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Macroporous Silica Monoliths Derived from Glyceroxysilanes: Controlling Gel Formation and Pore Structure

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Summary: Diglycerylsilane (DGS), a member of the family of sugar-based silanes, is converted into monolithic silica at low temperatures and at mild pH. These materials are suitable for the entrapment of proteins under conditions that generally offer protection against denaturation, particularly when compared to analogous silicas prepared from tetraethoxysilane (TEOS). However, the resulting monoliths did not have sufficient porosity to permit flow and, thus, could not be utilized as monolithic chromatographic supports for frontal affinity chromatography (FAC). It was demonstrated that poly(ethylene oxide) can be used to induce spinodal decomposition of the DGS-derived sol, prior to gelation, leading to a meso- and macroporous silica monolith after cure, as demonstrated by nitrogen sorption analysis. High molecular weight PEO is required for effective phase separation to take place: below 10000 MW, no such phase separation occurs under the conditions employed. The amount and molecular weight of PEO is critical to the timing of gelation. If too much PEO is present, or ionic strength is increased, gelation occurs before it is possible to fill the chromatography column with the sol, while too little results in a lack of macropores. Proteins entrapped in this material are shown to be of comparable stability to those prepared in the absence of PEO, and can be used to chromatographically screen, with MS detection, potential drug candidates by changes in retention resulting from ligand binding.

Keywords: biocompatible silica; macroporous; poly(ethylene oxide); protein-doped; sol-gel

Introduction

An evolving approach to the high throughput drug screening of compound libraries utilizes frontal affinity chromatography (FAC) [1,2]. In this technique, an immobilized protein associated with the disease of interest is exposed to the drug library using continuous elution.

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The technique requires that the only source of retention through the column is interaction with the protein. Although retention time through the column is associated with the ligand binding constant for the protein, the more important outcome of the technique is to reject from further consideration compounds that do not bind and, thus, will never be viable drug candidates.

A variety of methods have been used to immobilize proteins on columns including covalent attachment to beads of porous glass [3,4], agarose or sepharose [5-7] and cellulose [8]. An alternative to covalent linkage is the entrapment of the protein in a matrix that is accessible to small molecules, but from which the protein can not escape. We have previously described the entrapment of proteins in mesoporous silica [9] derived from diglycerylsilane (DGS). The resulting material, however, is not sufficiently porous for practical chromatographic application. We have also described entrapment of a protein (dihydrofolate reductase) into macroporous materials derived from DGS and PEO [10], but have not examined in detail the methods that can be used to control the morphology of the resulting material. In this paper, we describe the structural changes that accompany the addition of PEO of different concentrations and molecular weights to DGS derived silica. Of most importance are the morphological changes engendered by the addition of PEO, which are controllable and do not change the inherent biocompatibility of the material.

Experimental

DGS: was synthesized by literature methods [11]. PEO of average MW of *ca.* 2,000, 10,000 and 100,000, respectively, was obtained from Aldrich.

Equipment: The mixing of sols was facilitated with an ultrasonic bath. A Corning Model 308 pH meter was used to follow pH evolution in the sol. ^{1}H and ^{13}C NMR were recorded at room temperature on a Bruker AC-200 spectrometer; solid state ^{13}C and ^{29}Si CPMAS NMR spectra were recorded on a Bruker AV-300 at 75.47 and 59.62 MHz, respectively, with TMS and glycine used as external standards, respectively. FT-IR spectra were obtained on a Perkin-Elmer 283 spectrometer.

Gel preparation

DGS-derived silica gels: DGS (0.50 g, 2.40 mmol) was dissolved in H_2O (500 µL, 27.8 mmol) with ultrasonication at 0 °C for 20 min; a homogenous, transparent sol solution resulted to which H_2O (500 μL) was added. The time between the addition of the H_2O to the sol, and the sol losing its ability to flow, was recorded as the gel time (t_{gel}).

DGS/PEO derived gel: PEO was dissolved into water at different concentrations, using different molecular weights of PEO. The solubility of PEO decreases with the molecular weight. DGS (0.50 g, 2.40 mmol) was dissolved into H_2O (500 μL, 27.8 mmol) with sonication at 0 °C for 20 min. The PEO solution (500 μL) was added (Table 1, Figure 1A). The time at which the solution became totally opaque was recorded as phase separation time (t_{ps}), and the time when the opaque phase lost its ability to flow was recorded as gel time (t_{gel}).

