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A ^{13}C CP/MAS and ^{31}P NMR study of the interactions of dipalmitoylphosphatidylcholine with respirable silica and kaolin

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Abstract

The interaction of silica and kaolin with dipalmitoylphosphatidylcholine (DPPC) has been studied using ^{13}C and ^{31}P solid state nuclear magnetic resonance spectroscopy. These studies explore the molecular interactions of these respirable dusts with a model lung surfactant species to characterize silica toxicity in mixed systems. The choline head group of DPPC was found to remain mobile when adsorbed on kaolin, in contrast to an immobile head group on silica. Further, glycerol carbon intensities were greatly diminished relative to that of choline carbons, a result attributed to broadening effects. These preliminary findings suggest that silica toxicity may not be related to choline mobility as previously noted [J. Colloid Interface Sci. 172 (1995) 536–538].
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Keywords: DPPC; Silica; Kaolin; NMR; Silicosis; Lung surfactant; Silanol; Respirable dust; Dipalmitoylphosphatidylcholine

1. Introduction

Silicosis is an occupational lung disease caused by exposure to respirable silica, although the molecular mechanisms responsible for toxicity are controversial. Lysosomal membrane destruction of pulmonary macrophages is believed to account for silica's toxicity. Depasse and Warlus [1] attribute this destruction to surface-dissociated silanols (SiO^-) interacting with the positively charged $RN(CH_3)_3^+$ groups in cell membrane components such as DPPC, an interaction they claim may affect membrane protein adsorption and prophylaxis. Allison [2], however, relates toxicity to hydrogen-bonding interactions of surface silanols with phosphate groups in cellular membranes, changing membrane permeability. Chunbo et al. [3] agree, claiming that silica greatly decreases the mobility of phosphate species in phospholipid bilayers, and that the $RN(CH_3)_3^+$ head group becomes more mobile, freed from its association with the phosphate. They also attribute cell membrane disruption to these molecular changes which affect membrane permeability and bilayer fluidity. Furthermore, they saw that the cytotoxicity of silica-challenged cells was relieved by aluminum citrate, the aluminum ions successfully competing with silanols for phosphate sites in cell walls.

We have revisited this controversy in an attempt to understand the molecular basis for anomalous fibrogenic and cytotoxic behavior observed for silicon-containing respirable dusts. Toxicity appears dependent on surface silanol concentration for pure crystalline silicas, but not for mixed or amorphous silicas or silicon-containing clays such as kaolin which possess potential for surface impurities or competing chemistries [4,5]. Furthermore, Wallace et al. [6] claim that the primary response to dust intrusion into lungs is a prophylactic coating by lung surfactant, predominantly DPPC molecules. This coating renders these mineral dusts noncytotoxic. Subsequent enzymatic processes preferentially remove the surfactant coating and restore cytotoxicity for crys-

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; CP, cross polarization; MAS, magic angle spinning; BET, Brunauer–Emmett–Teller (adsorption isotherm); PSS, physiologic saline solution; SP, single pulse; CSA, chemical shift anisotropy.

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talline quartz, but the coating persists for kaolin. In vivo toxicity has been attributed to differing enzymatic coating digestion processes or rates [7]. The restoration of toxicity results from subsequent silanol interactions with chemically similar cell membrane sites. It is critical to understand the nature of these molecular interactions in coated and enzymatically altered DPPC/dust complexes to provide a basis for exposure assessment and treatment strategies.

NMR spectroscopy is a powerful tool to determine molecular structure and dynamics of biological species in solution. Solid state NMR is commonly applied to study molecular interactions of solids and of mixed in vitro systems such as biomolecular species/respirable dusts systems. The CP/MAS technique yields chemical structure and dynamic information of molecules in the solid state, although at poorer resolution than in solution. Molecular orientation can be further determined through chemical shift anisotropy analysis to yield further details describing molecular interactions. In this preliminary note, we report limited ^{13}C CP/MAS results with a bearing on the initial respirable dust/DPPC interaction. In this and subsequent studies, we examine the molecular interactions of respirable dusts with DPPC and other biomolecules present in the lung to develop a molecular basis for silica toxicity.

