TOXICOLOGY AND APPLIED PHARMACOLOGY 123, 62–67 (1993)

Binding of the Cationic Dye, Janus Green B, as a Measure of the Specific Surface Area of Crystalline Silica in Aqueous Suspension

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Received March 16, 1993; accepted June 30, 1993

Binding of the Cationic Dye, Janus Green B, as a Measure of the Specific Surface Area of Crystalline Silica in Aqueous Suspension. DANIEL, L. N., MAO, Y., VALLYATHAN, V., AND SAFFIOTTI, U. (1993). *Toxicol. Appl. Pharmacol.* 123, 62–67.

Twelve preparations of crystalline silica, with a wide range of particle sizes, were assayed by a new method, which measures surface adsorption of the cationic dye Janus green B to crystalline silica samples in a buffered aqueous suspension. The same samples were also assayed for total surface area by the Brunauer–Emmett–Teller (BET) method of surface adsorption of nitrogen gas. A strong linear correlation was found between the two methods of measurement ($r = 0.977$). Reproducible specific surface area measurements by the Janus green B adsorption method were made on 2-mg samples using ordinary visible wavelength spectrophotometric equipment, whereas the BET method necessitated sample sizes in excess of 100 mg and more specialized instrumentation. Five size-fractionated preparations from the same Min-U-Sil α-quartz sample showed an increase in BET surface area and Janus green B binding per unit weight with decreasing particle size. Among four standard α-quartz samples tested, Min-U-Sil 5 and F600 had the lowest specific surface areas, whereas DQ-12 and Chinese standard α-quartz had much higher surface areas. The synthetic silica preparations cristobalite and tridymite had intermediate surface areas. Binding by the cationic dye Janus green B is consistent with a surface charge mechanism and provides a useful new technique for the assessment of surface characteristics of crystalline silica samples. Its linear relationship to surface area suggests that the ratio of aqueous surface charge to surface area is constant for different crystalline silica preparations. Comparison of surface areas for different preparations of crystalline silica is important in understanding the relative activities of these preparations in studies on mechanisms of silicosis and silica-induced lung cancer.

Exposure to respirable particles of crystalline silica (SiO_2, hereafter silica) is the cause of significant respiratory illnesses in humans. Acute and chronic pulmonary silicosis have been observed among workers exposed to various crystalline forms of silica in a number of industries (Parkes, 1982). Lung cancer has been induced in rats by inhalation or by intratracheal instillation of silica (IARC, 1987; Saffiotti and Stinson, 1988; Muhle *et al.*, 1989). Epidemiologic studies have implicated silica exposure in humans as a possible carcinogen (Goldsmith *et al.*, 1986; IARC, 1987; Simonato *et al.*, 1990).

Crystalline silica is the second most abundant mineral in the earth's crust (after feldspar) and exists in a number of polymorphic structural forms. Quartz, the most common form of silica, is more compact than either tridymite or cristobalite, two polymorphs produced at higher temperatures (Klein and Hurlbut, 1985; IARC, 1987). Surface structures and hydration shells for these silica polymorphs differ as well (Unger, 1979). Distinct clinical forms of silicosis have been described following exposures to specific types of silica (Parkes, 1982), but the pathogenetic effects of surface structural differences among silica samples have not been characterized.

Particle size distribution and surface area have an important influence on the activity of silica in experimental model systems for silica toxicity. A number of studies have compared the effects of silica preparations by controlling for surface area (Wiessner *et al.*, 1988; Vallyathan *et al.*, 1992).

Surface area measurement of solid particles based on their physical adsorption of gases or liquids has been extensively studied (Gregg and Sing, 1967; Adamson, 1990). Measurement of silica surface area has been most commonly obtained by the gas adsorption technique, called the BET method from the initials of its authors (Brunauer *et al.*, 1938). Other methods include aqueous surface binding of poly(2-vinylpyridine-*N*-oxide) (PVPNO) to silanol groups (Nolan *et al.*, 1981), binding of Alcian blue in ethanol (Klockars *et al.*, 1990), and binding of 6-nitrobenzoindolinopyran in glycerin (Jozwiakowski and Connors, 1987).

