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Annealing of silica to reduce the concentration of isolated silanols and peak tailing in reverse phase liquid chromatography

Josh J. Newby[a], Michael A. Legg[b], Benjamin Rogers[a], Mary J. Wirth[a,*]

[a] Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47907, USA
[b] CNA Corporation, Alexandria, VA 22311, USA

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ABSTRACT

Non-porous, colloidal silica particles were annealed at three different temperatures, 800, 900 and 1050 °C. The adsorption of lysozyme, a probe of surface roughness, was consistent with progressively reduced surface roughness as temperature increased. The heat treated silica particles were rehydroxylated and then used to pack UHPLC columns. The cationic protein lysozyme was used to probe silanol activity, which exhibited progressively less tailing as the annealing temperature increased. FTIR spectroscopy confirmed that the abundance of isolated silanols on the surface was reduced by annealing at 900 °C or 1050 °C. FTIR also revealed that there was markedly increased hydrogen bonding of the isolated silanols to neighbors after rehydroxylation. These results combine to support the hypothesis that (a) isolated silanols on silica cause tailing in RP-LC and (b) nonplanar topography gives rise to isolated silanols.

1. Introduction

Peak tailing in HPLC is a long-standing problem in silica-based reversed phase liquid chromatography [1–4]. The type of peak tailing that is typically observed in RP-LC is termed nonlinear tailing, where the concentrations used in HPLC exceed the linear part of the adsorption isotherm [5,6]. The separation efficiency is reduced, as calculated using the Foley–Dorsey equation [7]. Tailing is most severe for analytes bearing amine groups because amines hydrogen bond to silanols on silica. Tailing is a serious problem since amino groups are pervasive in pharmaceuticals, peptides and proteins. Improvements in the quality of chromatographic silica gel two decades ago yielded Type B silica, which greatly reduced tailing [8]. The key to this higher performance was a fully hydroxylated surface that maximized the number of surface silanols [8], approximately 8 μmol/m^2 [9]. A high density of silanols enables these groups to preferentially hydrogen bond to one another rather than to the analyte. FTIR spectroscopy has shown that amines preferentially adsorb to isolated silanols on silica [10]. Type B silica was a great advance that improved peak symmetry by minimizing the number of isolated silanols, but tailing has not been satisfactorily eliminated.

It is believed that tailing persists because even fully hydroxylated silica has a small population of isolated silanols, which are evident in FTIR spectroscopy [11]. In this context, isolated silanols are those groups that are arranged in such a way that they cannot be involved in hydrogen bonding with neighboring silanols, as opposed to associated silanols that are in close enough proximity to hydrogen bond to neighboring groups. The idea, that isolated silanols represent the strong adsorption site, is not without controversy: caffeine, for example, adsorbs little to bare silica, yet it tails on silica that has been chemically modified with C_{18} [12]. On the other hand, measurements of retention factors for varying coverages of C_{18} have shown that the retention factor of caffeine maximizes at incomplete C_{18} coverage, indicating a synergy from simultaneous interaction with silanols and hydrocarbon [13]. Another quandary about isolated silanols being the cause of tailing is that these species disappear from the FTIR spectrum after reaction with chlorosilanes [14], hence it is not clear how they could cause tailing. Quantitative modeling of chromatographic peak shapes for a cationic dye has shown that the abundance of the strong adsorption sites is only on the order of 1 nmol/m^2 for Type B silica, and that tailing from these rare species occurs because the large partition coefficient compensates for the very low abundance [15,16]. While these results do not confirm that the strong sites are isolated silanols, they would explain why a species undetectable by FTIR could cause tailing. Because of the very low abundance, it is challenging to determine experimentally whether the strong adsorption sites are really isolated silanols, and if they are, why they persist despite extensive rehydroxylation to maximize neighboring silanols.

Studies performed using Corning 7980 fused silica, which is a highly pure form of silica, suggest a possible reason that isolated silanols remain on fully rehydroxylated silica gel: combining

* Corresponding author. Tel.: +1 765 494 5328; fax: +1 765 494 0239.
E-mail address: mwirth@purdue.edu (M.J. Wirth).

