Environmental Health Perspectives
Vol. 87, pp. 337–341, 1990

Evidence of an Oxidative Mechanism for the Hemolytic Activity of Silica Particles

by Bronwyn L. Razzaboni* and Pedro Bolsaitis*

The formation of reactive oxygen species resulting from the interaction of silica dust particles with red blood cell membranes was investigated; particularly, the effect of surface hydroxyl (silanol) group concentration on the rate of formation of such reactive oxygen species was investigated. The rate of formation was measured indirectly through the effect of catalase, a hemoprotein peroxidase, on silica-induced hemolysis. It was found that the addition of exogenous catalase to erythrocytes markedly reduces the hemolysis caused by silica particles. Furthermore, the amount of catalase required for deactivation of silica per unit area of particle surface is lower for fumed silica particles and calcined crystalline particles than for uncalcined, crystalline silica, suggesting a correlation between the concentration of OH groups at the silica particle surface and its potential for generation of H_2O_2. The addition of albumin, a copper chelator, also decreases hemolysis. These results suggest that the hemolysis caused by silica particles is at least partly related to the formation of H_2O_2 at the particle surface and its subsequent reaction with Cu^+ ions. The relationship between the concentration of surface silanol groups on the silica surface and the amount of catalase required to decrease hemolysis may also provide a method for testing potential fibrogenicity of respirable dusts.

Introduction

Reactive hydroxyl groups on silica surfaces are known to be cytotoxic and are believed to play a major role in silicosis (*1–3*). It has been proposed that this cytotoxicity results from hydrogen bonding to cell membranes and a resulting increase in membrane fluidity and permeability (*4*). Surface hydroxyl groups on silica surfaces have also been shown to interact with carbonyl oxygens in cell membranes (*1,5*). An extensive study by Nolan et al. (*6*) of the relationship between the negative surface charge resulting from ionized silanol groups and the hemolytic activity of crystalline α-quartz (Min-U-Sil) has shown a positive correlation between hemolytic activity and the surface concentration of silanol groups. These authors demonstrated that the hydrogen bonding of polyvinyl-*N*-oxide (PVPNO) with silanol groups, as well as the bonding of metal cations to the ionized SiO^- groups, results in significant decreases in hemolytic activity. Furthermore, it has been found that the complete removal of surface hydroxyl groups from the surface of respirable silica particles nearly eliminates the hemolytic potential of silica (*7*), suggesting that hydroxyl groups might be involved in other cytotoxic processes associated with silica-induced lung injury.

Reactive oxygen species have been implicated in cellular damage caused by respirable silica particles. Evidence for the presence of oxygen radical species ($O\bar{2}$, $OH^\cdot$, H_2O_2) generated by silica, especially on freshly formed surfaces, has been produced by various investigators. Kalbanev et al. (*8*) detected H_2O_2 in aqueous suspensions of quartz. Electron spin resonance and spin trapping by Shi et al. (*9*) confirmed the presence of $OH^\cdot$ radicals in aqueous suspensions of quartz, and Gulumian and van Wyk (*10*) suggested that both glass and quartz produce hydroxyl radicals in the presence of H_2O_2. Gabor et al. (*11*) detected the presence of malondialdehyde-reactive lipid peroxides (i.e., lipid-$O^\cdot$) in cultures of macrophages and respirable silica particles using the thiobarbituric acid (TBA) reaction test. Will (*12*), however, reported that iron enhances the TBA value of peroxidized lipids, and Scheulen and Kappus (*13*) found that catalase augmented malondialdehyde formation, thereby increasing the TBA values.

Clemens et al. (*14*) found that H_2O_2, in 10 mM concentration, induced lipid peroxidation of red blood cells and their cell components. This lipid peroxidation occurs by a Fenton reaction. The H_2O_2 reacts with a metal ion such as iron (Fe^{2+}) or copper (Cu^+) to form hydroxyl ($OH^\cdot$) radicals (*15,16*):

$$Fe^{2+} + H_2O_2 \rightarrow OH + OH^- + Fe^{3+} \quad (1a)$$

or

$$Cu^+ + H_2O_2 \rightarrow OH + OH^- + Cu^{2+} \quad (1b)$$

The hydroxyl radical is very reactive and oxidizes many types of organic chemicals. Biomolecules are also oxidized when present in close proximity to the source of hydroxyl radicals (*17*).

