ANOVA) followed by Tukey test. P values less than 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1. Characterization of CaCO₃ Particals

The toxicity of a nanomaterial is closely related to its size, shape and structure [24], so it is necessary to characterize material before the toxicity tests. As it is shown in the SEM image (**Figure 1**), the CaCO₃ particals is spherical with a diameter of 2 μm . The surface of the particles is very rough and consists of large number of nanometer-sized pores and channels. The specific morphology would offer a unique opportunity to capture biomacromolecules such as drugs which can permeate into the particles, or by the affinity to the carbonate surface, enabling very high substrate loading.

3.2. ROS Generation

ROS generation and oxidative stress are regarded as the best developed paradigms to explain the toxic effects of nanomaterials. ROS is a natural byproduct of the normal metabolism in cells. A proper amount of ROS could function as a second messenger in the signal transduction of healthy cells [25]. However, excessive ROS damages biomolecules, triggers the apoptosis pathway, and even further induces cell death.

Assessment of ROS generation following 12 h of exposure to CaCO₃ microparticles (0, 50, 100, 200, and 400 $\mu\text{g}/\text{mL}$) showed that the DCF-fluorescence intensity which represented the concentration of ROS increased slowly in a concentration-dependent manner (**Figure 2**). When the concentrations of CaCO₃ microparticles were

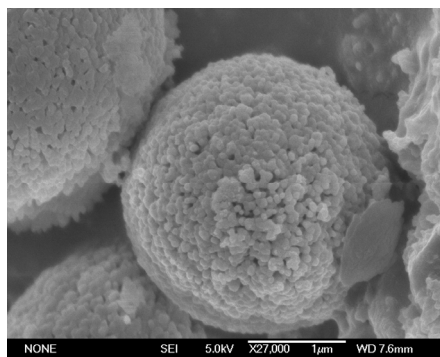


Figure 1. Scanning electronic microscopy (SEM) image of CaCO₃ particals.

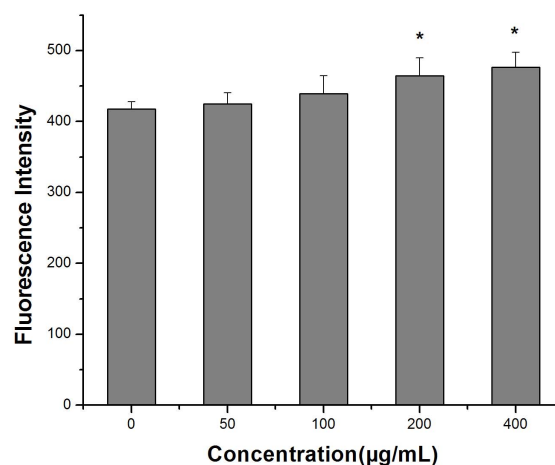


Figure 2. DCF-fluorescence intensity of HeLa cells after 12 h exposure to CaCO₃ suspensions. (* $p < 0.05$, compared with control).

more than 200 $\mu\text{g}/\text{mL}$, the fluorescence intensity was significantly enhanced (200, and 400 $\mu\text{g}/\text{mL}$, $p < 0.05$, compared with the control), which indicated that CaCO₃ microparticles may introduce excess ROS which may cause damage to cells at certain higher concentrations.

The surface chemistry and reactivity of nanometer-sized pores and channels are important considerations for ROS production. The interaction of active sites with molecular dioxygen may possibly lead to the formation of the additional ROS through dismutation or Fenton chemistry [26]. The toxic effects induced by nanoparticles via a mechanism involving the ROS generation had been reported in previous studies. Several nanoparticles such as Fe₂O₃ [27], TiO₂ [28], ZnO [29] and MnO₂ [30] nanoparticles were found to introduce irregular ROS levels in living organisms, subsequently leading to the toxicity. However, compared with these nanoparticles CaCO₃ need even higher concentration to introduce excess ROS.

3.3. Intracellular GSH Content

Reduced glutathione (GSH) is the major free sulfhydryl groups containing molecule in cells and is involved in detoxification of xenobiotics, removal of ROS and maintenance of oxidation state of protein sulfhydryl groups. It is the key antioxidant presenting in most of the cells. Cells under normal conditions have natural ability to scavenge ROS effects. However, under conditions of excess ROS production, the natural antioxidant defenses, such as glutathione (GSH) and antioxidant enzymes may be overwhelmed [31].