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single-molecule spectroscopy and AFM showed that strong adsorption to silica occurs at nanometer-scale roughness features [15–20]. In the study, DiI-C_{12}, a cationic, amphiphilic dye, showed nonlinear tailing similar to what occurs in HPLC for the same compound on commercial C_8 bonded phases [16]. In another study, DiI-C_{12} exhibited slower desorption rates at nanometer indentations on the fused silica that had been chemically modified with monomeric C_{18} [21]. In the same study, the dye molecules diffused rapidly in planar regions, but desorbed slowly at nanometer indentations [21]. A separate study revealed the lysozyme (pI ~11.0) binds to nanoscale scratches in fused silica under acidic conditions (pH <7) and thus could be used as a probe of nanoscale roughness [22]. Given the short distance for hydrogen bonding, 1.9–2.2 Å [23], asperities on the nanometer scale could conceivably promote isolated silanols. High resolution AFM images of commercial silica gels show that these materials are as smooth as polished fused silica on the nanometer scale, but they have occasional nanoscale ledges and other non-planar areas [24]. Since both topographical asperities and strong adsorption sites are rare, it is not a stretch to hypothesize that nonplanar topography could possibly be the origin of tailing.

To test for a relation between topography and tailing directly, the surface roughness would need to be varied experimentally. It has long been known that the surface of glass can be smoothed by high temperature annealing [25]. In annealing processes, the surface melts but the bulk does not, and this has the effect of smoothing the surface. Surface melting occurs at lower temperatures than bulk melting because surface atoms have fewer covalent bonds to break than do the atoms in the bulk. Annealing occurs at temperatures above 800 °C [26], whereas below this temperature, only surface dehydroxylation occurs. Annealing has been used to smooth out nanometer-scale surface roughness features on highly pure silica used in optics [27,28]. Annealing has also been used to sinter colloidal silica particles together to form the aggregate microspheres used as chromatographic silica gel [29]. The onset of sintering, and therefore annealing, of chromatographic silica is 900 °C [30], and temperatures as high as 1050 °C have been used [29]. Previous studies of heat treatments of silica determined the relative abundances of silanol groups as a function of temperature up to 800 °C by FTIR and NMR spectroscopies [31] and up to 1050 °C [14] and 1100 °C [32] by FTIR. There have been no studies to show whether annealing chromatographic silica affects the abundance of isolated silanols or chromatographic tailing.

The purpose of this paper is to investigate whether annealing of silica particles reduces peak tailing and isolated silanols. The particles are annealed at three different temperatures, 800, 900 and 1050 °C, thus spanning the onset of annealing. At temperatures above 900 °C, mobility of silanols is expected to be greatly enhanced, allowing for condensation of silanols that were previously unable to react due to geometric restraints. Both chromatography and FTIR are used to determine how tailing and isolated silanols are affected by surface roughness. Colloidal silica particles, which are the constituents of chromatographic silica gel, are used in these studies to ensure that the results apply to chromatographic silica gel. This study directly tests for any relation between the surface topography and the extent of tailing and the abundance of isolated silanols.

2. Experimental

Non-porous, colloidal silica particles were obtained from Fiber Optic Center (New Bedford, MA). All particles were calcined at 600 °C for 12 h in a furnace (Thermo Fisher Lindberg/Blue M) to drive out volatile regents remaining after the sol–gel reaction. Calcining and resuspension were preformed three times, which has been shown to minimize aggregates and minimize water content in colloidal silica [33]. Longer calcining times did not to make a difference in the FTIR spectra or particle diameters. After cooling, the particles were suspended by sonication in pure ethanol. The silica particles were then annealed in the same furnace to smooth the particle surfaces. For comparison, three different samples were annealed at 800, 900 and 1050 °C for 3 h. Longer annealing times did not make a substantial difference in the FTIR spectra. Annealed particles were found to be slightly smaller (<4%) than calcined particles in agreement with previous studies [14].

The annealed particles were rehydroxylated by refluxing in a 50:50 mix of concentrated nitric acid and ultrapure water for 3 h followed by rinsing with ultrapure water until the pH was neutral. Longer rehydroxylation times were not observed to affect the FTIR spectrum. The bare rehydroxylated silica particles (210 nm diameter) were packed into 100-μm I.D. fused silica capillaries for the measurements of lysozyme adsorption on three replicates at each annealing temperature. The packed capillaries were dried in a vacuum oven at 80 °C for several hours. Lysozyme (Sigma–Aldrich) was labeled with Alexa Fluor 546 (Invitrogen) and was prepared as a dilute solution (10 μg/ml) in a phosphate buffer (pH 7.4). The protein solutions were wicked into each of the packed capillaries and equilibrated for 5 min. After equilibration, the capillaries were rinsed by pressurized flow of water with 0.1% formic acid. Low pH was used because the pK_a of silanols on type B silica is near 6 in water [34], and the low pH rinse ensures a well protonated surface to minimize influence of SiO^- groups on adsorption. Optical micrographs were then obtained using an inverted fluorescence microscope (Nikon). Fluorescence was excited with a mercury lamp and was imaged through a filter cube (Omega Optical, QMAX-Yellow) onto a CCD camera (Cascade II). Images were acquired and processed using the WinView software package.

