Original Research

Carboxylate microsphere-induced cellular toxicity in human lung fibroblasts

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Abstract

Carboxylate microspheres (CMs) are mainly used in industrial, biomedical and various household products. In this study, we assessed the cytotoxic effects of CMs on human MRC-5 lung fibroblasts by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Oxidative stress was determined by measurements of reactive oxygen species and antioxidant (superoxide dismutase and catalase) levels and proinflammatory cytokines quantified by enzyme-linked immunosorbent assay. Morphological changes were examined by light microscopy, confocal microscopy and transmission electron microscopy. The lung fibroblasts were exposed to increasing concentrations of CMs (0.1–1000 μmol/L) for 24 h. The results showed significant changes in cell morphology with induction of cytotoxicity and oxidative stress observed in 10–1000 μmol/L concentrations *of* CM-treated fibroblasts. Ultrastructural examination revealed the presence of CMs inside the cytoplasm of treated lung fibroblasts. CMs also induced elevated interleukin (IL)-1, IL-6, IL-8, IL-10 and tumor necrosis factor α levels at higher concentrations. We have demonstrated that CMs significantly reduce cell viability in a dose-dependant manner in lung fibroblasts at 0.1–1000 μmol/L doses. The findings suggest that high doses of CMs have the potential to induce cellular toxicity to the lung *in vitro*.

Keywords: carboxylate microspheres, cytotoxic, oxidative stress, cytokines, lung fibroblasts

Experimental Biology and Medicine 2012; **237**: 635–643. DOI: 10.1258/ebm.2012.011310

Introduction

Microspheres are generally defined as small spherical particles or microparticles made of any material, with diameters in the micrometer range (1–1000 μmol/L). Microspheres have many applications in medicine such as encapsulation of drugs or protein-loaded microspheres which are delivered to the target area.[1,2] Biocompatible polymer solutions such as alginate microspheres that form stable hydrogels are used for continual delivery of therapeutic agents (including cells) for regenerative medicine.[3] Radioactive microspheres[4] and poly(lactide-co-glycolide) (PLGA) microspheres are suitable delivery vehicles for the treatment of cancer.[5] Chitosan and heparin microspheres provide a useful controlled release protein–drug system used in pharmaceutics.[6] Polystyrene microspheres have important applications in cell micropatterning for basic cell biology studies and the development of biosensors and lab-chip devices.[7] In contrast, carboxylate microspheres (CMs) like PLGA[5] and carboxylate-modified latex polystyrene microspheres are frequently used as colloid tracers in the laboratory.[8] Several other types of microspheres like gelatin/hydrogel matrix,[9] ganciclovir-loaded microsphere,[10] chitosan/hyaluronan scaffold integrated with PLGA[11] and scaffold-based microspheres,[12] are also used in drug delivery for bone and cartilage tissue engineering.[13–15]

Microsphere-mediated oxygen radicals are known to trigger activation of nuclear factor kappa B (NF-*k*B) and induce AP-1 up-regulation of intercellular adhesion molecules.[16] Reactive oxygen species (ROS) generated by macrophages have been shown to induce production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α),[17] which in turn can cause inflammation in organs such as the lung.[18] Murine alveolar macrophages exposed to silica microparticles are reported to produce ROS which have been suggested to mediate the release of TNF-α together with other cytokines and chemokines.[19] Several authors have reported oxidative stress, which can induce lipid peroxidation (LPx) and DNA damage,[20] as the mechanism of microsphere toxicity.[21] Increased generation

of ROS, decreased glutathione (GSH) and induction of oxidative stress-related genes and cell death by apoptosis has been seen in human bronchial epithelial cells exposed to nanoparticles.[22] Generation of ROS suggests that redox-active transition metals associated with microparticles and nanoparticles have the propensity to stimulate inflammatory processes in the lung,[23,24] causing deleterious effects such as injury and fibrosis.[25–27]

Interstitial lung disease (ILD) is a commonly encountered complication of systemic sclerosis (SSc).[28,29] SSc is characterized by tissue injury leading to excessive collagen deposition, with pulmonary disease as the leading cause of SSc-related death in patients.[30,31] ILD is subdivided into idiopathic interstitial pneumonia, of which idiopathic pulmonary fibrosis is one subset, and diffuse parenchymal lung diseases.[32] The principal defect is believed to be recurrent epithelial injury with resultant epithelial cell senescence and/or apoptosis. Epithelial injury can lead to the recruitment and activation of fibroblasts, which can be derived from resident fibroblasts, circulating fibrocytes or differentiation of epithelial cells, endothelial cells or pericytes into fibroblasts.[33] Elevated fibrocyte levels are known to be present in the blood and in the lungs of patients with pulmonary fibrosis.[34–37]

However, the toxicity and oxidative stress induced by CMs in the lung has not been well studied in the *in vitro* setting. In this study, we assessed the cytotoxicity, oxidative stress and morphological changes induced by CMs in normal human lung fibroblasts. This finding will give new insights into the mechanism underlying oxidative cell damage and morphological changes induced by CMs in cells.