To further explore the relationship between oxidative stress and CaCO₃ concentration, the GSH contents in HeLa cells were studied. As shown in **Figure 3**, with the increase of CaCO₃ concentration, GSH contents in HeLa cells showed a tendency to ascend at ≤ 100 $\mu\text{g}/\text{mL}$ and

descend $>100 \mu\text{g/mL}$. While at $200 \mu\text{g/mL}$ resulted in a significant decrease ($p < 0.05$). The data here suggested that, with ROS increase, the antioxidant defense system may be triggered and this effect may reach the maximum at $100 \mu\text{g/mL}$. However, antioxidant defense system may not be destroyed if CaCO_3 concentration was $<200 \mu\text{g/mL}$. Compared with other nanomaterials, CaCO_3 appeared to have good biocompatibility. For example, with only $10 \mu\text{g/mL}$ concentration, nano- SiO_2 [32] could induce significant ROS increase and GSH depletion, and nano-Ag [33] can produce some harmful effects to the antioxidant defense system at $50 \mu\text{g/mL}$.

3.4. DPC Assay

As shown in **Figure 4**, In the DPC assay, with the CaCO_3 concentration increased, DPC coefficient ascended, but no statistically significant changes were observed for all CaCO_3 exposures compared with the control.

Exposure of cells to CaCO_3 microparticles resulted in the generation of ROS (**Figure 2**). These ROS may be

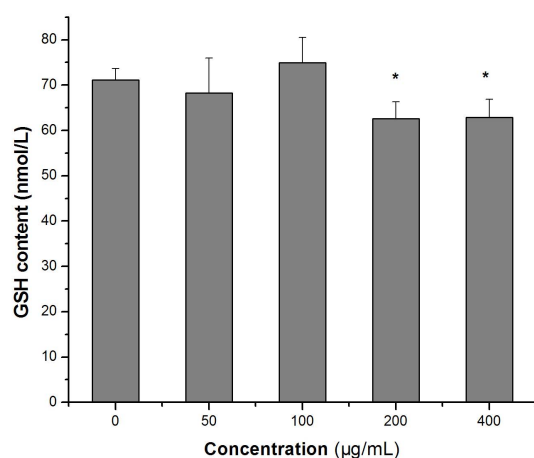


Figure 3. GSH content of HeLa cells after 24 h exposure to CaCO_3 suspensions. (* $p < 0.05$, compared with control).

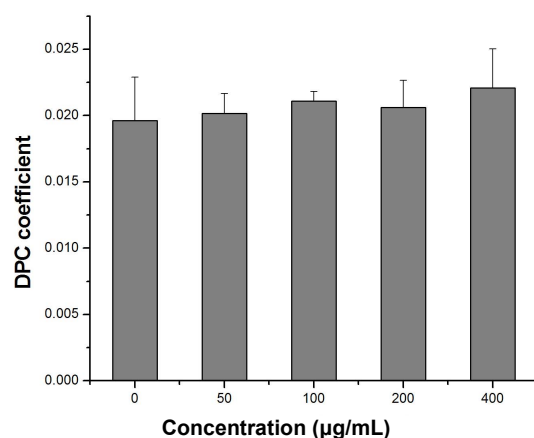


Figure 4. DNA and protein crosslink assay of HeLa cells after 12 h exposure to CaCO_3 suspensions.

localized within a short distance of each other and of the DNA. Many of ROS, including the extremely reactive hydroxyl radical, will be generated at high levels within small discrete regions known as spurs, blobs, and short tracks [34], which can react with DNA or protein and create crosslinks between proteins and DNA.

The covalent crosslinking of proteins to DNA was expected to interrupt DNA metabolic processes such as replication, repair, recombination, transcription, chromatin remodeling, etc. Unfortunately, the biological consequences of DPCs were hampered by the fact that no agent exclusively induced these lesions in genomic DNA [35]. Nonetheless, several studies had reported that the induction of DPCs by many agents correlated with genetic damage such as sister chromatid exchanges (SCEs), transformation, and cytotoxicity [36-40]. Thus, DPCs may contribute to the genotoxic effects. The results indicated that CaCO_3 particles with concentration of less than $400 \mu\text{g/mL}$ may have no mutagenicity and genetic toxicity.