RP-UHPLC was used to investigate the effect of annealing temperature on chromatographic tailing. RP-UHPLC was performed using chemically modified, annealed silica particles (790 nm average diameter) in stainless steel columns (2.1 mm I.D.). A monomeric C_4 stationary phase was made by refluxing the colloidal silica in 1% *n*-butyldimethylchlorosilane (Gelest) in toluene for 3 h, using pyridine (Sigma–Aldrich) as a catalyst. The chemically modified particles were made into a 5% by weight slurry, sonicated, filtered through a 5-μm stainless steel mesh (TWP, Berkeley, CA), then sonicated again and pressure-packed into the columns. For RP-UHPLC, a 1.2 μM lysozyme solution was used as an analyte. The chromatograph was an Accela UHPLC system (Thermo Fisher) with a PDA monitoring at 210 nm. Shallow gradient conditions were employed (73:27 water:acetonitrile to 68:32 water:acetonitrile over 10 min) for a 200 μl/min flow rate.

FTIR spectroscopy was performed using a Tensor 37 FTIR spectrometer (Bruker) on particles before and after rehydroxylation. Since FTIR is sensitive to the composition of the particle interior, particles of 120 nm diameter were used for FTIR measurements. This was done to match the particle diameters used for wide-pore silica gel. This reduces the chance that the spectrum of the particle interior would contribute more than it did in previous studies of the FTIR of chromatographic silica gel. The samples were thin films of 120 nm silica particles deposited using drawdown deposition. In drawdown deposition, the colloidal slurry is coated onto a polished silicon wafer using a Mayer rod with an automated coater (Industry Tech). The Mayer rod spreads a thin (5 μm), uniform layer of the colloidal slurry onto the silicon wafer. The coated wafers were allowed to dry at room temperature and were then subjected to the annealing conditions listed above. The thin films were annealed using the same conditions as for the other measurements. All samples were dried in a vacuum oven prior to FTIR analysis to minimize absorption of water to the surfaces.

Spectra were recorded for 1024 scans at a resolution of 4 cm^{-1}. A bare silicon wafer was used as the background reference. Spectra were recorded in transmission mode using a Brewster's angle sample holder at 55° incidence with vertically polarized light to minimize interference fringes. The spectrometer was continuously purged with dry nitrogen to minimize absorption due to water, as well as spectral interferences from water vapor and carbon dioxide in the spectral regions of interest. The software provided by the manufacturer was used to automatically subtract the vapor phase spectra of any water and carbon dioxide.

3. Results and discussion

Lysozyme was used to probe surface topography of bare silica because it has been shown to adsorb irreversibly to submicroscopic indentations [22]. When measuring the adsorption of lysozyme to silica, packing silica into a capillary provides an easy method for ensuring equal amounts of material. This is reliable since the surface area of a capillary is negligible when compared to the surface area of silica. Fig. 1 shows fluorescence images from capillaries packed with rehydroxylated particles, before and after adsorption of lysozyme, where the images before adsorption are used as the blanks. These images are representative of the three replicate capillaries made for each annealing temperature. There was some adsorption of lysozyme to the protective polymer coating of the capillary, and this is indicated by the red arrows for each image. The capillaries did not pack uniformly against the walls, sometimes leaving void spaces, and this is attributed to there being agglomerates of particles after the annealing. Two examples of voids at the inner walls are indicated to the figure using blue arrows. Despite these imperfections, it is obvious that the adsorption of lysozyme is decreased with higher annealing temperature. To extract quantitative data from these images, the fluorescence intensity was measured along the center line in each capillary, the blank was subtracted, and the result was then normalized to the average result for the 1050 °C sample. The normalized, blank-corrected fluorescence intensities from irreversibly adsorbed lysozyme were different from one another within 95% confidence: 3.1 ± 0.2 at 800 °C, 1.5 ± 0.2 at 900 °C, and 1.0 ± 0.2 at 1050 °C. The images and data thus show a clear trend confirming that an increase in annealing temperature gives a smoother silica surface, as expected. These results show that smoothing effect is more pronounced when reaching the annealing threshold of 900 °C than it is after exceeding the annealing threshold by heating to 1050 °C.