Materials and methods

Preparation of microspheres

Carboxylate microspheres-yellow (CMs-Y) in 2 mmol/L sodium azide in 10 mL solution, size 20 nmol/L (Invitrogen Corporation, Carlsbad, CA, USA) were suspended in the Roswell Park Memorial Institute (RPMI) culture medium at concentrations of 0.1, 0.5, 1, 10, 100 and 1000 μmol/L dispersed by ultrasonic vibration for 15 min. To ensure that the microspheres were uniformly dispersed before use, the suspension was subjected to vortex agitation for 1 min.

Cell culture

Human fetal lung MRC-5 fibroblasts (ATCC CLL-171) purchased from the American Type Culture Collection (Rockville, MD, USA) were cultured in RPMI medium supplemented with 10% fetal calf serum (Gibco, Carlsbad, CA, USA). Cultures were grown in the presence of 100 U/mL penicillin/streptomycin in a 5% CO_2 incubator at 37°C.

Evaluation of cell viability

Cell viability was measured using 0.4% trypan blue dye.[38] MRC-5 fibroblasts were seeded at a density of 2.5×10^5 cells/well in 96-well plates (Nunc, Rochester, NY, USA) and grown for 24 h at 37°C in a 5% CO_2 incubator. CMs-Y were serially diluted (0.1–1000 μmol/L) with culture media in a 96-well microtiter plate and then incubated for 24 h. After exposure, the trypsinized cells were centrifuged at $1000 \times g$ for 5 min and 200 μL of cell suspension supernatant was mixed with 30 μL trypan blue. After a 3-min incubation at 37°C, viable cells in the culture were counted by a haemocytometer and the percentages of stained cells determined.

Cytotoxicity analysis by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The cytotoxic effect of CMs-Y was evaluated by modifying the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay from previously published methods.[39] The cells were seeded in a sterile 96-well plate (2.5×10^5 cells/well) and incubated for 24 h at 37°C with 5% CO_2. Different doses of serially diluted (0.1–1000 μmol/L) CMs-Y were suspended in culture media in a 96-well plate and incubated for 24 h. The MTT mixture (20 μL/well) was added 4 h before the end of each exposure period of CMs-Y. The absorbance of the formazan product was read at 595 nm (enzyme-linked immunosorbent assay [ELISA] reader). Wells consisting of cells and medium alone served as controls, and three replicates ($n = 3$) were used for each experiment.

Lactate dehydrogenase assay

Cytotoxicity was also measured using the lactate dehydrogenase (LDH) assay. MRC-5 fibroblasts were seeded (2.5×10^5 cells/mL) in a 96-well plate 24 h before the following treatment of different doses of CMs-Y (0.1–1000 μmol/L) dissolved in culture medium, and medium without microsphere served as a control. After incubating cells with each treatment for 48 h, release of the cytosolic enzyme LDH (Roche, Nutley, NJ, USA) as a quantification of cell cytolysis was measured using a microplate reader at 490 and 650 nm. Experiments were repeated at least three times ($n = 3$) and the values were expressed as a percentage of the media control.

ROS measurement by ELISA

Intercellular ROS was measured using 2,7-dichlorofluorescein diacetate dye (DCFH-DA, Invitrogen Corporation) detected by a fluorescence ELISA reader (Bio-Rad, Hercules, CA, USA) at an excitation of 485 nm and an emission of 530 nm. In this experiment, MRC-5 fibroblasts (1.0×10^6) were grown in six-well plates for 48 h to reach 85% confluence. They were exposed to CMs at 0.1–1000 μmol/L concentrations for 24 h in culture medium before introducing DCFH-DA (10 μmol/L) to the cells, and the mixture was then incubated for 30 min in the dark at 37°C. The intracellular ROS level was expressed by the ratio of fluorescence intensity over the quantity of protein.[40,41]