*Energy Laboratory, Massachusetts Institute of Technology, Cambridge, MA 02139.
Address reprint requests to P. Bolsaitis, Energy Laboratory, Massachusetts Institute of Technology, Cambridge, MA 02139.

Although evidence exists that links silica surfaces and hydroxyl radicals to hemolytic activity, it has not yet been shown that free radicals result from the silica-cell membrane contact. Results of our previous research (*7*) suggest that this issue merits further investigation. The interaction of silica with cell membranes may produce free-radical species and cause the subsequent penetration of these radicals into the cell. We have not found any systematic investigation of the formation of free radicals from hydroxylated silica surfaces in water, but there is circumstantial evidence that free radicals may be formed under such conditions.

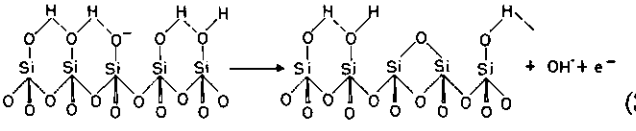
Silicon can form bonds of variable angles and compounds of many different coordination numbers. Also, the oxygen contained in silica may react to form oxides, hydroxides or, when in contact with organic molecules, a variety of alcohol, enol, phenol, and other organic groups. This gives rise to a large variety of possible stereochemistries for the reactions of silica with biochemical species (*18*).

Silica, when present in water, hydrolyzes to form a complex interface. The hydrolysis may be postulated to start from an ideal hydrated Si^{4+} cation in water, through various levels of hydroxylation and oxidation to an amorphous, crosslinked, polymeric SiO_2, and, eventually to crystalline surfaces, e.g., quartz:

$$[Si(OH_2)_4]^{4+} \rightleftharpoons [Si(OH)_4] + 4H^+ \rightleftharpoons SiO_n(OH)_{4-2n} \rightleftharpoons SiO_{2(\text{polymeric})} \rightleftharpoons SiO_{2(\text{crystalline})} \quad (2)$$

The amorphous surface layer of hydrated SiO_2, which is present even in crystalline material in contact with water, is believed to lack much structural regularity. The surface silica atoms may be bonded to one or more OH groups and a few negative surface charges may exist (*18*).

Evidence exists for the formation of free radicals when silicon-oxygen bonds are mechanically broken (*19*), thus it may be equally possible that such radicals could form under suitable catalytic or stereochemical conditions near biochemical molecules. We have not found any studies of the possible mechanism for free-radical formation on silica surfaces by a chemical path. It is known that the silica surface tends to be negatively charged, and radicals can readily be formed by oxidation-reduction processes where electrons are transferred between ions in solution and the solid matrix. The released electrons may also be trapped near holes in the solid matrix. This form of electron trapping is enhanced by a glassy surface structure and the presence of impurity atoms in the lattice (*20*) and $OH^{\cdot}$ radicals may be formed by mechanisms such as the one shown in Eq. (3).



(3)

In the present experiments, two enzymes were used to test possible oxidative mechanisms in hemolysis resulting from reactive oxygen species generated at the silica surface. Catalase (CAT), a hemoprotein peroxidase present in all mammalian cells, rapidly decomposes H_2O_2 to form H_2O and O_2. The addition of catalase to remove H_2O_2 present in erythrocytes has also been shown to decrease the conversion of oxyhemoglobin (existing as Fe^{2+}-hemoglobin) to methemoglobin (Fe^{3+}-hemoglobin) (*16*), suggesting an interaction between H_2O_2 and iron ions. Superoxide dismutase (SOD) is an enzyme present in red blood cells predominantly as CuZnSOD (*21*). The role of SOD is to scavenge O_2^- radicals, which help to cause lipid peroxidation.