In the present study, we undertook the development of a new binding assay, because most of the above methods require the use of specialized instruments or of organic solvents. We addressed the need for a simple and rapid method to obtain surface area measurements applicable to small silica samples in an aqueous environment.

0041-008X/93 $5.00

MATERIALS AND METHODS

Silica preparations. Seven α-quartz preparations used in this study were derived from Min-U-Sil.[1] A sample of Min-U-Sil 5 (MQZ) was obtained through the Illinois Institute of Technology Research Institute in 1985, characterized by X-ray diffraction as >99% pure, and used for experiments on carcinogenesis in animals, cell toxicity and transformation, and DNA damage *in vitro*. A sample was obtained by treatment of MQZ with hydrofluoric acid etching (HFMQZ) and similarly used (Daniel *et al.*, 1993). Five Min-U-Sil size-fractionated samples (<2, <5, <10, 5–7, and 5–10 μm) were prepared from Min-U-Sil 10 using a Donaldson particle classifier.[2] Silica samples were analyzed for purity by X-ray powder diffraction using an automated powder diffractometer.[3] The samples were found to be 98–99% pure quartz. Particle size determinations were done with scanning electron microscopy in concert with automated image analysis. Samples prepared on filters[4] were viewed at a magnification of 1000×, and a minimum of 1000 particles from 20 fields were measured at random for area equivalent diameters.

Three other quartz standard preparations were used. DQ-12 (characterized by Robock, 1973) and F600 were obtained from F. Pott, University of Düsseldorf, and Chinese standard α-quartz (characterized by Fu *et al.*, 1984) was obtained from the University of Nanjing, China.

Samples of cristobalite and tridymite were synthesized and purified at the Illinois Institute of Technology Research Institute. These samples were assayed for purity by X-ray fluorescence spectroscopy and for particle size distribution by optical microscopy and Coulter counting.

BET surface area measurement. The specific surface area measurements were made on 1-g samples of silica using the nitrogen adsorption isotherm technique of Brunauer *et al.* (1938). The samples were treated at 200°C for 2hr and the specific surface area/pore volume distributions were measured.[5] Free space was determined with helium.

Janus green B assay. Janus green B (3-(diethylamino)-7-[[*p*-(dimethylamino)phenyl]azo]-5-phenylphenazinium chloride) was obtained as a dry powder[6] (70% dye). Monobasic and dibasic sodium phosphate[7] were used to make molar stock solutions, which were titrated to pH 7.4 by mixing. All buffers were treated with a chelating resin[8] before use.

Janus green B was dissolved in 10 mM phosphate buffer as a 100 μg/ml stock solution. This solution was filtered through a 0.2 μm cellulose nitrate filter[9] and was stable at room temperature for up to 2 weeks without forming a precipitate. A standard curve of Janus green B at concentrations from 15 to 28.5 μg/ml, at a wavelength of 595 nm (absorbance maximum), was obtained for 10 samples. Absorbance was found to be linearly correlated to concentration of Janus green B in this range and corresponded to a mean correlation relationship of 0.0444 OD units = 1 μg/ml (SD = 0.00057).

Prior to each assay, silica samples were dried in an oven at 110°C for 24 hr. A 1 mg/ml suspension in 10 mM phosphate buffer (pH 7.4) was prepared in a tube and sonicated in a cleaning sonicator[10] for 2 min. Two milliliters of the fresh suspension were added to 2 ml of a freshly diluted 50 μg/ml Janus green B solution in a 10-ml polystyrene tube and tightly capped. Samples were then incubated at 37°C in a shaker incubator for 1 hr. Following centrifugation at 1800*g* for 10 min at 25°C, the upper 3.2 ml of supernatant was divided into two 2-ml plastic tubes and centrifuged 15 min at 12,000 rpm in a microcentrifuge. For each duplicate supernatant sample (0.9 ml), absorbance was measured at 595 nm.[11] Duplicate samples were compared for consistency and the average between the two readings was subtracted from the absorbance of a control specimen of Janus green B exposed to buffer, which did not contain silica and which was similarly centrifuged. Using this separation method, background absorbance by silica-treated buffer alone was found to be less than 0.002 for all silica samples, when compared to the buffer blank. Therefore, no correction was necessary for absorbance at 595 nm by residual silica. The amount of Janus green B bound for each reaction was computed, based on the standard curve of Janus green B absorbance versus concentration.

