Study of the Stability of a Paramagnetic Label Linked to Mesoporous Silica Surface in Contact with Rat Mesothelial Cells in Culture

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Stable radicals detectable by electron paramagnetic resonance (EPR) may be of use in the investigation of early events in cell–particle toxicity. Piperidine-N-oxyl derivatives (nitroxides), covalently linked to the surface of a high surface area silica (used as model solid for the technique), served as probes in the investigation of the effects of incubation of silica particles with mesothelial cells. A mesoporous silica (MCM-41), prepared by precipitation from a micellar solution, was the most appropriate silica-based particle for this purpose, as its channels allow direct contact with small molecules but not with macromolecules. The cytotoxicity of this amorphous silica is very low, allowing relatively high particle loading in the cell cultures. Both the high surface area of the sample and the large amount of inorganic material extracted from the cell culture provide enough material to run reasonably intense EPR spectra. Computer-aided analysis of the EPR spectra of silica-bound nitroxides provided information on the sensitivity of the labeled silica monitoring different environments, e.g., to follow the path of particles in a mammalian cell culture. Upon contact of the particles with mesothelial cells, the mean distance among the labels at the silica surface decreased as a consequence of the release of oxidizing and/or radical moieties from the cells. — Environ Health Perspect 105(Suppl 5):1031–1036 (1997)

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Introduction

The molecular mechanisms underlying pulmonary toxicity of solid particulates are still unclear (1,2). Even in the case of the most infamous hazardous minerals such as asbestos or crystalline silicas, the chemical nature of the solid surface responsible for the pathogenic response is still under debate (3–7). The chemical reactions implied in pathogenesis take place mainly at the solid–liquid interface, but the specific role of the solid surface is not well understood. Molecular interactions between the solid surface and the cells determine inflammation, immune cell recruitment, and release of cytokines and oxidants, which play crucial roles in the pathogenic response (8). A mechanistic approach to the chemical basis of the pathogenic response to solid particles is often lacking. In a large number of biological investigations, in fact, little attention has been given to the molecular mechanisms whereby some solid particles elicit a given biological response. It is generally agreed that toxicity stems from various aspects of the solid particle, namely its form (fibers are more dangerous than isometric particles), its chemical composition (iron associated with asbestos is implicated in carcinogenicity) and its biopersistence, i.e., the length of time a particle resides within a given biological compartment. Moreover, various particle–tissue interactions take place in subsequent steps, involving different surface functionalities. Therefore, overall pathogenicity is the consequence of several physicochemical properties (5,6).

Attention has been focused recently on early events in cell–particle toxicity for two main reasons: a) identification of early markers is the best way for prevention of disease (9); and b) investigation of the primary molecular message between the particle surface and the cells enables identification of chemical functionalities implied at the surface, thus allowing design of new safer materials. This is particularly important at present; asbestos has been banned from several countries and the long-term safety of various asbestos substitutes has still to be proven (10).

To investigate the first step in cell–particle interactions, markers monitoring the local topography must be placed at the particle surface. We attempted a new research route based on labeling with a paramagnetic moiety that possesses an electron paramagnetic resonance (EPR) spectrum extremely sensitive to the surrounding medium (11). Such spectra in a cell culture ideally monitor: a) adsorption of endogenous matter; b) adhesion of cell membrane to the solid; and c) internalization of the particle—the most likely events taking place when particles or fibers are in direct contact with cells. Piperidine-N-oxyl derivatives (nitroxides) are stable radicals that have been successfully employed for investigations in several biological systems when linked to various molecules (12). To date, they have not been used in connection with particle toxicity. This paper explores methods to employ nitroxide spin labels linked to silica-based particles or fibers in the investigation of biopersistence of the...
Materials and Methods

Preparation of Labeled Silica

Chemicals. MCM-41 is an amorphous mesoporous silica, obtained by precipitation of silica from a micellar solution, synthetized in École Nationale Superiéure de Chimie de Montpellier (Montpellier, France) (14). Spin label 1-oxyl-2,2,6,6-tetramethylpiperidine and all the reagents used in the labeling procedure, carbonate buffer, (3-aminopropyl)trimethoxysilane, ethanol, methanol, NaBH₃CN, and the dye fluorescein isothiocyanate (FITC, isomer I) were from Sigma (St. Louis, MO). All the reagents were used as received.

Silanization. Before silanization, silica particles were washed thoroughly with dilute hydrochloric acid to remove contaminants and trace amounts of iron. The substrate was then thoroughly washed with deionized water.

The particles were first silanized to fix a propylamine chain to the surface. The solid particles were previously sonicated then derivatized by adsorption of freshly hydrolyzed (3-aminopropyl)trimethoxysilane from degassed aqueous alkaline solution (15). This reaction is illustrated in Figure 1. Final drying was necessary to improve covalent bonding of the alkylic ligands at the surface.