Materials and Methods

Crystalline α-quartz (Min-U-Sil), with a median particle diameter of 5 μm, was obtained from U.S. Silica Inc. (Berkeley Springs, WV) and amorphous, fumed silica (Cab-O-Sil) from Cabot Corporation (Tuscola, IL). BET (Brunauer, Teller, and Emmett) adsorptometry (adsorbed gas used was 30% nitrogen in helium) was used to determine the specific surface area of each dust. The specific surface areas of Min-U-Sil and Cab-O-Sil were found to be 5.2 m^2/g and 200 m^2/g, respectively. After calcination at 800°C for 19 hr, the specific surface area of Min-U-Sil changed to 4.78 m^2/g. For use in the erythrocyte assays, the Min-U-Sil was made up in the form of a stock mixture of 4 mg silica/mL calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS). The saline solution was obtained from Sigma Chemical Company (St. Louis, MO). The fumed silica was prepared similarly, but the stock mixture had a concentration of only 1 mg silica/mL DPBS.

All proteins [CAT from bovine liver, SOD from bovine erythrocytes, bovine serum albumin (BSA), and acetylated bovine serum albumin (AcBSA)] were obtained from Sigma Chemical Company (St. Louis, MO). To test the specificity of its reaction with H_2O_2, catalase was heat-inactivated by boiling at 80°C in DPBS and then drying at 110°C. Stock solutions of 1 mg/mL DPBS for CAT, heat-inactivated CAT, and SOD, and 20 mg/mL DPBS for the albumins, were made up on the day of testing. Bovine erythrocytes were purchased from the Colorado Serum Company (Denver, CO). Before incubation, the erythrocytes were washed twice in DPBS, centrifuged at 990*g*, and the supernatant removed.

The proteins were added to 2 mL of 2% erythrocytes for 10 min to give preincubation concentrations of 10 to 300 μg/mL for CAT and SOD, and 100 μg to 4 mg/mL for the albumins. At time zero, enough Min-U-Sil (either calcined or noncalcined) was added to the erythrocyte-protein mixture to obtain a final concentration of 0.5 mg/mL; fumed silica was added to give a final concentration of 0.015 mg/mL. The total volume of the suspensions was 4 mL. Negative controls were prepared by mixing 2 mL of 2% erythrocytes and 100 μg/mL of CAT or 200 μg/mL SOD for preincubation and adding 2 mL of DPBS at time zero. Positive controls consisted of erythrocytes mixed with distilled water. The samples were incubated for 30 min at room temperature with inversion of tubes every 10 min. The amount of hemo-

globin released was determined by measuring the optical density (OD) of hemoglobin on a Bausch and Lomb Spectrometer (Spec 20) at a wavelength of 540 nm. The percent inhibition of hemolysis was calculated as follows:

$$\% \text{ Hemolysis} = [(OD_{(b)} - OD_{(nc)})/OD_{(pc)}] \times 100 \quad (4a)$$

$$\% \text{ Inhibition} = 100 - \% \text{ hemolysis} \quad (4b)$$

All experiments were done in duplicate and repeated two to five times. The results are expressed as the mean ± the calculated standard deviation.

Results

The effect of adding CAT and heat-inactivated CAT to mixtures of erythrocytes and crystalline silica (Min-U-Sil) on hemolysis is shown in Figure 1. These results demonstrate that the inhibition of hemolysis is specifically related to the enzymatic action of CAT rather than other physical effects such as adsorption of CAT to the particle or cell surface. Figure 2 compares similarly the results obtained with two different proteins, CAT and SOD, which again demonstrates the effect of the specific enzymatic action of CAT.

The apparent relationship between the surface concentration of surface hydroxyl groups on the dust particle and the amount of H_2O_2 produced is shown in Figure 3. Cab-O-Sil, with a surface area approximately equal to that of a Min-U-Sil, requires less catalase to achieve the same degree of inhibition of hemolysis as Min-U-Sil: 50 μg (140U)/mL CAT is needed to reduce by approximately 81% Cab-O-Sil induced hemolysis, whereas 200 μg (560U)/mL catalase is required to achieve approximately 76% inhibition of hemolysis induced by Min-U-Sil. Infrared results show that fumed silica has a lower concentration of surface silanol groups than the crystalline material (7).