PVPNO assay. Binding of silica to PVPNO was determined using the method of Nolan *et al.* (1981). Individual samples were prepared and tested in a manner similar to that used for the Janus green B assay. Acrylic cuvettes were used in place of polystyrene ones because of their transparency in the uv region. The absorbance maximum of PVPNO (in 10 mM phosphate buffer) was 270 nm, as reported by Nolan *et al.* (1981).

A standard curve for absorbance versus concentration was linear between 20 and 50 μg/ml. The latter concentration was used as the test concentration for solutions exposed to silica. Silica in suspension was exposed to PVPNO at a final concentration of 2 mg silica/ml. The amount of PVPNO bound per milligram of silica (after subtraction for background absorption of the centrifuged supernatant for silica alone in buffer) was calculated, based on the standard curve of PVPNO absorbance versus concentration.

RESULTS

Visual inspection of solutions of Janus green B, after silica was suspended in them and sedimented, showed loss of color from the solution and a corresponding dark coloration of the sediment (Fig. 1). Because the surface of silica in aqueous suspension is negatively charged and Janus green B is positively charged (Fig. 2), it is likely that the dye binds by a charge attraction mechanism, but direct correlation of the binding with the ζ potential (Adamson, 1990) was not performed in the current study. The dark coloration of the sediment, however, does indicate that Janus green B is not reduced upon binding to the silica surface, since Janus green B in its reduced form is colorless (Kelley *et al.*, 1984). The avid binding was found to be slowly reversible by repeated washing of successive pellets with fresh buffer. Changes in pH between 4 and 9 had no effect on Janus green B solubility at the concentration used.

Janus green B at a concentration of 25 μg/ml had a visible wavelength maximum absorbance near 595 nm in 10 mM phosphate buffer at pH 7.4 (Fig. 3). Absorbance at 595 nm was used to derive a standard curve of Janus green B absorbance versus concentration (Fig. 4). Measurement of changes in absorbance of a 25 μg/ml solution of Janus green B after exposure to 500 μg/ml silica in suspension was used to calculate the amount of Janus green B bound per milligram of silica. Triplicate samples were weighed separately

[1] Pennsylvania Glass Sand Corp., Pittsburgh, PA.
[2] Accucult particle classifier, Donaldson, Majal Division, St. Paul, MN.
[3] Phillips.
[4] Nucleopore, Pleasanton, CA.
[5] Gemini 2360 unit, Micrometrics, Norcross, GA.
[6] Aldrich.
[7] Sigma.
[8] Chelex, Bio-rad, Richmond, CA.
[9] Nalgene.
[10] Branson model 220, Shelton, CT.

[11] Beckman DU-7.

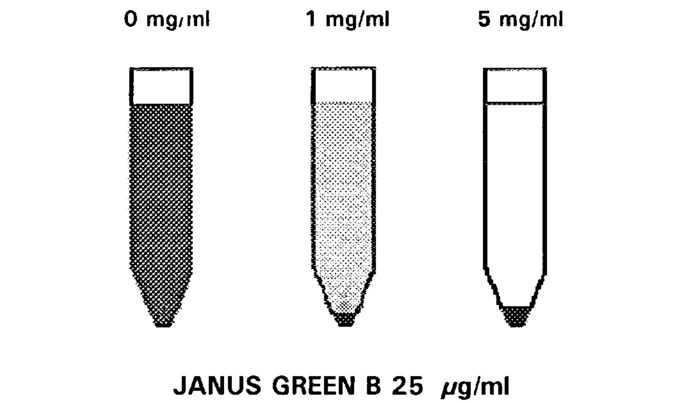

FIG. 1. Diagram of Janus green B binding reaction with 0, 1, and 5 mg/ml of Min-U-Sil. The dark green color of a 25 μg/ml solution was almost completely clarified by a 5 mg/ml suspension of Min-U-Sil. The silica sediments at the bottom of the tubes were darkly colored. Min-U-Sil sedimented in buffer alone was light gray in color (not shown).

and the final supernatants of each assay read in duplicate. Duplicate readings were not significantly different (data not shown) and an average of the two readings was taken as the sample value. Table 1 shows amounts of Janus green B binding per silica unit weight.