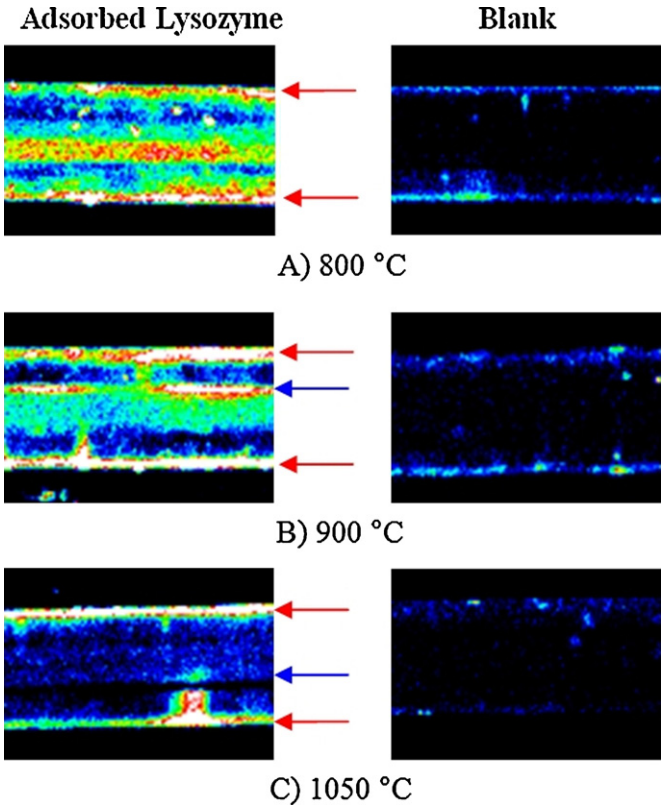

Fig. 1. False-color fluorescence images for lysozyme, labeled with Alexa Fluor 546, adsorption to colloidal silica annealed at (A) 800 °C, (B) 900 °C and (C) 1050 °C. All images are on the same intensity scale. Fluorescence on the outermost perimeters (red arrows) is attributed to lysozyme absorbed to the protective polyimide coating on the outside of the capillary, which itself fluoresces slightly. Fluorescence near the inner capillary walls (blue arrows) is attributed to void in the packing material. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

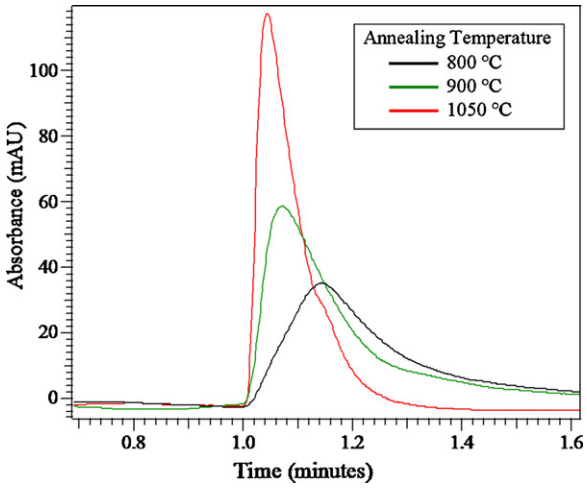

Fig. 2. Observed tailing of the cationic protein lysozyme. Here, the red trace was acquired from a surface annealed at 1050 °C, the green trace from 900 °C and the black trace from 800 °C. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

To determine whether the progressive surface smoothing is accompanied by a progressive decrease in tailing, reversed-phase chromatography was performed. Large particle sizes (790 nm) were used in this study in order facilitate the use of a standard UHPLC pump system by reducing the back pressure that would accompany smaller particles. To accentuate the effects of the silica on the peak shape, no endcapping was performed. The chromatograms for the three columns are shown in Fig. 2. Clearly, the tailing of lysozyme was greatest for the silica annealed at the lowest temperature (800 °C), and the tailing was least for the silica annealed at the highest temperature (1050 °C). The center of the peak shifts to progressively shorter times with annealing, which is consistent with progressively less retention to silanols.