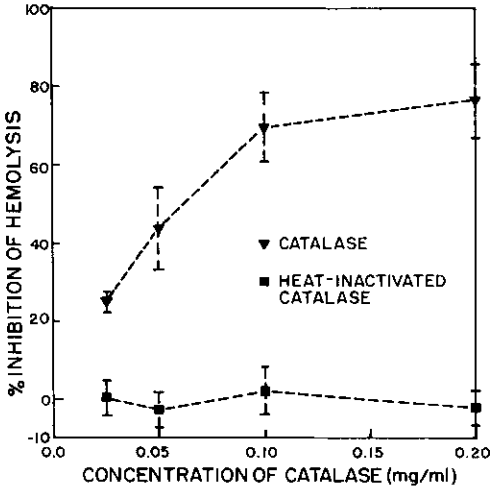


FIGURE 1. Effect of CAT on hemolysis. Two percent erythrocytes were preincubated for 10 min with untreated or heat-inactivated CAT at the concentrations indicated. At time 0, 0.5 mg respirable Min-U-Sil/mL was added. Incubation period was 30 min at room temperature. Each value is the mean of three to five different experiments. Error bars represent SD.

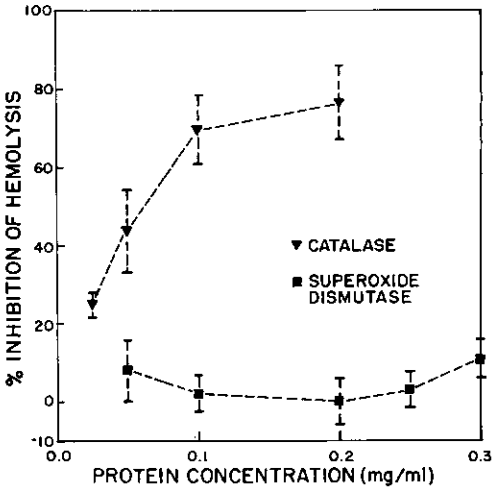


FIGURE 2. Effect of CAT and SOD on hemolysis. Two percent erythrocytes were preincubated for 10 min with CAT or SOD at the concentrations indicated. At time 0, 0.5 mg respirable Min-U-Sil/mL was added. Incubation period was 30 min at room temperature. Each value is the mean of three to five different experiments. Error bars represent SD.

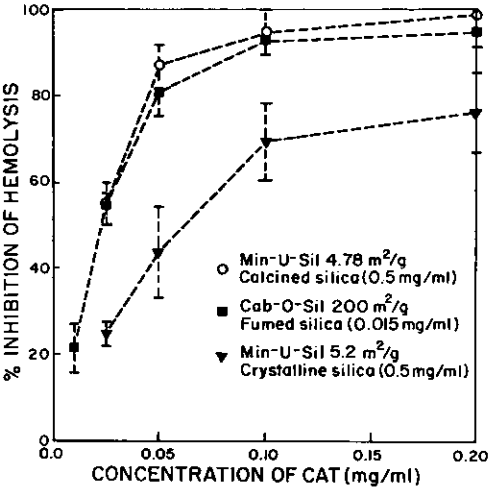


FIGURE 3. Relationship between concentration of surface silanol groups and amount of CAT required to cause inhibition. Two percent erythrocytes were preincubated for 10 min with CAT at the concentrations indicated. At time 0, 0.5 mg calcined (800°C, 19 hr) or uncalcined Min-U-Sil/mL was added or 0.015 mg respirable Cab-O-Sil was added. Incubation period was 30 min at room temperature. Each value is the mean of three to five different experiments. Error bars represent SD.