BET surface areas for the samples are also shown in Table 1. As expected, for size-graded MQZ samples surface areas increased with decreasing particle size. Large differences in surface area were found among various α-quartz standard preparations in use in the United States (MQZ, HFMQZ), Germany (DQ-12, F600), and China (CSQZ). Correlation of BET surface area with Janus green B binding to the silica samples was linear (r = 0.977) (Fig. 5), according to the formula $y = 1.52x + 4.25$, where y represents Janus green B bound/mg and x represents BET surface area in m^2/g.

Particles other than crystalline silica were tested and did not conform to the same linear relationship of Janus green B binding and BET surface area. Silica gel (Sigma), which was assayed by the manufacturer at 500 m^2/g, bound only 220.9 μg Janus green B/mg. Using the above formula, the predicted surface area would have been only 144.4 m^2/g. Other measured and predicted (in parentheses) surface

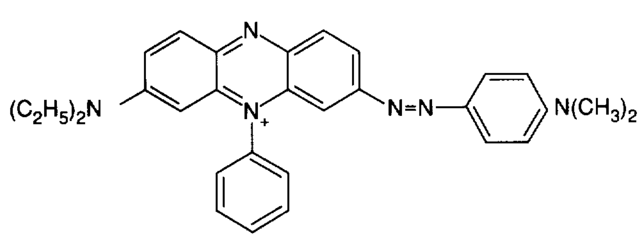

FIG. 2. Janus green B.

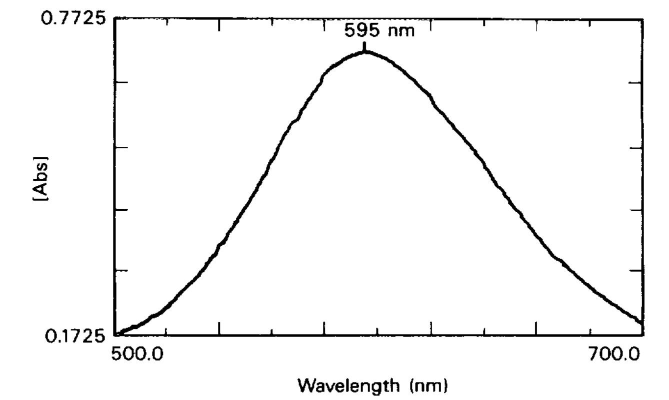

FIG. 3. Absorbance spectrum of Janus green B (25 mg/ml) in 10 mM phosphate buffer (pH 7.4).

areas were alumina, 4.0 (1.6); barite, 5.8 (1.3); and feldspar, 2.2 (3.4). Differences in the relationship of Janus green B binding capacity and BET surface area for these four samples may be related to a finite pore size or a reduction in surface area by hydration (of the amorphous silica gel), and to differences in the negative surface charge characteristics of the other three samples, which had silica contents of 0.6, 3.1, and 15.2%, respectively.

Results of the quantitative binding assay of silica to PVPNO also showed a linear correlation to BET surface area (Fig. 6), as well as a correlation with Janus green B binding; however, these correlations were not as strong (r = 0.922 and r = 0.961, respectively).