Labeling with Stable Radicals (Electron Paramagnetic Resonance Active). Derivatized silicas were spin labeled by reductive amination (Figure 1) using an excess of 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl (TEMPONE) with NaBH₃CN as reductant in methanol for 24 hr following the preparation method previously reported (15).

Labeling with Fluorescent Labels. Derivatized silicas were labeled by an additional reaction (Figure 1) using an excess of FITC (isomer I) following the preparation method previously reported (16).

Cellular Tests

Cell Culture. Rat pleural mesothelial cells were cultured in standard conditions (17) in RPMI 1640 medium supplemented with 50 μg/ml streptomycin, 50 U/ml penicillin, 10% fetal calf serum (FCS), and 1 mM HEPES. These cells grow as a monolayer adherent to the bottom of the tissue culture surface. Under routine conditions, cells were cultured in 75 cm² flasks containing 12 ml culture medium.

Contact between Cells and Silica Particles. When cells reached confluency, the culture medium was removed and replaced with a suspension of MCM-41 particles at a concentration of 42 μg/ml in RPMI 1640 medium supplemented with

![Figure 1](image)

**Figure 1.** Schematic representation of reactions at the MCM-41 surface: silanization, linking of TEMPONE (nitroxide) to the aminopropyl chain, and linking of FITC.
Acellular performed spectra cytotoxic of interactions Resonance EPR rpm) Parallel medium and collection medium were transferred to EPR tubes. The samples were then dried overnight by vacuum process. All EPR spectra were recorded within 48 hr from deposition in EPR tubes.

A zero time preparation was also performed by mixing cells and particles directly in the centrifuge tube. This sample was then processed in the same manner as the other time preparations. Time of contact between cells and silica in this case was 5 min.

Acellular Tests
Parallel acellular experiments were conducted by using the same concentrations of silica at the same conditions of incubation and collection as the cell tests, minus the cells.

Electron Paramagnetic Resonance Measurements
The intensity of EPR spectra depends on the success of the labeling, amount of material introduced in the EPR cell, and specific surface of the material. The higher the specific surface, the lower the amount of material required to obtain a good spectrum. The spectra recorded on the labeled mesoporous silica were therefore very intense. EPR spectra were recorded with either a Varian E109-EPR spectrometer (Palo Alto, CA), working in X band (about 9.5 GHz) with a double resonant cavity, or with an Adani PS100-X EPR spectrometer (Minsk, Belarus).

Analysis of Electron Paramagnetic Resonance Spectra
Simulation of the experimental EPR spectra was performed by a well-established procedure (18). This analysis allows the evaluation of the dipolar broadening, $\Delta H_p$, through spectral computation, which allows one to calculate the mean distance (d, in Ångstroms) among the probes at the surface from the equation (19):

$$\Delta H_p = 3 \times 10^4 d^3$$

The probe–probe distance is the most relevant parameter to characterize the differences between samples in the presence and absence of cells, and for different times of incubation in cell cultures.

Results and Discussion
Figure 2 shows the EPR experimental spectra of the labeled MCM-41 under various experimental conditions. Details on the spectral characteristics, obtained by computer simulation, are beyond the scope of this paper. Only the qualitative results of these calculations are reported here. The spectrum obtained from labeled MCM-41 and fully dried after preparation is shown in Figure 2A. Even with 100 μg MCM-41, an amount commonly used in cellular tests, the intensity of the EPR signal is sufficiently large to indicate an acceptable label loading level. The presence of even small amounts of water markedly modifies the spectrum. The spectrum of a wet sample (Figure 2B) is typical of a nitroxide fixed at the surface but free to move (11). The spectrum reveals that on contact with water, the label is displaced from the surface by water molecules that interact more strongly than the label itself with the surface [heat of adsorption of water higher than 50 kJ mol⁻¹ (20)]. The displaced label thus protrudes into the water phase and is free to move than in the dried form.

If ethanol (Figure 2C) replaces water, the increase in mobility with respect to the dry sample (Figure 2A) is lower than in water (Figure 2B) because of the lower affinity of the alcohol with the silica surface. When immersed in cyclohexane (Figure 2D), the strong interaction between the nitroxide moiety and the surface of MCM-41 is barely perturbed by the solvent, so that the radical mobility is nearly equivalent to that of the dried sample (Figure 2A). As expected from literature reports (21), these silica labels reveal the polarity of the surrounding medium, thus providing information on their localization.

Figure 2. Electron paramagnetic resonance spectrum of the propyl nitroxide labeled MCM-41 silica (A) dry; (B) wet (water) (C) in ethanol; (D) in cyclohexane; (E) wet sample aged for 5 months.

When kept in a refrigerated, dry, oxygen-free atmosphere, nitroxide moiety is fairly stable. A spontaneous decay of the radical is visible only over long periods of time; Figure 2E shows a sample from Figure 2A after drying in air for 5 months. In this case the major change is a marked decrease in spectral intensity.