3.5. CCP Assay

The measurement of CCP showed that the carbonyl content in proteins in HeLa cells increased in a concentration-dependent manner (**Figure 5**), and $400 \mu\text{g/mL}$ of CaCO_3 treatment resulted in a significant increase of carbonyl contents in proteins ($p < 0.05$) which indicated that CaCO_3 microparticles may have harmful effects on these biomacromolecules at the highest concentrations tested.

Oxidative stress and reactive oxygen species (ROS) play an important role in the progression of a number of human diseases [41]. The generation of ROS may occur through a large number of physiological or nonphysiological processes, which include their generation as

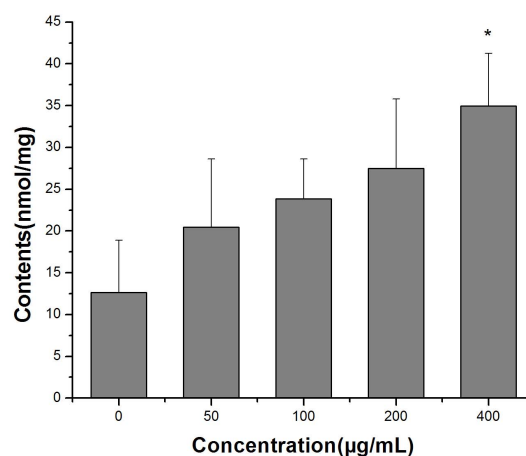


Figure 5. Carbonyl content in proteins (CCP) of HeLa cells after 24 h exposure to CaCO_3 suspensions. (* $p < 0.05$, compared with control).

by-products of normal cellular metabolism, primarily in the mitochondria. Excess ROS may damage all types of biological molecules. Oxidative damages to proteins, lipids, or DNA may all be seriously deleterious and may take place concomitantly [42].

3.6. Cytotoxicity of CaCO₃ Microparticles

Drug delivery systems require biocompatible inorganic matrices that permit safe retention as well as controlled drug delivery. CaCO₃ appear to fit these conditions.

To evaluate the CaCO₃ microparticles induced cytotoxicity, an MTT assay was conducted to determine the viability of the treated Hela cells. As shown in **Figure 6**, even at the concentration of 400 µg/mL, the viabilities of the treated cells were 89.6%. Compared with MnO₂ [30] at the same concentration the viabilities of Hela cells were only 32.45%. The results showed that porous spherical CaCO₃ microparticles had only a little cytotoxicity and could be applied as relatively safe drug vehicles.

4. Conclusion

Results showed that at the high level of CaCO₃ exposure, DCF-fluorescence intensity and carbonyl content in proteins (CCP) presented a significant increase compared with the control, DNA and protein crosslinks (DPC) coefficient were also ascended, but no statistically significant changes were observed. Furthermore, GSH contents in Hela cells and cell viability were showed a significant decrease compared with the control. But compared with other nanomaterials, CaCO₃ appeared to have good biocompatibility. Therefore this paper implied that CaCO₃ microparticles could be applied as relatively safe basic materials for drug vehicles, but with the caveat that the effects of high dosages should not be ignored when at-

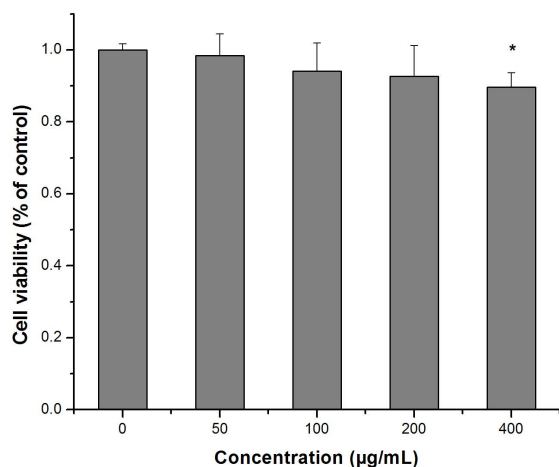


Figure 6. Results of MTT assay of Hela cells after 36 h exposure to CaCO₃ suspensions. (* $p < 0.05$, compared with control).

tempting to maximize therapeutic activity by increasing the concentration.

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