DISCUSSION

The current study provides a new method for measuring the specific surface area of crystalline silica quickly and easily using very small amounts of material. Reproducible duplicate measurements were obtained on a large number of

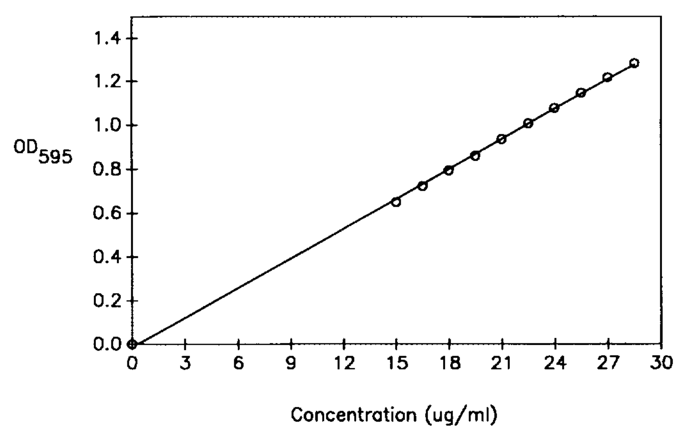

FIG. 4. Standard curve of Janus green B absorbance.

TABLE 1
Surface Area and Janus Green B Binding

Sample	Mean BET area (m^2/g)	STD	Mean JGB bound (μg/mg silica)	STD
Min-U-Sil 10 derived				
<2 μm	6.77	0.01	13.16	0.32
<5 μm	4.67*	ND	9.26	0.47
<10 μm	1.47	0.07	6.49	0.15
5–7 μm	1.70	0.03	5.81	0.07
5–10 μm	0.80*	ND	4.48	0.38
Min-U-Sil 5 derived				
MQZ	3.15	0.01	10.52	0.15
HFMQZ	2.98	0.05	10.57	0.31
Other α-quartz				
DQ-12	8.48	0.08	18.06	0.09
F600	3.65	0.02	10.52	0.10
CSQZ	11.67	0.03	21.52	0.14
Other polymorphs				
Cristobalite	3.93	0.12	9.34	0.50
Tridymite	5.24	0.25	12.46	0.24

* Single reading only.
Note. ND, not determined.

2-mg samples run concurrently. Use of smaller volumes and small cuvettes might allow even greater sensitivity. In principle the assay is sensitive enough to be applied to repurified silica samples isolated from pathological specimens (Hemenway *et al.*, 1990) and thus provides a means of accurately assessing the tissue burden and size characteristics of silica actually present in the lung or intracellularly in phagocytic cells, for example. However, currently available methods of silica repurification may alter the surface adsorption characteristics of silica and necessitate modification of the procedures. We have observed a 10–30% increase in Janus green B adsorption following grinding of

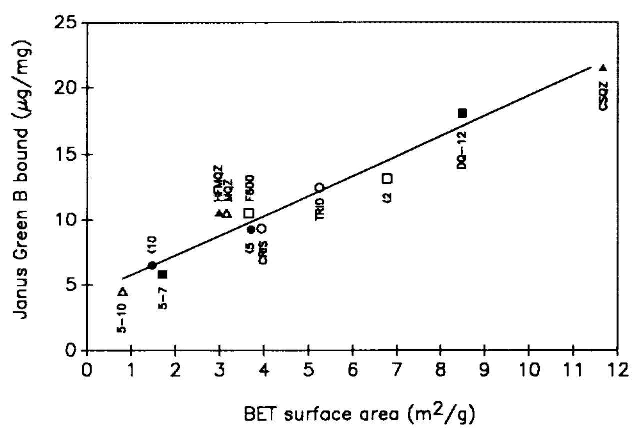

FIG. 5. Correlation of BET surface area with Janus green B binding.

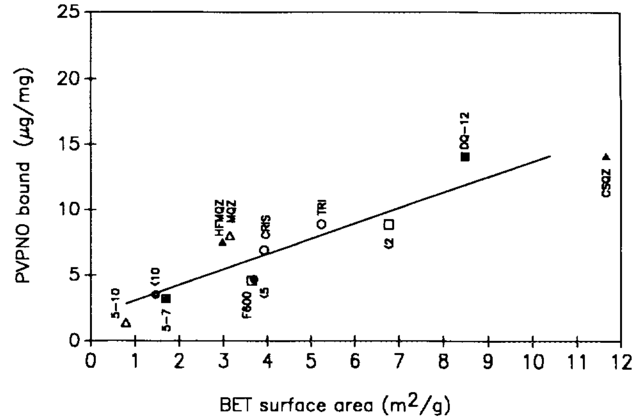

FIG. 6. Correlation of BET surface area with PVPNO binding.

silica in an agate mortar. Aging of this ground silica in air or water leads to a gradual decrease in Janus green B binding activity (data not shown).