SEM: After gelation, 5 mL of water was added to the vial containing the gel in order to remove uncured constituents of the reaction. The samples were again washed with water after 1 hour, then dried in air for another 14 days. The fracture surfaces of the samples were observed by JEOL 840 Scanning Electron Microscopy (SEM).

TGA: The sample was treated as noted above. After aging for 14 days, the gel was crushed and washed 3 times (1/2 hour with water with sonication) then soaked in water for 24 h. Thermogravimetric analysis (TGA) was performed using a THERMOWAAGE STA409. The analysis was measured under air, with a flow rate of 50 cm^3/min. The heating rate was 5 °C/min from room temperature to 750 °C.

N_2 sorption experiments: The nitrogen sorption isotherm, surface area and pore radius were measured with NOVA 2000 from Quantachrome Instruments. The samples were prepared as for the TGA. Then the sample was degassed with a final vacuum in the order of 10 millitorr (or less) for 5 hours at 100 °C, a temperature below that of PEO decomposition. BET surface area was calculated by the BET (Brunauer, Emmett and Teller) equation [12]; the pore diameter from nitrogen adsorption-desorption isotherms was calculated by the Wheeler formula [13]. All the data were calculated by the software provided with the instruments.

Results and Discussion

DGS exists as series of low molecular weight structures that appear to interconvert in the absence of catalysts, as has previously been noted [14]. The compound was co-hydrolyzed with PEO of different molecular weights (2000, 10,000, and 100,000, respectively), and allowed to gel. As can be seen from Figure 1A, both PEO concentration and MW played a role in the gelation time and the morphology of the resulting silica monolith. As is the case with DGS alone (o), with 2000 molecular weight PEO, gelation occurred from optically transparent sols (Δ) to give optically transparent gels. Clear monoliths (□,o) could also be prepared from DGS with low concentrations of higher molecular weight PEO (10,000 MW PEO ([PEO] < 0.02 g/mL sol) or 100,000 MW PEO ([PEO] < 0.01 g/mL sol). However, at higher concentrations of high MW PEO phase separation occurred prior to gelation to give an opaque sol that gelled into an opaque, macro- and mesoporous monolith. Both PEO polymers at these higher molecular weights are soluble in water. Thus, the difference in concentration at which phase separation occurs is likely related to the relative amount of bound water that the two different molecular weight materials possess, the viscosity of the phases before and after separation, and also to the free energy of mixing of the two polymers (silica and PEO) when using different molecular weights of PEO.

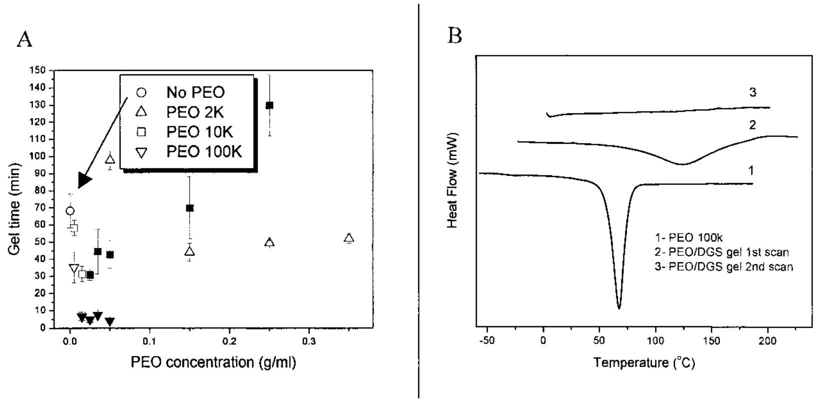


Figure 1. A: Gel time of DGS doped with different molecular weight and different concentration of PEO solution. Open symbols indicates a transparent gel at gel time, and the filled symbols means phase separation to an opaque mixture occurred before gelation. B: DSC analysis of 1) PEO 100k; b) 100k PEO/Silica 1st scan; c) PEO/silica 2nd scan.

It is apparent from Figure 1A that the molecular weight of the PEO has an impact on gelation kinetics. At low concentrations, the rate of gelation is inversely proportional to the molecular weight of the PEO. Previous work with glycerol alone [11] followed a similar trend: the higher the concentration of polyol or of polyether, the slower are the gelation kinetics. The retarded gelation may be ascribed both to steric stabilization [15] of the evolving silica structures and chemical stabilization of hydrophilic intermediates by the PEO chain.