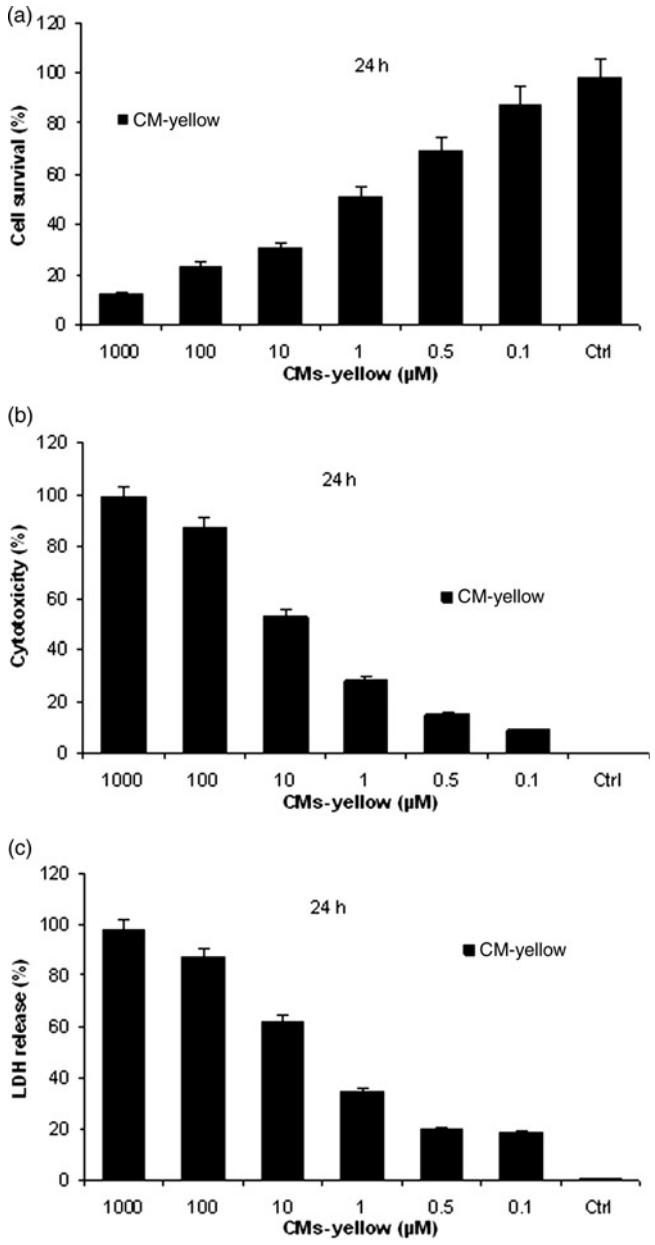

Figure 1 Cytotoxicity of carboxylate microspheres-yellow (CMs–Y) on human lung fibroblast cells (MRC-5) evaluated *in vitro*. (a) Exponentially grown lung fibroblasts were treated with various concentrations of CMs-Y ranging from 0.1, 0.5, 1, 10, 100 to 1000 μmol/L. The cell viability was measured using trypan blue dye and the percentage of cell survival was analysed after 24 h. (b) Cytotoxicity was determined by the MTT assay with lung fibroblasts exposed to different doses of CMs-Y (0.1–1000 μmol/L) for 24 h. (c) CMs-Y-induced cytolytic activity was measured using lactate dehydrogenase (LDH) enzymes released in the culture medium. Data represent mean $\pm$ SD ($n = 3$, n indicates the number of wells of a plate for each experimental condition)

Confocal microscopy

Following CMs-Y (0.1, 0.5 and 1 μmol/L) exposure, cells were thoroughly washed in 1 $\times$ phosphate-buffered solution, and fixed for approximately 30 min using 1 mL of 4% paraformaldehyde per well. After fixation, cells were stained using 4,6-diamidino-2-phenylindole for nuclear staining at 4°C before viewing under the laser scanning confocal microscope (JEOL Ltd, Tachikawa, Tokyo, Japan).

Morphological studies by transmission electron microscopy

Transmission electron microscopy of CMs-Y-treated MRC-5 fibroblasts were performed as previously described.[42,43] Cells (2×10^5 cells/mL) were seeded onto sterile cover glass. Cells were initially fixed in 2.5% glutaraldehyde followed by osmification with 1% osmium tetroxide. Samples were then dehydrated with an ascending series of ethyl alcohol (concentration 25–100%) before subsequent embedding in araldite resin. Semithin and ultrathin sections were cut using a glass knife. The ultrathin sections were doubly stained with uranyl acetate and lead citrate before viewing under an Olympus EM2085 microscope (Philadelphia, PA, USA).

Analysis of catalase activity

Cells were lysed with 1 mL of lysis buffer containing 10 μL of protein inhibitors and incubated for 10 min at 4°C. The cells were centrifuged at 10,000 $\times$ g for 15 min. Catalase (CAT) activity was measured by estimating the decrease in the H_2O_2 concentration for 15 s and reading the absorbance at 240 nm on an ELISA plate reader as described by Claiborne.[44] In brief, the reaction volume (1 mL) contained 1:1 volume ratio of sample homogenate and sodium phosphate buffer 50 mmol/L (pH 7) and 15 mmol/L H_2O_2 (omitted in the control). One unit of CAT enzymatic activity was equal to a decrease in absorbance of 0.001/min read at 240 nm.

Superoxide dismutase assay

The superoxide dismutase (SOD) activity was determined as described by Beauchamp and Fridovich.[45] In brief, the enzyme extract was added to a cocktail containing 50 mmol/L sodium phosphate at pH 7.8, 13 mmol/L methionine and 2 mmol/L riboflavin with 100 mmol/L ethylenediaminetetraacetic acid in the presence of 75 mmol/L nitroblue tetrazolium, before the absorbance was read at 560 nm.

Determination of cytokines (ELISA)

The quantitative determination of the proinflammatory cytokines interleukin (IL)-1, IL-6, IL-8 IL-10 and TNF-α were measured using a human ELISA (Anogen, Mississauga, Ontario, Canada) in cell culture supernatant of MRC-5 fibroblasts treated with CMs-Y (0.1–1000 μmol/L), with the controls. One hundred-microliter samples were introduced into a sterile 96-well microtiter plate (Nunc) containing pre-coated antibody and incubated for 1 h at room temperature before the plates were washed with 1 $\times$ wash buffer for 5 min. One hundred microliters of conjugate were dispersed to each well and incubated in the dark for 1 h at room temperature. After an 1-h incubation with 100 μL of substrate, 100 μL of stop solution was introduced into each well and mixed well. The plate was read at 450 nm using a Molecular Devices plate reader equipped with SoftMax Pro software (Research Instruments, Herndon, VA, USA) and cytokine concentrations were calculated using a standard curve.