The concentration of silanol groups can also be reduced by thermal treatment (7). Calcination of silica dust results in a substantial reduction of hemolytic activity (associated with a reduction of silanol groups at the surface) before an appreciable change in specific surface area by sintering takes place. Changes in specific surface area with calcination temperature are illustrated in Figure 4. Figure 3 shows that the reduction of the surface silanol group concentration by calcination

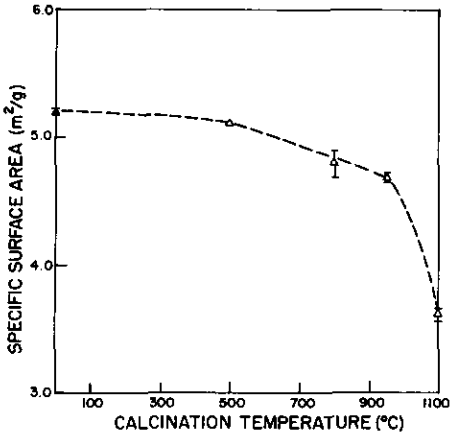


FIGURE 4. Effect of calcination temperature on specific surface area of Min-U-Sil. Surface area was measured with BET adsorptometry using 30% nitrogen in helium. Calcination was conducted at 500, 800, 950, or 1100°C for 18 to 72 hr. (Samples were stored in a dessicator between calcination and hemolysis testing.) Each value is the mean of five to six readings. Error bars represent SD.

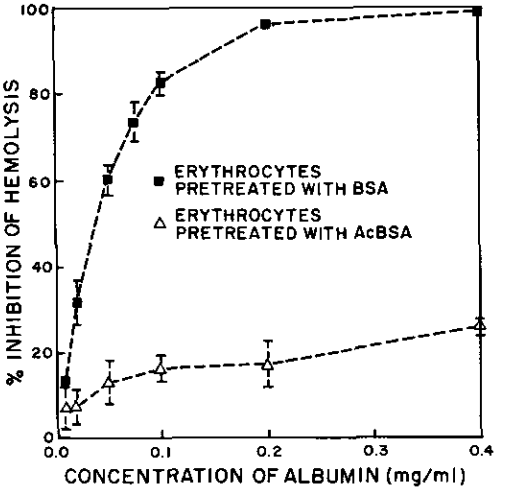


FIGURE 5. Effect of albumin (BSA) and acetylated albumin (AcBSA) on hemolysis. At time 0, 0.5 mg respirable Min-U-Sil/mL was added. Incubation period was 30 min at room temperature. Each value is the mean of three to five different experiments. Error bars represent SD.

is paralleled by an obligatory decrease in catalase concentration required for inhibition of hemolysis. The amount of catalase required to achieve a given level of reduction of hemolysis caused by calcined silica dust was approximately half of what was required to achieve a similar reduction of hemolysis caused by uncalcined material.

Results obtained from the addition of BSA and AcBSA are shown in Figure 5. The addition of albumin to the silica and erythrocytes caused decreases in normal hemolysis, whereas the AcBSA provided only slight protection to the erythrocytes (400 μg/mL AcBSA gives approximately 26% protection to lysis versus 99% protection by BSA). This suggests that the albumin is chelating copper ions and that the acetylation process causes damage to the Cu^+ binding sites on the protein.

Discussion

Previous results obtained in this laboratory demonstrated a correlation between the concentration of silanol groups on the surface of silica particles and erythrocyte damage (*7*). The present investigation shows that the addition of catalase to respirable silica and erythrocytes significantly protects the cells from the damaging effects of the dust. Denatured catalase did not cause a decrease in hemolysis, implying a specific role for the enzyme. These results suggest that H_2O_2 is produced in the process of silica damage.

SOD was also tested for its effect on the silica-erythrocyte system. No lessening of erythrocyte lysis was observed by the addition of SOD, but this result does not eliminate the possible involvement of the O_2^- radical as part of the cell-damaging process. SOD produces H_2O_2 in its catalytic role of eliminating O_2^- radicals; e.g.,

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

This H_2O_2 is believed to then irreversibly inhibit SOD by binding to its copper cofactor (*22*):

$$\text{Enz—}Cu^{2+} + H_2O_2 \rightarrow \text{Enz—}Cu^+ + 2H + O_2^- \quad (6)$$

In addition, Gutteridge and Wilkins (*15*) reported that both cupric- and ferric-salt-dependent reactions with H_2O_2 are markedly inhibited by catalase and only slightly inhibited by SOD or albumin. The TBA test was also conducted in an attempt to find malondialdehyde-reactive lipid peroxides but without conclusive results (not shown), possibly due to interference by both iron and catalase.