The morphological consequences of phase separation, prior to gelation, are profound. As the materials are ultimately to be used for chromatographic applications, the flow rate of aqueous solutions through the monolith is a critical parameter of the gels. When clear sols gel, they form optically clear mesoporous gels through which flow is significantly retarded. By contrast, sols that undergo phase separation lead, after gelation, to opaque gels that have very low resistance to flow. The relative flow rates are associated with significant differences in morphology of the two classes of monoliths. As shown in Figure 2, opaque monoliths containing high MW PEO are highly open macroporous and mesoporous materials.

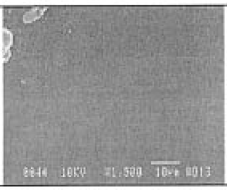
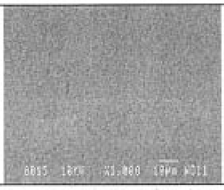
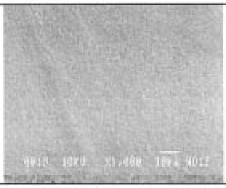
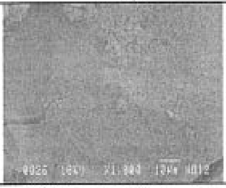
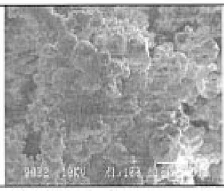
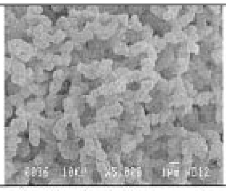
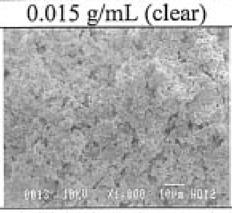
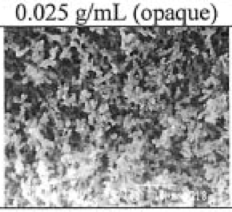
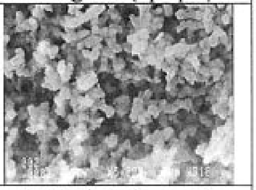
MW PEO			
2k			
	0 mg/mL (pure DGS, clear)	0.05 g/mL (clear)	0.25 g/mL (clear)
10k			
	0.015 g/mL (clear)	0.025 g/mL (opaque)	0.25 g/mL (opaque)
100k			
	0.015 g/mL (opaque)	0.025 g/mL (opaque)	0.05 g/mL (opaque)

Figure 2. SEM image of DGS with PEO of different molecular weights.

After cure, all the gels contain considerable amounts of PEO, even after extensive washing. As shown in Table 1, when present in the sol at low concentrations almost all the PEO is retained within the resulting gel: that is, PEO is associated exclusively with the curing phase. As the concentration of PEO increases, PEO partitions into both the non-curing and curing phases after spinodal decomposition. At low concentrations of PEO, the monolith exhibits a larger weight loss was observed than can be accounted for by PEO alone. As we have shown with monoliths prepared from DGS, this is a result of the presence of entrained glycerol that is not removed by water washing, but which can be removed thermally.

PEO is a crystalline polymer with a readily measured endothermic peak at 68 °C, corresponding to the melting point (of PEO 100k). The macroporous silica containing PEO (100k, 19%, prepared from 0.025 g/mL) was analyzed by DSC (Figure 1B). There is clearly no peak corresponding to the melting point of PEO: that is, the entrained PEO is amorphous and likely dispersed effectively throughout the silica. The broad peak at 124 °C corresponds to the evaporation of the residual water and entrained glycerol, as demonstrated from the absence of such peaks after the sample was cooled and rescanned (Figure 1B, curve 3).

The morphology of PEO/silica mesostructures was further studied by nitrogen sorption experiments, including BET surface area and average pore size. As shown in

Table **2**, an increase in PEO content is generally associated with lower available surface areas. This is consistent with the formation of larger macropores (macropores cannot be effectively measured with nitrogen porosimetry) that would produce a lower surface area per unit volume of material. We note that these data were made on crushed gels that were dried at 100 °C, but that were not sintered. Thus, these measurements provide guidance about the nature of the gels containing PEO. The addition of PEO is associated with an increase in the average mesopore diameter, but on the basis of the data presented, there is no correlation between pore size and either concentration or PEO MW.

Table 1. PEO percentage in DGS derived silica by TGA.[a]

PEO/Sol[b] (2K) g/mL	PEO wt.%		PEO/Sol (10K) g/mL	PEO wt. %		PEO/Sol (100K) g/mL	PEO wt. %	
	Calc.	TGA		Calc.	TGA		Calc.	TGA
						0.005	3.3	8
						0.015	9.1	12
			0.025	14.2	17	0.025	14.0	19
			0.035	20.5	23	0.035	18.0	21
0.05	24.1	25	0.050	24.2	29	0.050	23.6	25
0.15	45.4	34	0.150	45.2	33			
0.25	55.0	39	0.250	54.6	34			
0.35	60.8	23						
0.45	64.4	32						

[a] Some water must accompany the presence of PEO, which accounts for the examples, at low PEO%, where the PEO% exceeds the amount incorporated in the recipe.