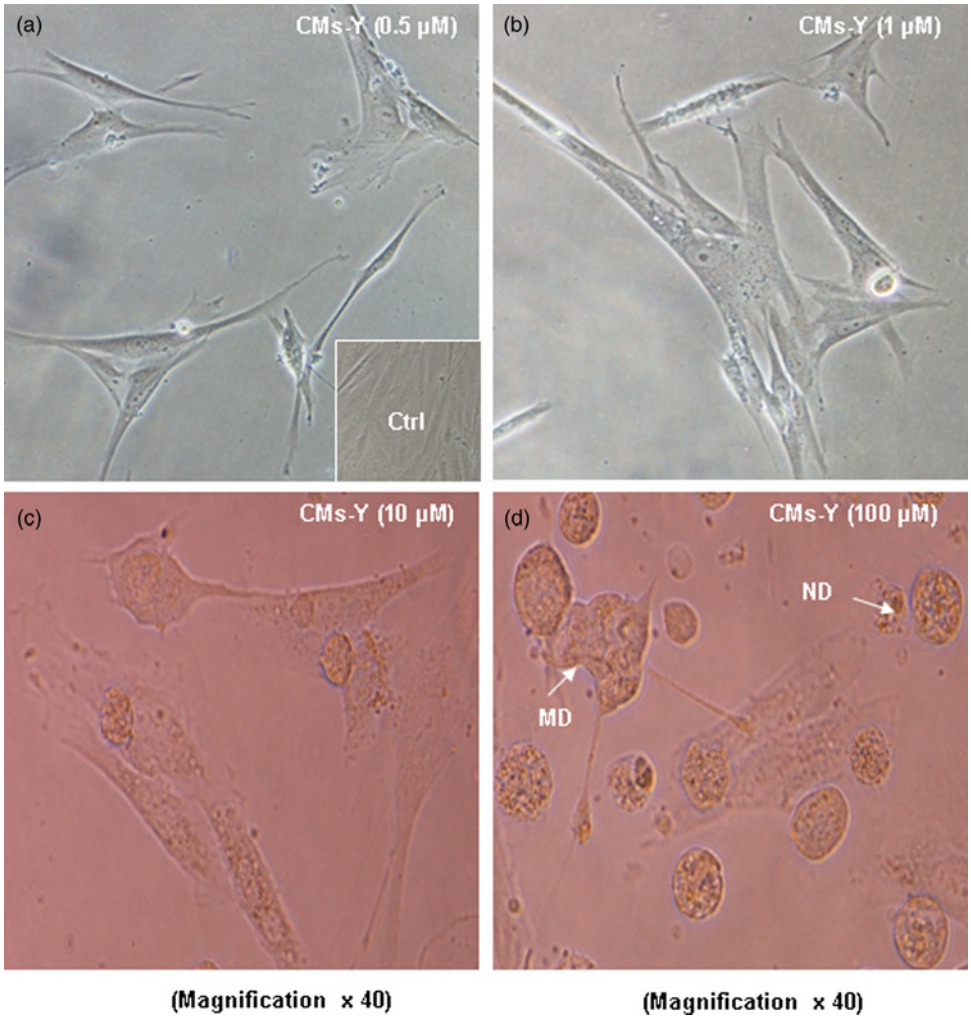

Figure 2 Light microscopy analysis of human lung fibroblasts. (a and b) Exposure of human lung fibroblast cells to lower concentrations of carboxylate microspheres-yellow (CMs-Y) (0.5–1 μmol/L) for 24 h. (c and d) Lung fibroblasts treated with higher concentrations of CMs-Y particles (10–100 μmol/L) induced morphological changes such as membrane damage and nucleus disintegration apoptosis. Ctrl, control; ND, nucleus disintegration; MD, membrane damage (magnification $\times$ 40). (A color version of this figure is available in the online journal)

Statistical analysis

Data were analyzed as mean $\pm$ SD of three independent experiments ($n = 3$). Statistical analysis was performed using the one-way analysis of variance for three or more samples and Student's *t*-test for comparison of two samples. Values were determined to be significant at $P < 0.01$ and $P < 0.05$.