Another significant result is the proportionality found between the number of surface silanol groups and the amount of catalase required to inhibit hemolysis. Fumed and calcined crystalline silica are less hemolytic and have a lower concentration of surface silanol groups compared to the crystalline material (*7*). The amorphous and calcined materials, when compared to crystalline particles of equal surface area, require proportionally less catalase for an equivalent inhibition of hemolysis. This result suggests that the surface hydroxyl groups of silica are involved in the formation of H_2O_2.

The data presented also imply that metal ions are involved in the hemolysis process, possibly by reacting with H_2O_2 in a Fenton reaction. A role for copper (Cu^+) ions appears to exist in the reaction of red blood cells and silica because albumin, a copper (Cu^+) chelator (*16,23*), markedly reduced hemolysis by the silica. Halliwell and Gutteridge (*16*) and Halliwell (*23*) noted that albumin significantly inhibited copper-stimulated peroxidation in erythrocytes by preventing the reaction of Cu^+ ions and H_2O_2. Halliwell and Gutteridge (*16*) added that albumin does not prevent Fe^{2+}-dependent formation of $OH^\cdot$ radicals.

Summary and Conclusions

The present investigation provides the following evidence on the mechanism of silica-induced hemolysis: The hemolysis caused by silica particles appears to involve hydrogen peroxide as the active intermediate agent. This is the simplest explanation for the reduction of hemolysis when catalase is added to the erythrocyte-silica particle system. The action of catalase in deactivating silica surfaces is not achieved by physical adsorption to cell membranes, but by its enzymatic action. The amount of catalase required to quench the hemolysis is directly proportional to the concentration of silica surface silanol groups present in the system. A possible mechanism for hydrogen peroxide-induced hemolysis is a Fenton reaction occurring inside the erythrocytes [Eqs. (1*a*, 1*b*)]. Catalase inhibits the reaction of copper and iron salts with H_2O_2. The observation that albumin causes a significant decrease in hemolysis indicates that copper ions may be principally involved in the reaction.

In conclusion, it is known that silica reacts with carbonyl oxygens on proteins and in cell membranes (*1,5*). This interaction between available surface silanol groups and carbonyl oxygens may produce H_2O_2 in amounts that exhaust the protective mechanisms of the cell, and thereby causes hemoglobin denaturation leading to cell membrane rupture. Although the specific reaction leading to the formation of H_2O_2 has not yet been defined, the findings reported here explain the variable hemolytic activity of different silica surfaces as well as the role of reactive oxygen species in the process of erythrocyte damage by respirable size silica particles.

The authors acknowledge the support received from the Generic Technology Center for Respirable Dust through project number USDI-TPSU-MIT 5142-283.