2. Materials and methods

2.1. Respirable dusts

Min-U-Sil 5 respirable quartz dust (U.S. Silica Corporation, Berkeley Springs, WV) was determined by X-ray diffraction to be 99.5% α-quartz with 98% of particles smaller than 5 mm area equivalent diameter. The specific surface area of this silica dust was 3.97 m^2/g as measured by BET N_2 gas adsorption. A sized fraction of the respirable kaolin dust (Georgia Kaolin Mills, Augusta, GA) used was at least 95% aluminosilicate with 99% of the fraction <5 mm area equivalent diameter. Crystalline quartz was not detected by X-ray diffraction. The specific surface area of this kaolin dust was 13.25 m^2/g as measured by BET.

2.2. Mixed DPPC/dust preparation

DPPC (Calbiochem, San Diego, CA) was ultrasonically dispersed into a physiologic 0.165 M NaCl solution at 5 mg DPPC/ml PSS, followed by centrifugation at 1500*g* for 10 min to remove nondispersed DPPC. Silica or kaolin were mixed in this dispersion at a ratio of 0.1 g DPPC/g silica or 0.2 g DPPC/g kaolin and then centrifuged at 1500*g* for 10 min. After decanting, the 0.2 to 0.4 g wet paste was packed in a 7 mm rotor and spun at low speed (800 Hz) for several minutes. Clear water was siphoned from the rotor, and the still damp solids were spun at 5.5 kHz. Prior studies [8] of these preparations had shown that the silica adsorbs about 60 mg DPPC/g and kaolin about 150 mg DPPC/g as multilayers. Approximately 20 mg DPPC/g silica and 80 mg DPPC/g kaolin provide a bilayer covering that is stable to rinsing and fully suppresses hemolytic activity.

2.3. NMR spectroscopy

Solid-state ^{13}C NMR studies of mixed DPPC/dust systems were made in a 7 mm Bruker CP/MAS probe (Bruker Biospin Corporation, Billerica, MA) at 295 K. SP experiments typically were performed using 5 μs 90° pulses at 75.45 MHz with high power ^1H decoupling during signal acquisition to obtain quantitative resonance chemical shift. Recycle delays were 3–4 s. Spectra are the result of signal-averaging 16,384 to 32,768 acquisitions. CP/MAS experiments were carried out in matched 60–80 kHz B_1 fields typically with 4 ms contact times and 4 s recycle delays. CP experiments were 2–5 times more sensitive than SP experiments. CP experiments are used to enhance sensitivity relative to SP and to observe species within 0.5 nm of ^1H nuclei. ^{13}C chemical shifts were referenced externally to hexamethylbenzene (^{13}C $\delta_{CH_3} = 17.4$ ppm). ^{31}P NMR was obtained at 121.494 MHz, externally referenced to 85% D_3PO_4 in D_2O (^{31}P $\delta = 0.00$ ppm). The reference high resolution ^{13}C NMR study of DPPC in $CDCl_3$ solution was performed in a 5 mm inverse detection Nalorac probe (Nalorac Corporation, Martinez, CA). The spectrum was obtained by signal averaging 8192 scans over a 25 kHz spectral width using a single pulse with decoupling pulse sequence. ^{13}C nuclei were excited using an 11 μs 90° pulse. Resonance widths were <2 Hz. Chemical shifts were referenced internally to $CDCl_3$ at 77.23 ppm.

3. Results and discussion

^{13}C NMR spectra of DPPC in solution, as a neat solid, and adsorbed on respirable dust materials are presented in Fig. 1. Spectra are divided into two regions. Carbons in the nonpolar palmitoyl tails are observed in the aliphatic hydrocarbon region (14–35 ppm). (The palmitoyl carbonyl resonance is an exception, found offscale at 173 ppm.) DPPC's polar head group contains aliphatic carbons bonded to heteroatoms (O, N), which are found in the 50–75 ppm region. These resonances are sensitive to intermolecular interaction between the heteroatoms and solvents or surface hydroxyls of respirable silica and kaolin. A 4× expansion of this region is inserted into each spectrum.