Results

In vitro cytotoxicity

Cells exposed to CMs-Y (0.1–1000 μmol/L) showed gradual reduction of cell viability and an increased level of cytotoxicity after 24 h (Figure 1a). Higher doses (10, 100 and 1000 μmol/L) of CMs-Y treatment induced a remarkable reduction of cell growth and percentage of toxicity (Figure 1b). The total percentage of total cell counts was significantly higher in the lower dose treatments of CMs at 0.1, 0.5 and 1 μmol/L, than for the cells exposed to 10–1000 μmol/L doses of CMs ($P < 0.05$). There was a concomitant increase of LDH being released into the medium at higher doses of CMs-Y treatment (Figure 1c). There were no morphological changes observed at 0.5–1 μmol/L doses of CMs-Y-treated cells by light microscopy (Figures 2a and b), but higher doses caused disruption of cellular membrane and nuclear disintegration (Figures 2c and d). Fluorescence microscopic examination showed that the CMs-Y accumulated densely in the cytoplasm after a 24-h incubation without altering the cell and nuclear morphology (Figures 3a–d). Ultrastructural examination revealed aggregation of CMs-Y inside vesicles in the fibroblasts, which may contribute to the cytotoxicity of MRC-5 cells (Figures 4b and c compared with Figure 4a). Treatment with lower doses of CMs-Y appears not to have an acute effect on the cells after 24 h of treatment.

ROS production and LPx

The effects of CMs-Y-induced H_2O_2 generation and the levels of LPx (MDA) in the lung cells are shown in Table 1. There was a dose-dependent increase of ROS production when exposed to CMs-Y. Concomitantly,

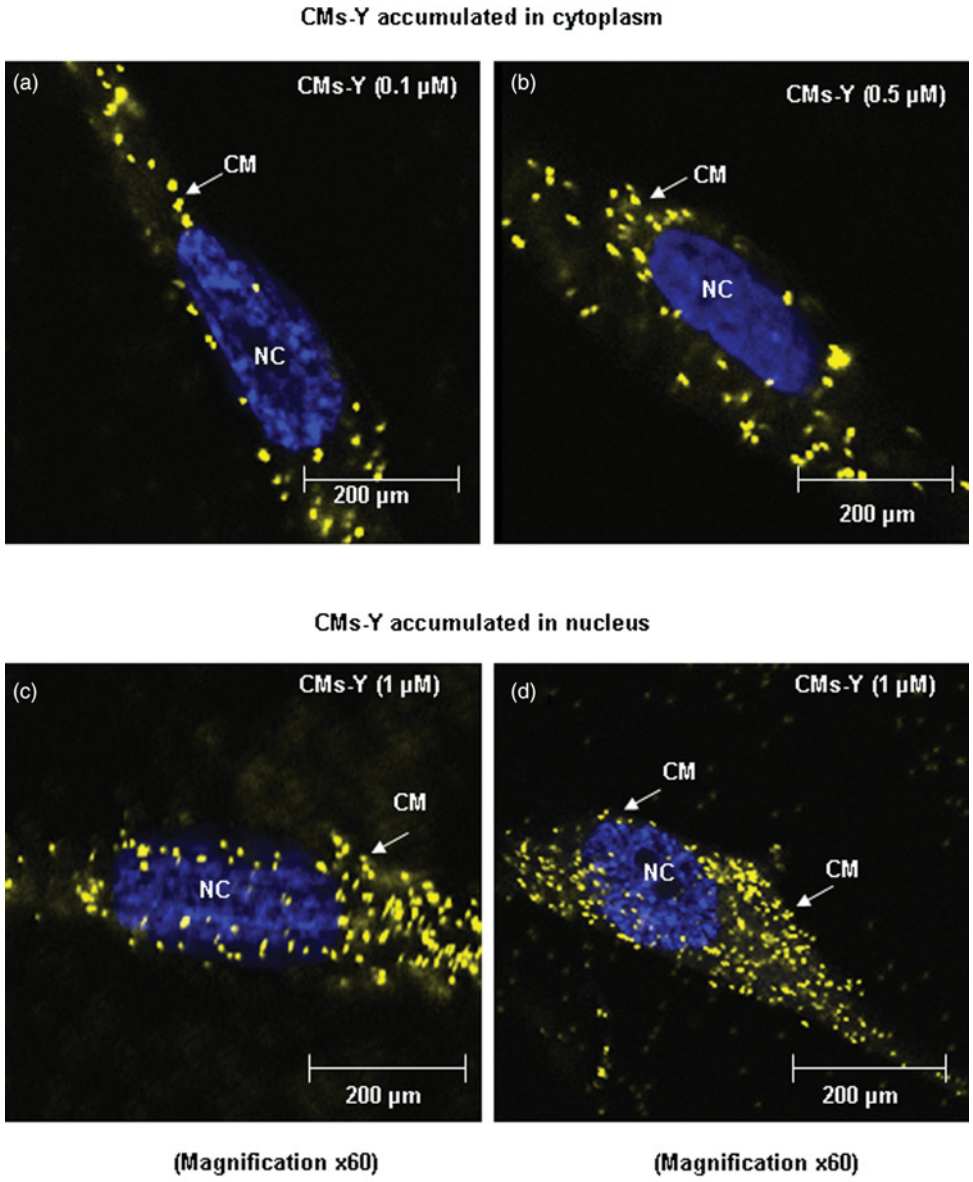

Figure 3 Fluorescence microscopy of lung fibroblasts exposed to CMs-Y. (a and b) Cells exposed to 0.1 and 0.5 μmol/L carboxylate microspheres-yellow (CMs-Y). (c and d) Cells exposed to 1 μmol/L CMs-Y. Microspheres mostly accumulated in the cytoplasm and nuclei of cells at higher concentrations. NC, nucleus; CM, cell membrane (magnification $\times$60). (A color version of this figure is available in the online journal)

microsphere-treated MRC-5 fibroblasts also exhibited a significantly increased level of lipid hydroperoxide production at high concentrations.