REFERENCES

1. Nash, T., Allison, A. C., and Harington, J. S. Physio-chemical properties of silica in relation to its toxicity. Nature 5033: 259–261 (1966).
2. Stöber, W., and Brieger, H. On the theory of silicosis. Arch. Environ. Health 16: 706–708 (1968).
3. Allison, A. C. Interaction of silica and asbestos with macrophages. In: Biochemistry of Silicon and Related Problems (G. Bendz and I. Linquist, Eds.), Plenum Press, New York, 1978, pp. 337–355.
4. Cao, C. J., Liu, S. J., and Lin, K. C. The injurious effect of quartz on cell membranes and the anti-injurious effect of aluminum citrate against quartz. In: Abstracts of VII International Pneumoconioses Conference, Pittsburgh, PA, 1988, p. 109.
5. Kriegseis, W., Biederbick, R., Boese, J., Robock, K., and Scharman, A. Investigations into the determination of the cytotoxicity of quartz dust by physical methods. In: Inhaled Particles IV, (W. H. Walton, Ed.), Pergamon Press, Oxford, 1977, pp. 345–359.
6. Nolan, R. P., Langer, A. M., Harington, J. S., Oster, G., and Selikoff, I. J. Quartz hemolysis as related to its surface functionalities. Environ. Res. 26: 503–520 (1981).
7. Pandurangi, R. S., Seehra, M. S., Razzaboni, B. L., and Bosaitis, P. Surface and bulk infrared modes of crystalline and amorphous silica particles: a study of the relation of surface structure to cytotoxicity of respirable silica. Environ. Health Perspect. 87: 327–336 (1990).
8. Kalbanev, I. V., Berestetskaya, I. V., and Butyagin, P. U. Mechanochemistry of quartz surface. Kinet. Katal. 21: 1154–1158 (1980).
9. Shi, X., Dalal, N. S., and Vallyathan, V. ESR evidence for the hydroxyl radical formation in aqueous suspension for quartz particles and its possible significance to lipid peroxidation in silicosis. J. Toxicol. Environ. Health 25: 237–245 (1988).
10. Gulumian, G. and van Wyk, A. Free radical scavenging properties of poly-vinylpyridine n-oxide: a possible mechanism for its action in pneumoconiosis. Med. Lav. 78: 2: 124–128 (1987).
11. Gabor, S., Anca, Z., and Zugravu, E. In vitro action of quartz on alveolar macrophage lipid peroxides. Arch. Environ. Health 30: 499–501 (1975).
12. Will, E. D. The effect of inorganic iron on the thiobarbituric acid method for the determination of lipid peroxides. Biochem. Biophys. Acta 84: 475 (1964).
13. Scheulen, M. E. and Kappus, H. The activation of oxygen by bleomycin is catalyzed by NADPH-cytochrome P-450 reductase in the presence of iron ions and NADPH. In: Oxygen Radicals in Chemistry and Biology (W. Bors, M. Saran, and D. Tait, Eds.), Walter de Gruyter, Berlin, 1984, pp. 425–433.
14. Clemens, M. R., Einsele, H., and Waller, H. D. Lipid peroxidation of erythrocytes monitored by the formation of volatile hydrocarbons. In: Oxygen Radicals in Chemistry and Biology (W. Bors, M. Saran, and D. Tait, Eds.), Walter de Gruyter, Berlin, 1984, pp. 341–349.
15. Gutteridge, J. M. C., and Wilkins, S. Copper salt-dependent hydroxyl radical formation damage to proteins acting as antioxidants. Biochem. Biophys. 759: 38–41 (1983).
16. Halliwell, B., and Gutteridge, J. M. C. Free Radicals in Biology and Medicine. Clarendon Press, London, 1985.
17. Kappus, H. Oxidative stress in chemical toxicity. Arch. Toxicol. 60: 144–149 (1987).
18. Williams, R. J. P. Introduction to silicon chemistry and biochemistry. In: Silicon Biochemistry: Ciba Foundation Symposium 121. John Wiley and Sons, New York, 1986, pp. 24–39.
19. Vallyathan, V., Shi, X., Dalal, N. S., Irr, W., and Castranova, V. Generation of free radicals from freshly fractured silica dust: potential role in acute silica-induced lung injury. Am. Rev. Respir. Dis., 138: 1213–1219 (1988).
20. Treinin, A. Trapped radicals in inorganic glasses. In: Radical Ions (E. T. Kaiser and L. Keran, Eds.), John Wiley and Sons, New York 1968, pp. 525–578.
21. Stansell, M. J., and Deutsch, H. F. Preparation of crystalline erythrocuprein and catalase from human erythrocytes. J. Biol. Chem. 240: 4299–4305 (1965).
22. Hodgson, E. K., and Fridovich, I. The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: inactivation of the enzyme. Biochemistry 14: 5294–5299 (1975).
23. Halliwell, B. Albumin—an important extracellular antioxidant? Biochem. Pharmacol. 37: 569–571 (1988).