Since previous work implicated isolated silanols as the cause of tailing [8,11], FTIR can be used to determine whether the abundance of isolated silanols is reduced by annealing. Previous studies of porous silica materials show a decrease in overall silanol concentrations as a function of temperature [31,32]. It is logical to assume that non-porous particles will respond in a similar fashion. In the current study, smaller colloidal silica particles of 120 nm in diameter were annealed at varying temperatures, and the FTIR spectra were acquired. The smaller particles were used because they have a larger surface-to-volume ratio, reducing any contribution to the FTIR spectra from silanols inside of the non-porous particles. These particles have the same surface-to-volume ratio as those used to

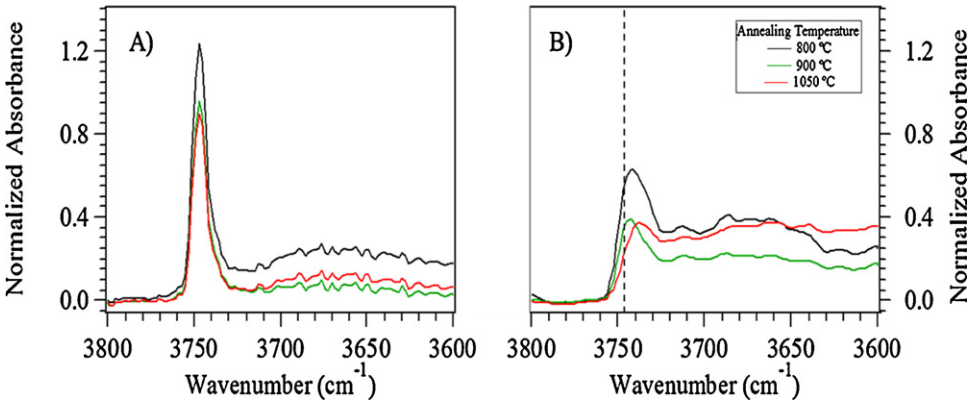

Fig. 3. (A) FTIR spectra of isolated silanols on colloidal silica using the same vertical scale and spectral range. Each spectrum has been normalized to peak height for the spectrum of the 1050 °C sample. (B) Same samples after rehydroxylation. Each spectrum in both parts (A and B) is the average of three replicates, each having 2% precision. Here, the red trace was acquired from a surface annealed at 1050 °C, the green trace from 900 °C and the black trace from 800 °C. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

manufacture wide-pore silica gel [35], so the spectra are representative of what one would see for chromatographic silica gel. Free, isolated silanols appear in the FTIR spectrum as a sharp band at 3750 cm^{-1}, whereas associated (vicinal) silanols and germinal silanols are observed to be a broad feature at 3200–3700 cm^{-1}. The FTIR spectra in Fig. 3A show the magnitude of the silanol peak, averaged from three replicate measurements for each of the three annealing temperatures. The results show that the abundance of isolated silanols is decreased by 26 (±2) % at onset of annealing at 900 °C. There is no detectable reduction in the abundance of isolated silanols, within the 10% experimental error, when increasing the annealing temperature to 1050 °C.

Silica is rehydroxylated before it is used in chromatography, and a comparison of two papers shows that rehydroxylation shifts the spectrum of the isolated silanols to the red by about 5 cm^{-1} [8,11]. These earlier results thus indicate that the new silanols created from the hydrolysis of siloxane bonds during rehydroxylation are close enough to the isolated silanols to shift their spectra to the red, but are still beyond the optimal hydrogen bonding distance. Fig. 3B shows the FTIR spectra for the silica particles treated at the three different annealing temperatures. Each sample was dried in a vacuum over before FTIR analysis, which minimizes any water peak. After annealing at 800 °C, rehydroxylation is shown to red-shift the isolated silanol peak by a few wavenumbers and to reduce its peak intensity by a factor of 2. After annealing at 900 °C, rehydroxylation also red-shifts the isolated silanol peak by a few wavenumbers, and its peak intensity is another factor of 2 lower. Annealing at 1050 °C, produces less of a change in the peak intensity, but rehydroxylation red-shifts the isolated silanol peak a few more wavenumbers, indicating even closer approach to neighboring silanols. Varying annealing temperature thus shows two effects: (1) reducing the abundance of isolated silanols with the onset of annealing at 900 °C and (2) red-shifting the isolated silanol peak for the higher annealing temperature of 1050 °C.