[b] The concentration reported on the table refers to the final concentration of PEO in the sol, after mixing with DGS (0.5 g) in water (0.5 mL).

Given that our objective is to entrain proteins in silica for bioaffinity chromatography applications, it is important to determine if proteins are functional in DGS/PEO based materials. Figure 3 shows that columns prepared from DGS and 10k PEO with entrapped dihydrofolate reductase [10] are able to retain known substrates and inhibitors of the protein, while compounds that do not bind the protein elute in the void. Thus, these columns have the potential to be exploited for high throughput screening of compounds that can bind to entrained proteins.

Table 2. Nitrogen adsorption data of DGS derived silica doped with PEO (2K).

	2k		**10k**		**100k**	
PEO/sol g/ml	**BET surface Area m^2/g**	**Average Pore Size nm**	**BET surface Area m^2/g**	**Average Pore Size nm**	**BET surface Area m^2/g**	**Average Pore Size nm**
0	674.9	4.4	617.8	4.5	620.9	4.7
0.005					474.5	7.1
0.015					433.8	5.9
0.025			679.4	4.8	554.7	2.9
0.035			326	3.6	460.11	3.0
0.05	374.8	8.8	9.658	26.1	195.8	3.2
0.15	110.7	6.6	11.93	12.0		
0.25	68.08	6.6	5.747	11.7		
0.35	174.3	6.2				
0.45	106.5	3.4				

Seminal work by Nakanishi pioneered the concept of adding polymers to sols to control gel morphology [16]. With an appropriate amount of sufficiently high molecular weight PEO, spinodal decomposition occurs prior to gelation and a range of morphologically different silica gels is available after sintering.

Polyethylene oxide has a long standing reputation for stabilizing most proteins [17, 18]. Thus, in principle, the protein stabilizing silicas that we have previously described [9, 10, 11] should be expected to remain biocompatible with the inclusion of PEO in the sol. However, it is clear that subtle changes in viscosity and solvency can change the polymerization profile, hence it was necessary to test this principle. As importantly, the PEO that remains in the silica cannot act either to occlude the protein from contact with the drug library or change the retention characteristics of the resulting silica surface: it is critical that the main mechanism for retention of the compounds in the column is binding to the protein. As shown above, proteins entrapped in PEO doped silica remain active and accessible to external analytes.

The replacement of $Si(OEt)_4$ with DGS does not substantially change the morphology of the resulting macroporous gels. While the morphologies formed are subtly effected by the specific recipes, it is possible to prepare meso- and macroporous materials using Nakanishi's precedent but in the presence of glycerol and proteins in the sol. As can be seen in Figure 3, the column effectively discriminates between compounds that don't bind (coumarin and fluorescein), folic acid (micromolar substrate) and pyrimethamine and trimethoprim (nM inhibitors). Thus, the PEO used for structural control of the silica neither significantly changes retention of compounds on the column, nor inhibits access to the entrained protein.

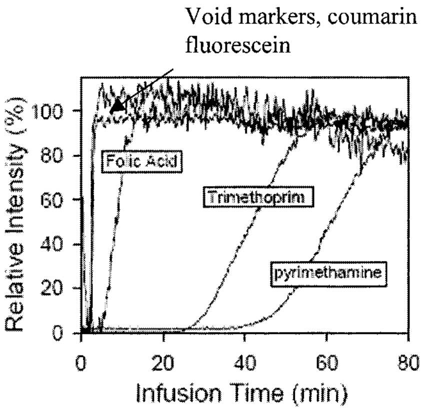

Void markers, coumarin and fluorescein

Figure 3. Separation of compounds by protein affinity using frontal affinity chromatography.

Conclusion

Highly porous, biocompatible silicas can be prepared from sugarsilanes and high molecular weight PEO. The PEO acts to facilitate spinodal decomposition, before gelation, into a silica rich phase that cures, and a second phase that is removed by washing with water. Much of the PEO remains entrained in the silica, which exhibits larger mesopores and lower surface area than silica prepared in its absence. The resulting silica monoliths are biocompatible. The monoliths, containing entrained proteins, can be used as affinity supports to separate drug candidates on the basis of protein binding.

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