Antioxidant enzyme activities

CMs-Y treatment induced more antioxidant enzyme activity at lower concentrations (0.1–1 μmol/L) than the controls (Figures 5a and b). The activity of SOD in the cells treated with 10, 100 and 1000 μmol/L CMs-Y was significantly lower than those of the control cells.

Cytokine protein secretion (ELISA)

Lung fibroblasts exposed to 10, 100 and 1000 μmol/L CMs-Y had higher levels of inflammatory cytokine IL-1, IL-6, IL-8, IL-10 and TNF-α production than the lower doses (0.1–1 μmol/L) (Figures 6a and b). A higher level of TNF-α expression is considered to be an important factor in the development of inflammation.

Discussion

Although microspheres have been used for a variety of applications,[12,14,46] recent findings clearly show that microspheres are a potential occupational hazard.[47,48] In this study, exposure of lung fibroblasts to CMs caused an increased level of cytotoxicity after 24 h. Higher doses of CMs-Y treatment induced a significant inhibition of cell growth and increased the percentage of toxicity. This is in agreement with Sivadas *et al.*,[49] who showed that PLGA exerted potential toxicity in lung cells. Toxic effects observed with conjugated microspheres on epithelial cells

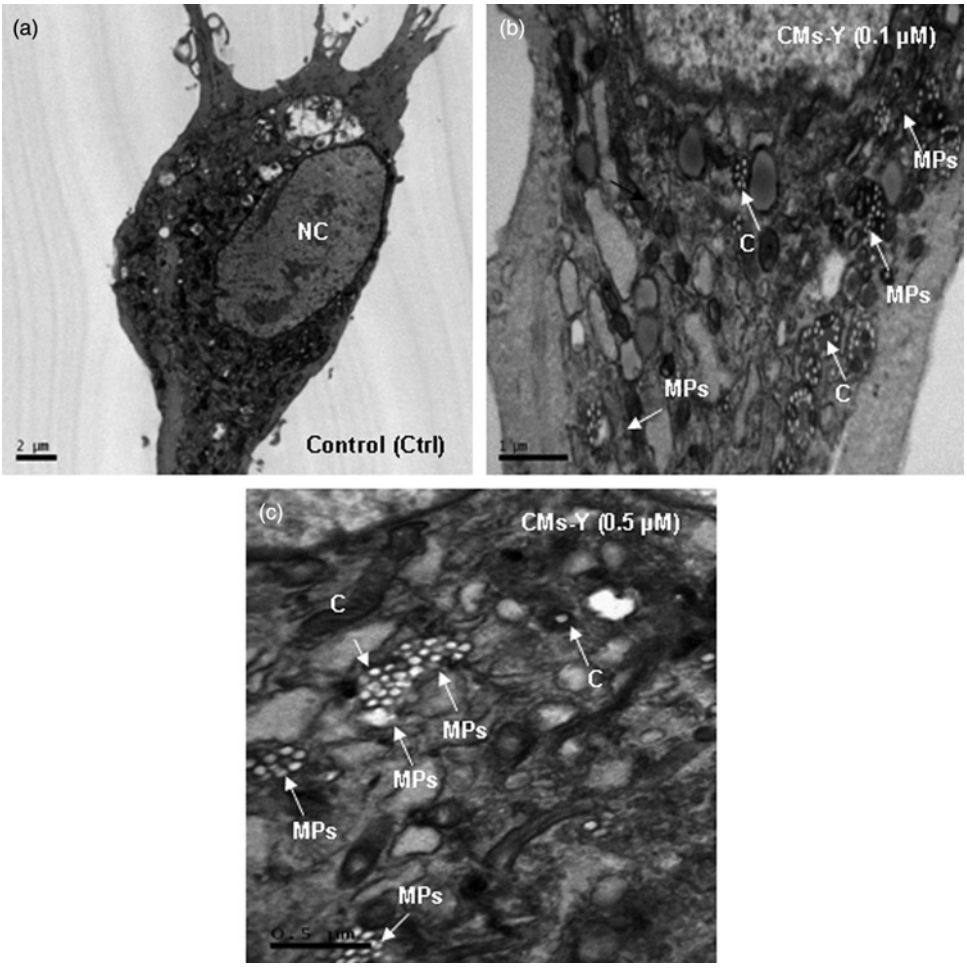

Figure 4 Transmission electron microscopy observation of fibroblasts. MRC-5 cells exposed to carboxylate microspheres-yellow (CMs-Y) particles. (a) Control showing the normal architecture of the cells without any treatment. (b and c) Cells were exposed to 0.1 and 0.5 μmol/L of CMs-Y particles after 24h. CMs-Y were internalized in cells and localized in cytoplasm, mostly in membrane compartments. NC, nucleus; MPs, microspheres; C, cluster

include induction of cell death.[50] Evaluation of cytotoxicity of glucose oxidase encapsulated in alginate/chitosan hydrogel microsphere (ACMS-GOX) formulation in murine breast cancer cells has also revealed a dose- and time-dependent cytotoxicity to cells.[21]