Carbon resonance assignments have been previously reported, obtained in D_2O emulsion or in the solid state [9–11]. Glycerol carbon resonances are assigned G-1 (63.6 ppm), G-2 (70.7 ppm), and G-3 (63.2 ppm), where the number denotes the structural position relative to the phosphate group (see Fig. 1a). Choline resonances are likewise designated and assigned C-1 (59.5 ppm), C-2 (66.7 ppm), and the trimethylammonium carbons C-3 (54.7 ppm).

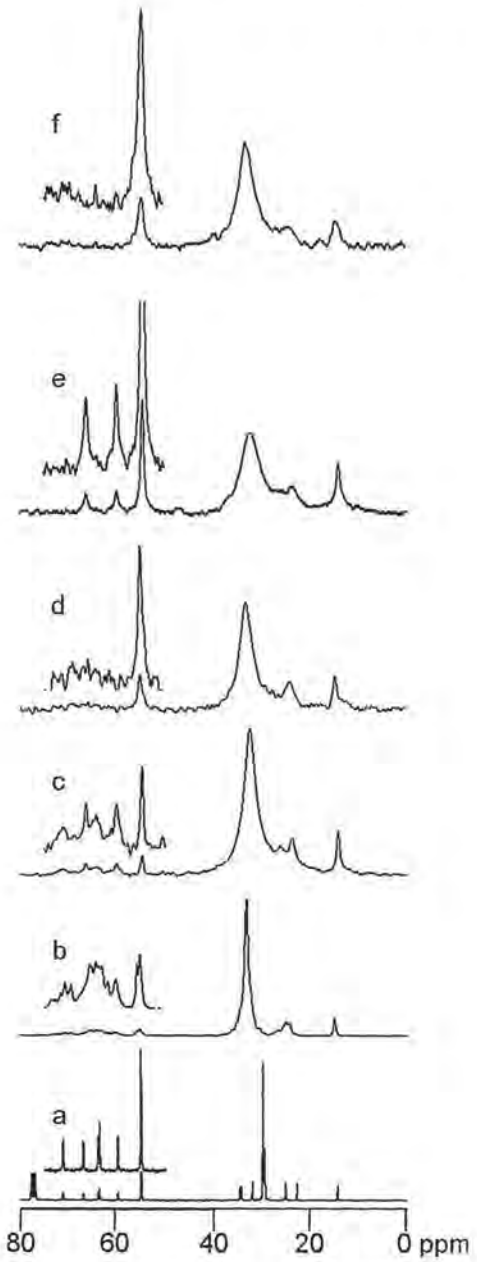

Fig. 1. ^{13}C NMR spectra of DPPC in various phases: (a) in $CDCl_3$ solution obtained using high resolution NMR; (b) as a neat solid; (c) adsorbed on kaolin; (d) adsorbed on silica (b–d obtained using CP/MAS NMR); (e) adsorbed on kaolin; and (f) adsorbed on silica (e–f obtained using SP/MAS NMR).

Resonances in the DPPC solid state spectrum (Fig. 1b) show the effects of differing intermolecular association when compared to the solution spectrum. Chemical shift changes ($\Delta\delta$) are evident for the palmitic acid carbons, from 29.9 ppm to roughly 32 ppm as a result of nonpolar solvent removal. Similar downfield shifts (~1 ppm) are found for C-1, C-3, G-1, and G-3 resonances, though G-1 and G-3 have not been unambiguously assigned. Upfield shifts are observed for G-2 and C-2 resonances. Bruzik et al. [9] report ^{13}C CP/MAS NMR of DPPC solids vs temperature and describe chemical shift, intensity, and line width changes in this region through two phase transitions at 307 and 310.5 K. Guo and Hamilton also present ^{13}C MAS NMR spectra for hydrated DPPC in the representative phases [11]. In the gel phase at 298 K, the choline carbons are sharp resonances while the glycerol carbon resonances are significantly broadened. These resonances are lost in the ripple phase at 311 K, but much sharper for the liquid crystalline phase at 318 K. All the glycerol and choline carbon resonances are present in our CP/MAS spectra at ambient temperature in their studies. Our assignments are consistent with this ambient L_b phase at roughly the same experiment temperature. The C-3 and G-2 resonances in Fig. 1b show some splitting, suggesting a distribution of glycerol/choline inter- and intramolecular interactions within this mixed crystalline material.