It may be possible to determine surface area for other particulates based on Janus green B binding. As shown, alumina, barite, and feldspar also bind Janus green B, but the relationship of Janus green B binding to surface area was not studied for these materials.

In our hands, quantitative binding of silica could be more easily obtained to Janus green B than to PVPNO, because the former's absorbance maximum is not in the ultraviolet region (where significant absorption by residual silica in the supernatant was found to occur). In preliminary experiments, two other cationic dyes, rhodamine B and rhodamine 123, were found to be less suitable for a quantitative binding assay because of their less avid binding to silica (data not shown) and because of significant absorption by the silica suspension supernatant blank in the wavelength region of rhodamine maximum absorption. Compared to the structures of the rhodamines, Janus green B (see Fig. 2) is a larger molecule with six nitrogen atoms as compared with only two for the rhodamines. Furthermore, four nitrogen-containing areas of the Janus green B molecule are spaced at intervals of approximately 0.5 nm. These nitrogen-rich areas may share positive charge through resonance of the aromatic structure and favor interaction with multiple anionic groups on the silica surface simultaneously. Interestingly, the distance between surface oxygen groups of cristobalite and tridymite has also been calculated to be about 0.5 nm (Unger, 1979). Our finding of a constant relationship between Janus green B binding and BET surface area for all crystalline silica polymorphs tested is consistent with a surface charge distribution for α-quartz similar to that of cristobalite and tridymite.

Two other methods, Alcian blue (Klockars *et al.*, 1990) and 6-nitrobenzo-indolinopyran (Jozwiakowski and Connors, 1987), measure binding of silica in nonaqueous solvents and were not compared in the current study. Compared to the BET method, surface area measurement by Janus green B adsorption requires much less material and a minimum of specialized equipment, but has the disadvantage of compromising the purity of the tested aliquot.

The mechanisms of cellular injury and of carcinogenesis in experimental animals, caused by silica, are under investigation. Several studies have attempted to relate the toxic effects of silica to its surface properties. Early on, attention was drawn to chemical interactions involving surface silanol groups (Nash *et al.*, 1966). Covering of the silica surface by the organic polymer PVPNO markedly reduces silica toxicity *in vitro* (Nolan *et al.* 1981; Klockars *et al.* 1990; Englen *et al.*, 1992) and *in vivo* (Goldstein and Rendall, 1987). Hemolytic activity of quartz is also reduced by binding of metal salts to the negative surface charges of silica (Nolan *et al.*, 1981). Toxicity to alveolar macrophages (Brown *et al.*, 1988) and other cells (Miller *et al.*, 1987; Lesur *et al.*, 1992) has been correlated with the production of free radicals from the silica surface, either directly or through Fenton chemistry involving trace iron impurities (Vallyathan *et al.*, 1988; Fubini *et al.*, 1989; Doelman *et al.*, 1990; Daniel *et al.*, 1993). Recent studies comparing cell toxicity of silica preparations have attempted to control for differences in particle size distribution by normalizing the dose according to total BET surface area (Wiessner *et al.*, 1988; Vallyathan *et al.*, 1992).

The current study provides a useful new technique for the assessment of surface characteristics of crystalline silica samples. Binding by the cationic dye Janus green B is consistent with a surface charge mechanism. Its linear relationship to surface area suggests that the ratio of aqueous surface charge to surface area is constant for different crystalline silica preparations. Comparison of surface areas for different preparations of crystalline silica is important in understanding the relative activities of these preparations in studies on mechanisms of silicosis and silica-induced lung cancer.

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