Light microscopic examination provided evidence of damage to the lung fibroblasts by CMs-Y accompanied by the release of LDH, a marker of cytotoxicity, into the medium. At the ultrastructural level, CMs-Y were observed to be mostly gathered in clusters inside cellular vesicles. Several recent studies demonstrated the pathophysiological responses found in animal models, including increased generation of ROS as an initiating factor of toxicity in cells exposed to various nanoparticles or microparticles.[51–53]

ROS is known to be important for the induction of apoptosis in inflammatory cells.[54] Microsphere-induced cell damage is believed to be due to the secondary generation of ROS.[39,40] In this study, ROS level was gradually increased by CMs-Y exposure in a dose-dependant manner in MRC-5 cells, which could increase oxidative stress, leading to cellular dysfunction and cell death.[55] ROS are known to activate members of the mitogen-activated protein kinase (MAPK) family.[56–58] In general, p38 MAPK and c-jun N-terminal kinase induce apoptosis signals CMs-Y under stressful conditions.[59] ROS have been shown not only to damage cells by peroxidizing lipids, disrupting DNA and proteins, but also to exert signaling functions and modulate gene transcription.[20,45] Lipid peroxides play an important role in the development of pathological changes in cells and tissues.[60,61] Overproduction of ROS would break down the delicate balance of oxidative stress and antioxidant defense in the cells, culminating in LPx. The toxic effects of ROS can be neutralized by antioxidative enzymes such as CAT and SOD.[62,63] However, in this study, we observed a reduction in the activities of CAT and SOD when exposed to higher concentrations of CMs, thereby exacerbating oxidative stress in the cells.

Studies have shown that expandable microspheres can induce pulmonary toxicity and cause inflammatory reactions in the lung[64,65] by stimulating the expression of inflammatory cytokines.[66–68] Poly (lactide-co-glycolide) and gelatin microparticles are known to enhance IL-8 cytokine release in the cells.[49] We also found that CMs-Y (10–1000 μmol/L)-exposed cells had higher levels of inflammatory cytokine production, *viz.*, IL-1, IL-6, IL-8, IL-10 and TNF-α than controls. These cytokines are capable of inducing inflammation and contributing to chronic pulmonary diseases.[69]

Table 1 Generation of reactive oxygen species (ROS) and lipid peroxidation (LPx) in lung fibroblasts after exposure of carboxylate microsphere (CMs-yellow)

CMs-yellow	Control	0.1 μmol/L	0.5 μmol/L	1 μmol/L	10 μmol/L	100 μmol/L	1000 μmol/L
H_2O_2 generation (μmol/mg protein min)	19.33 $\pm$ 2.96	61.98 $\pm$ 2.29	76.85 $\pm$ 3.84*	82.26 $\pm$ 3.32*	92.10 $\pm$ 3.87*	94.98 $\pm$ 4.86**	120.23 $\pm$ 4.67**
MDA content (μmol/g protein)	16.24 $\pm$ 1.17	18.96 $\pm$ 1.12	26.41 $\pm$ 1.79*	30.62 $\pm$ 2.19*	39.46 $\pm$ 2.64*	47.33 $\pm$ 2.72**	62.90 $\pm$ 3.27**