CP/MAS spectra are presented for DPPC adsorbed on kaolin (Fig. 1c) and DPPC adsorbed on silica (Fig. 1d). The palmitic chain region is similar to the neat solid DPPC spectrum, but significant differences are observed in the glycerol/choline region. For DPPC on kaolin, all choline carbon resonances are singular and sharp, while the glycerol carbon resonances appear broadened (line width ~3 ppm vs 1 ppm). $\Delta\delta$ again is about +1 ppm for G-1, G-3, C-1, and C-3, but −1 ppm for G-2 and C-2. For DPPC on silica, the only surviving glycerol or choline carbon resonance is C-3. Figs. 1e and 1f present the corresponding SP/MAS spectra. The choline carbon resonances remain sharp, but the glycerol resonances are missing for DPPC on kaolin. The C-3 resonance again is the lone survivor in this region for DPPC on silica.

The behavior of resonances C-1 and C-2 in this series is evidence of a strong interaction between the choline chain in DPPC and surface hydroxyl species on silica. In contrast, C-1 and C-2 behave as if part of a chain unassociated with the kaolin surface, similar to the behavior of the palmitic acid chains. The clear presence of C-3 as well as the palmitic acid carbons in all spectra demonstrates that the loss of the other glycerol or choline carbon resonances is not a signal-to-noise issue. Bruzik et al. [9] report a similar situation: a complete loss of signal intensity for these carbons at elevated temperatures in pure DPPC. In that work, loss of signal intensity is attributed to two factors: ineffective ^{1}H decoupling (as explained by Lyerla et al. [12]), and inefficient cross polarization. Both factors are a function of regional molecular motion. Inefficient cross polarization efficiency does not account for the loss of signal intensity in SP/MAS spectra in Fig. 1f. Regional chain motion on the order of the ^{1}H decoupling power would leave the ^{13}C–^{1}H dipolar coupling in an intermediate correlation time regime which would diminish the observed signal, as line broadening, or complete loss in the case of extreme broadening. The dynamics of the ^{1}H–^{13}C dipolar interactions is more complicated in CP/MAS experiments, which may account for the presence of residual although broadened glycerol carbon signal intensity in those CP/MAS spectra. The absence or broadening of glycerol carbon resonances indicates strong interactions between glycerol oxygens or palmitic acid carbonyls and dust surface

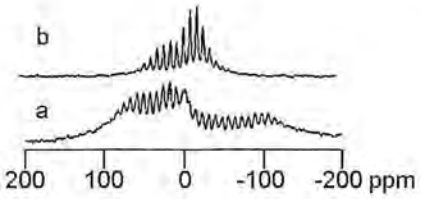

Fig. 2. ^{31}P NMR spectra of DPPC: (a) adsorbed on silica; (b) adsorbed on kaolin. Rotor spin speed was 990 Hz.

hydroxyls. These interactions are significant on both kaolin and silica.

The sample preparation technique ensured DPPC surface coverage for each dust, but also inferred that additional bilayers may be present as well. Nonsurface DPPC species were, however, not observed in ^{13}C NMR spectra. Rinsing techniques were apparently effective in only leaving a single DPPC monolayer on the dust surface.