Spectra obtained from labeled MCM-41 in contact with a mesothelial cell culture for 5 min and with the same amount of cell culture medium (RPMI 1640 supplemented with 1% FCS, 1 mM HEPES, and antibiotics [50 g/ml streptomycin, 50 U/ml penicillin]) are reported in Figure 3A and B. The main difference between the two spectra is line width, which arises from the spin–spin interactions. The decrease in line width on contact with the cell culture reflects radical annealing. As a result, the spectrum of particles in the cell culture was roughly equivalent to the spectrum of the original sample when aged 5 months (Figure 2E). A similar spectrum was also found following adsorption of dipalmitoylphosphatidylcoline.

This spectrum similarity indicates that a reaction occurs, which modifies or anneals nitroxide radicals rapidly on contact with cells. Annealing may be caused by a reaction between the oxidizing species present in the cells (e.g., the lipid peroxidation in cell membranes) or produced (active oxygen species) by the cell as a defense response.
and the labeled nitroxides. In this respect the modifications registered in the spectrum with cell contact is a measure of both cell activity and cell–particle interaction.

MCM-41 particles were incubated for concurrent time periods in the cell culture and the cell culture medium.

Figure 4 shows some significant experimental spectra (full lines) and the correspondent computed signals (dotted lines) of material obtained upon incubation of MCM-41 particles with the cell culture (Figure 4A) and with the cell culture medium (Figure 4B). Incubation times of 5 and 30 min were selected because time affects spectral changes. The unique parameter that tracks changes in line shape is the intrinsic line width. Dipolar broadening extracted from the intrinsic line width allowed evaluation of the mean distance (d, in Ångströms) among the probes at the surface. (See “Analysis of Electron Paramagnetic Resonance Spectra.”) The variation of d as a function of incubation time is reported in Figure 4C for both the cell culture and cell culture medium. The contact of silica with cells strongly increases the mean distance among the labels.

Several factors may modify the spectrum, including interaction of the labels with cell membrane debris, redox reactions with various cytoplasmic components, and direct annealing of the nitroxide radicals caused by the production of activated cells. Most nitroxide chains are on the inner surface of the particle, located in channels 5-nm wide, where macromolecules or cellular debris cannot enter unless in a monomolecular dissolved state. Therefore, the interpretation proposed for radical suppression originated by cell response to contact with the particle (internalization) seems the most probable one. Experiments using fluorescein-tagged MCM-41 have suggested a particle internalization. Confluent rat mesothelial cells were incubated at 37°C with three concentrations of silica (17, 33, and 66 μg/ml) for 6 and 24 hr and investigated with light microscopy. MCM-41 appeared to be present in the cytoplasm and concentrated around the nucleus, which suggested particle uptake (Figure 5). These results were observed after 6 hr incubation and persisted at 24 hr. In agreement with this hypothesis, electron microscopy of the same culture (data not shown) showed evidence of silica particle presence in internalization vacuoles. The effects investigated using EPR have yet to be ascribed to labeled particles located within the cells.

**Conclusion**

The electron paramagnetic spectra of nitroxides covalently linked to the surface of silica particles appear to be effective for monitoring early cell response to contact with a solid surface. This method, tested on a very peculiar type of high surface area mesoporous silica (MCM-41) would seem applicable to virtually any particle or fiber surface provided that silica is a major constituent of the solid and consistent patches of silanols are available. Using the same chemical procedure, spin labels have in fact been linked to a large variety of crystalline and amorphous silica as well as to glass fibers (22). Because of the much lower surface area of these materials with respect to MCM-41, their use in cell culture requires a much larger number of cells to obtain enough material for EPR measurement. The procedure adopted yields reasonably stable surface covalent bonds, which are not hydrolized to a large extent in aqueous media. The contact of the particles with mesothelial cells at noncytotoxic doses appears to elicit an immediate defense response from cells through release of oxidizing and/or radical annealing agents. Further work is required to confirm these data on a dose–response basis and extend the proposed method to highly toxic particles or fibers and other cell lines. In this respect, the method appears promising for systematic investigation of early cell response to a large variety of particles. The sensitivity of the spin label to the presence of free radicals allows detection of the initial steps yielding oxidative stress.

**Figure 3.** Electron paramagnetic resonance spectra of the propyl nitroxide labeled MCM-41 silica (A) incubated 5 min in a mesothelial cell culture; (B) incubated 5 min in the cell culture medium.

**Figure 4.** Experimental (full lines) and simulated (dotted lines) EPR spectra of dry pellets obtained upon incubation for 5 and 30 min of MCM-41 particles in (A) the cell culture, and (B) the cell culture medium. (C) Mean distance (d, in Ångströms) among the probes as a function of the incubation time in the cell culture (●) and the cell culture medium (○).
Figure 5. Micrographs of rat pleural mesothelial cell culture incubated for 24 hr with (A) 33 μg/ml using fluorescence light microscopy or (B) 66 μg/ml (using light microscopy) of MCM-41 labeled with a fluorescent tag. Aggregates of silica are concentrated in the perinuclear region. (Magnification ×1300).

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