Values are presented mean $\pm$ SD. $n = 3$; *$P < 0.01$ and **$P < 0.05$ level

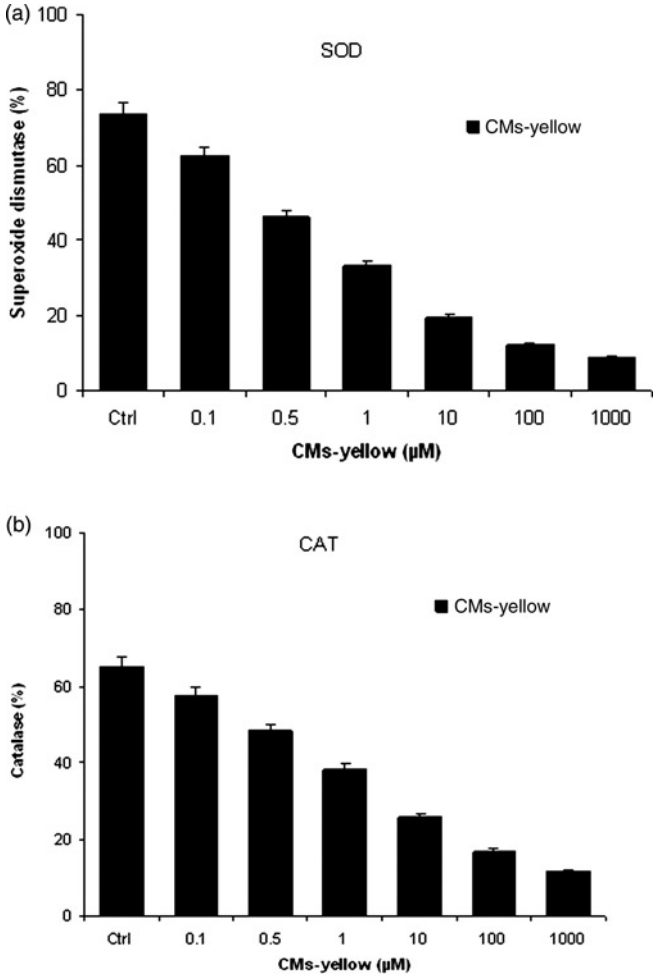

Figure 5 (a and b) Antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were quantified from the fibroblast culture supernatants of fibroblasts exposed to carboxylate microspheres-yellow (CMs-Y). The CMs-Y-treated cells showed a gradual reduction of SOD and CAT in a dose-dependent manner (0.1–1000 μmol/L)

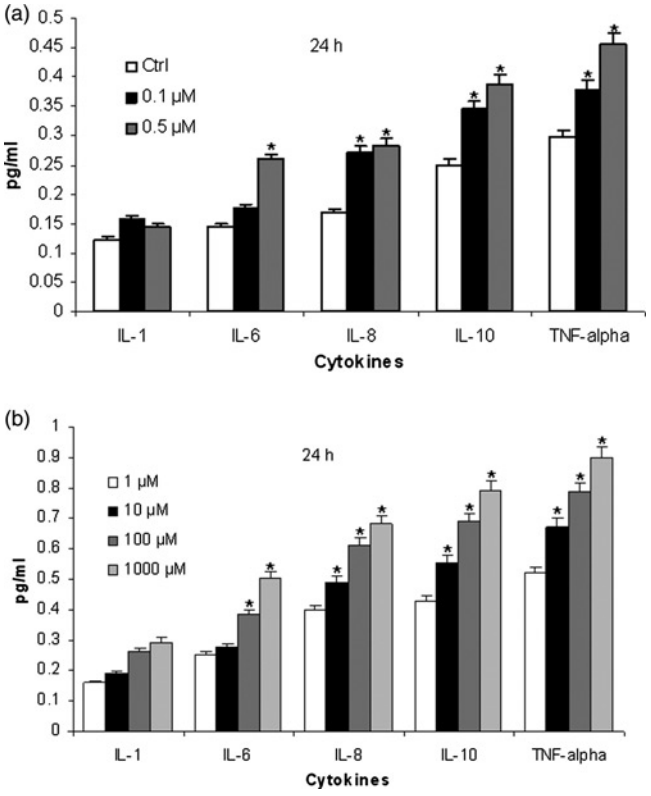

Figure 6 (a and b). The production of proinflammatory cytokines interleukin (IL)-1, IL-6, IL-8, IL-10 and tumor necrosis factor (TNF)-α produced by lung fibroblasts exposed to carboxylate microspheres-yellow (CMs-Y) (ranging from 0.1 to 1000 μmol/L). Level of significance was *$P < 0.01$ versus control

TNF-α is a major cytokine known to initiate inflammatory processes in the respiratory system.[18] For instance, lung alveolar macrophages elaborate a broad spectrum of proinflammatory mediators such as TNF-α as well as ROS.[70] TNF-α is required for the host defence against intracellular damage and exerts its cytotoxic effects via generation of intracellular ROS which stimulate signal transudation pathways. This could lead to a rapid activation of stress- and MAPKs and NF-κB,[71,72] which result in a coordinated expression of genes of several inflammatory mediators, including cytokines. The NF-κB family of transcription factors plays a central role in regulating the expression of a variety of inflammatory genes that have been linked to cytotoxicity. However, NF-κB controls not only inflammation, but also apoptosis in many cells.[73]

Although transforming growth factor-β (TGF-β) was not evaluated in this study, it is a known and important mediator of fibrosis.[33,74] TGF-β is a pleiotropic cytokine that affects cell proliferation, differentiation and apoptosis and is involved in homeostatic functions, playing a major role as a 'master switch' of pulmonary fibrosis.[75] The major effects of TGF-β include inhibition of epithelial cell proliferation, induction of fibroblast proliferation and expression of genes encoding components of the extracellular matrix (ECM), as well as decreasing the expression of metalloproteinase genes.[33] TGF-β can stimulate fibroblast conversion into contractile myofibroblasts, which actively produce collagen and other ECM proteins, and may serve as an inducer of epithelial-to-mesenchymal transition,

leading to fibrosis.[76] In summary, evaluating the toxicity and biosafety of CMs is of importance to human health. We have shown that CMs at higher concentrations may induce cellular toxicity *in vitro*. This study should be extended to investigate the effect of CMs in *in vivo* models.

Authors contributions: RPS designed and conducted the experiments, performed the data analysis and drafted the manuscript. C-TN was involved in sample preparation and performed electron microscopy. B-HB provided input to the experimental protocols and made amendments to the manuscript, and FW provided scientific comments and suggestions. All authors read the manuscript before submission.

ACKNOWLEDGEMENT

This work was funded by Singapore Bioimaging Consortium (SBIC), A-STAR SBIC RP C-013/2007, Singapore.

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(Received September 16, 2011, Accepted March 12, 2012)