In ^{31}P NMR studies, DPPC presented a single phosphate resonance at −0.66 ppm in $CDCl_3$, but at +3.0 ppm in D_2O. Chemical shift is an established indicator of phosphate pH (or pD) [13], which was determined to be 7.3 in D_2O. The upfield shift observed in the $CDCl_3$ spectrum is consistent with a protonated phosphate in a nonpolar solvent. In the solid state ^{31}P NMR studies, the single resonance observed at +0.8 ppm was analyzed for CSA in a nonspinning solid-state NMR study ($\delta_{11} = +105$ ppm, $\delta_{22} = +28$ ppm, $\delta_{33} = -130$ ppm, CSA = 235 ppm, $\delta_{iso} = +1.0$ ppm). These values are similar to those reported for anhydrous DPPC at 20 °C ($\delta_{11} = +98$ ppm, $\delta_{22} = +34$ ppm, $\delta_{33} = -134$ ppm, CSA = 232 ppm, $\delta_{iso} = +0.3$ ppm) and broader than hydrated DPPC-H_2O ($\delta_{11} = +81$ ppm, $\delta_{22} = +25$ ppm, $\delta_{33} = -110$ ppm, CSA = 191 ppm, $\delta_{iso} = -1.3$ ppm) [14]. Guo and Hamilton report further narrowing (CSA = 67) to an axially symmetric pattern for gel phase DPPC hydrated in degassed D_2O (70 wt%). The isotropic chemical shift val-