The quantitative data for the lysozyme adsorption, lysozyme peak tailing, and the FTIR intensities and spectra shifts for the isolated silanols are summarized in Table 1, including 95% confidence intervals. The trend for each one is the same: better performance is attained for higher annealing temperature. Fig. 4 illustrates a plausible interpretation of how smoothing the surface through annealing could reduce the abundance of isolated silanols and also red-shift the FTIR peaks of the isolated silanols. Given the preliminary evidence that strong adsorption occurs at nanoscale topographical features, we postulate that the isolated silanols are at nanoscale bumps on the surface. Such bumps would be the simplest way of isolating a silanol group because it raises the –OH group above its neighboring silanols to place it beyond the optimal hydrogen bonding distance. Fig. 4 depicts that annealing reduces the heights of the bumps, and once rehydroxylation creates neighboring silanols, these groups are now closer to the optimal hydrogen bonding distance of the isolated silanols. The shorter hydrogen bonding distance imparts a red shift to the FTIR peak of the isolated silanol, and also makes it less adsorptive, thereby reducing tailing. In science, even a battery of measurements does not conclusively prove an interpretation, instead, what can be said is that there is now more evidence that supports the hypothesis that isolated silanols are the strong adsorption sites and that the isolated silanols occur at sites of nonplanar topography.

This interpretation prompts us to revisit the previous interpretation of the lysozyme adsorption in regions of nonplanar topography [22]. The original work correlated irreversible adsorption of lysozyme with AFM images, and lysozyme was chosen for the previous work because it is notoriously sticky to bare silica. The speculation at that time was that a more three-dimensional adsorption site would permit more interactions with the protein than would a planar adsorption site. Now that the isolated silanols appear to be located at these same regions, another interpretation is that isolated silanols are the cause for the irreversible adsorption of lysozyme. It is also possible that both of these factors contribute to the irreversible adsorption of lysozyme in nonplanar regions

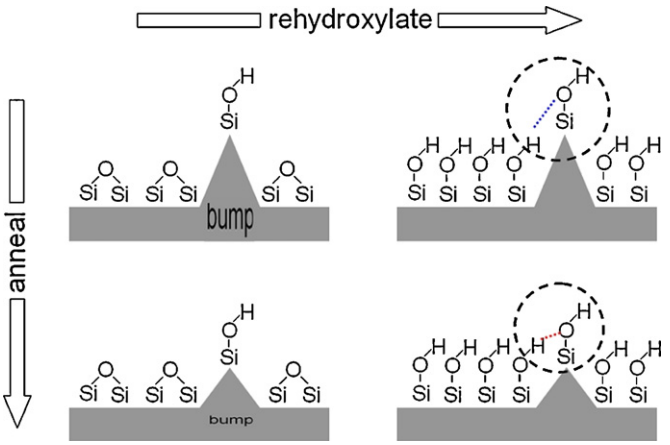

Fig. 4. Conceptual model for interpretation of data. Annealing reduces the heights of roughness features, and rehydroxylation creates neighboring silanols. The circles draw attention to the hydrogen bonding distances for the isolated silanols, which become shorter with annealing.

Table 1
Summary of data: For each of the three annealing temperatures, the values are listed for (A) the relative fluorescence intensity of adsorbed lysozyme, (B) the peak asymmetry, as measured by the ratio of trailing to leading half-width at 10% of the peak height, (C) the relative abundances of the isolated silanol peaks before rehydroxylation, and (D) the peak position for the isolated silanols after rehydroxylation. The uncertainties are the 95% confidence intervals, calculated from three replicate measurements.

Annealing temperature	A. Lysozyme adsorptivity	B. Peak asymmetry	C. SiOH abundance	D. SiOH peak position (cm^{-1})
800 °C	3.1 ± 0.2	5.5	1.32 ± 0.09	3739
900 °C	1.5 ± 0.2	4.4	1.05 ± 0.03	3743
1050 °C	1.0 ± 0.2	2.5	1.00 ± 0.09	3743

because this protein is strongly basic, giving many terminal amino groups to interact with multiple isolated silanols.

4. Conclusions

The three different experiments, lysozyme adsorptivity, chromatographic tailing in RP-UHPLC, and the abundance and spectral shift of the isolated silanols indicate that annealing of silica at higher temperature reduces chromatographic tailing and also reduces the abundance and adsorptivity of isolated silanols. The results lend further credence to the notion that isolated silanols are the strong adsorption sites that cause tailing of amines in RP-LC for bonded phases on silica. The results support the hypothesis that nonplanar surface topography gives rise to the isolated silanols. This work indicates that peak tailing can be reduced by annealing chromatographic silica gel.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.05.080.

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