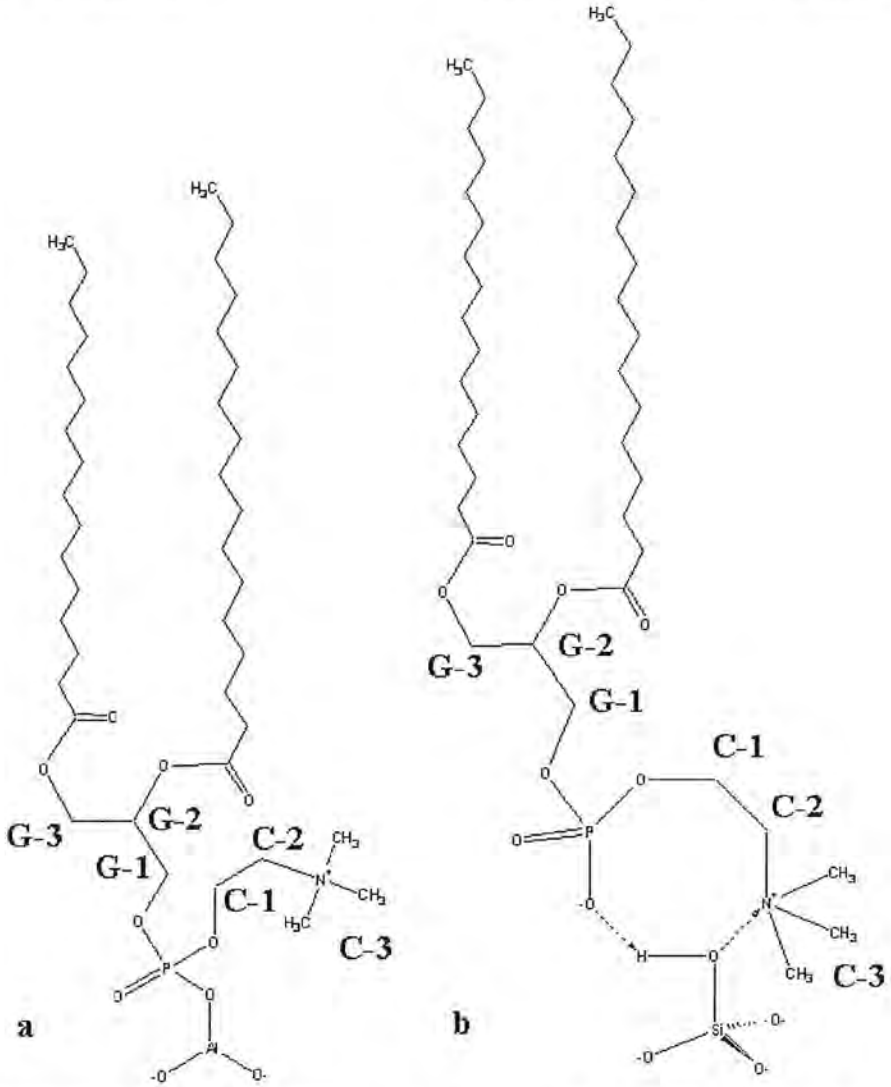

Fig. 3. Proposed respirable dust surface interactions with DPPC: (a) surface adsorption on kaolin; (b) surface adsorption on crystalline silica. Hydrogen bonding between glycerol or phosphate ester oxygen or choline nitrogen and surface hydroxyls are indicated with dashed arrows. Covalent Al–O–P bond formation is indicated with a solid line.

ues indicate that the phosphate in the nonhydrated solid crystalline material is always associated with a positively charged species, which could be a proton, the choline head group or a surface site.

CSA studies of DPPC adsorbed on dusts surfaces (Fig. 2) revealed significant differences for kaolin ($\delta_{11} = +55$ ppm, $\delta_{22} = -21$ ppm, $\delta_{33} = -55$ ppm, CSA = 110 ppm, $\delta_{iso} = -7.0$ ppm), but essentially equivalent values for silica ($\delta_{11} = +95$ ppm, $\delta_{22} = +11$ ppm, $\delta_{33} = -130$ ppm, CSA = 235 ppm, $\delta_{iso} = -8.0$ ppm) relative to DPPC solid. When adsorbed on either respirable dust, a strong upfield isotropic shift was observed, indicating that the phosphate site is still in a highly acidic environment in either case. The observed decrease in asymmetry at the phosphate is consistent with increased mobility for the choline chain.

DPPC's phosphate was found to be strongly coordinated to surface hydroxyls upon adsorption on respirable dusts, in contrast to its coordination with the $RN(CH_3)_3^+$ choline head group observed in pure solid and dissolved DPPC samples. The glycerol backbone also experienced motional restriction upon adsorption to dusts, leading to signal broadening and loss not observed in pure solid DPPC ^{13}C NMR studies. The choline end group appeared to be similarly attached on silica, but it appeared to be freely mobile on kaolin, leading to a significant change in the asymmetry observed at the phosphate site. A rough schematic of these interactions is presented in Fig. 3. Phosphate species are known to dissociate aluminols to form aluminum phosphates. Murashov and Leszczynski [15] have performed computational studies which predict covalent Al–O–P bond formation, with a heat of formation = 13 kJ/mol. Covalent bond formation would release a mobile hydroxide ion that could charge compensate a mobile choline end group. Corresponding studies of phosphate and silanol interactions predict an intact silanol hydrogen bonded to the phosphate with a heat of formation roughly 4 kJ/mol. The silanol remains intact, and the choline group remains coordinated with surface oxides by electrostatic forces.

These initial NMR results reveal a difference in the surface interactions of the choline head group as DPPC initially coats respirable dust surfaces. They demonstrate that solid-state NMR studies can substantially contribute to the understanding of the chemical interaction of silica, kaolin, and mineral/DPPC mixed systems. They describe the initial dust/surfactant interactions, but certainly do not offer a complete explanation for observed differences in silica and kaolin toxicity. Both dusts have been identified as noncytotoxic while coated with DPPC, but become cytotoxic again when that coating is removed. Studies of the secondary biomolecular responses that affect the coating stability are under way to provide a molecular basis for overall differences observed in kaolin and silica toxicity and disease potential. More thorough examinations of the strength and geometry of these interactions are proceeding.

4. Summary

We present ^{13}C and ^{31}P solid state NMR analyses of mixed DPPC/dust systems. Studies reveal restricted molecular motion in the choline region of the DPPC molecule upon silica adsorption, but mobile choline groups on kaolin. These tentative findings suggest that molecular orientation of DPPC adsorbed on the differing dust types may account for differences in pulmonary disease anomalies previously observed. Further studies are needed to determine if orientation can account for differences in lysosomal activity and secondary effects responsible for differences observed in cytotoxicity and chronic disease processes.

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