Dye leaching from a doped sol–gel is eliminated by conjugation to a dendrimer

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Abstract

The extent of leaching of dye molecules following encapsulation into sol–gel glasses was investigated. Covalent attachment of erythrosin isothiocyanate (EITC) to a polyamidoamine generation 4 (PAMAM) dendrimer, having a molecular weight of 14,215 Da, eliminated all detectable leaching from hydrated monoliths prepared from tetramethylorthosilicate (TMOS). In contrast, a prior study showed that significant leaching takes place when the dye is conjugated to 70 kDa dextran. To eliminate dye leaching via attachment to a macromolecular carrier, an inherently globular macromolecule having an organized secondary structure is therefore required. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The sol–gel process is a low temperature route for production of optically transparent, microporous glasses [1]. Heat-labile species, such as organic chromophores and proteins can be entrapped merely by addition to the sol prior to gelation [2–9]. These features have led to extensive research and development on chemical and biochemical sensing devices based on organically-doped sol–gels [9–21]. Rapid diffusion of an analyte into a sol–gel and reaction with an entrapped indicator is essential for the device to respond rapidly [16,18,21]. However, just as the analyte can enter the gel, a low molecular weight indicator (MW ≤ 1000) that is soluble in the liquid that fills the pores can also leach from the gel. Decreasing the average pore size, through adjustment of sol composition and gelation/aging conditions can reduce the extent of leaching [22–24]. However, analyte diffusion may be hindered as well, resulting in a longer response time.

The use of a (silicon) alkoxide derivative of the indicator, which becomes covalently incorporated into the polycondensed network, is an effective strategy to prevent indicator leaching [24–27]. An alternative is to increase the size of the indicator by attaching it to an inert, macromolecular carrier [28–30] or encapsulation in a supramolecular assembly [31]. Prior investigations have shown that attachment of water soluble dyes to 70 kDa dextran reduces, but does not eliminate, the leaching of encapsulated dyes from hydrated TMOS monoliths [28,29]. However, globular proteins with molecular weights significantly <70 kDa (e.g. myoglobin) do not undergo leaching [29,32]. These observations suggest that attaching a dye to a globular macromolecular carrier having an organized secondary structure may be effective in preventing dye leaching from a doped sol–gel. A
PAMAM dendrimer [33,34] is an attractive choice for a carrier since it is an inherently globular structure. In this study, we have investigated the stability of erythrosin-dendrimer conjugates entrapped in hydrated TMOS monoliths. The results show that this strategy quantitatively eliminates dye leaching.

2. Experimental

2.1. Modification of PAMAM with erythrosin-5-isothiocyanate

Starburst polyamidoamine generation 4 (PAMAM) is a dendrimer having a molecular weight of 14,215 g/mol and 64 amine groups in the outer shell [34]. A solution of PAMAM dissolved in methanol at 24.8% (w/w) was received as a gift from Prof. R.M. Crooks, Texas A&M University. The PAMAM (0.162 g of methanol solution) was dissolved in 4 ml of 0.20 M sodium carbonate (Chempure, 99.62%) to produce a 10 mg/ml solution buffered at pH 9.0. Erythrosin-5-isothiocyanate (EITC, Molecular Probes) was dissolved in dimethylformamide (Fisher, 99.9%) at 10 mg/ml. While stirring the PAMAM solution, 0.5 ml of the dye solution was added. The dye:PAMAM molar ratio in the reaction mixture was 2:1 [35]. The stirring was continued for 3 h at room temperature. Unreacted EITC was separated from dendrimers by gel filtration on Sephadex G-10–120 using a mobile phase of 0.020 M phosphate buffer, pH 6.2.

2.2. Sol–gel monoliths

Tetramethylorthosilicate (TMOS, 99.4%, Aldrich) gels were prepared in 1 cm, disposable acrylate cuvets as described previously [29]. Briefly, sols were prepared from volumetric percentages of the following reagents: 33% TMOS, 7% Type 1 reagent grade water (Barnstead Nanopure, 18.2 M Ω/cm), 0.5% 0.04 M HCl, and 58% 0.020 M phosphate buffer (pH 6.2). The total volume of the components per cuvet was 3.028 ml (assuming no change of volume upon mixing and gelation). The TMOS was added to a premixed solution of water and HCl. Hydrolysis was allowed to proceed without mechanical mixing for 30 min at room temperature, which yielded a single-phase sol. The buffer solution (containing the dissolved dye to be entrapped, either EITC or EITC-PAMAM) was then added with mixing. The cuvet was then covered with parafilm and allowed to gel, which required a few minutes at room temperature. Monoliths were aged for 24–48 h at 4°C prior to initiating leaching experiments. The amount of added dye per cuvet was selected to yield an initial (pre-leach) absorbance in the range of 0.15–1.7. Blank (dye-free) gels were prepared and treated under identical conditions.

2.3. Fixed volume leaching experiments

After aging, an initial visible absorbance spectrum of each gel was measured, then the gel was carefully removed from the cuvet in which it was formed. Fixed volume leaching experiments were performed by immersing each gel into a 50 ml volume of leaching solution. Periodically the monolith was withdrawn, placed into a cuvet, and the visible absorbance spectrum was recorded. The monolith was then returned to the leaching vessel in order to maintain a constant volume of leaching solution. Blank gels were subjected to the same conditions. All leaching solutions contained 0.020 M phosphate. Four types of leaching conditions were investigated, either singly or in sequential combinations: (i) pH 6.2; (ii) pH 3.0; (iii) pH 1.5; and (iv) pH 6.2 containing 500 mM KCl.

The degree of dye leaching was measured using absorbance spectroscopy. Absorbance spectra were acquired at 1 nm resolution using a Hitachi U-2000 spectrometer. Background spectra were obtained using blank (dye-free) gels. Background spectra were found to be drift some what during gel aging and the subsequent leaching trials, which required that the absorbance values measured for dye-doped gels be corrected. For comparison purposes, absorbance spectra of EITC-PAMAM and EITC dissolved in the various leaching solutions were also measured. All measurements were performed in triplicate.

3. Results and discussion

The major objective of this study was to determine if attaching a dendrimer to a water soluble, organic dye could inhibit leaching of the dye from a hydrated TMOS monolith. EITC was selected as the test dye
for several reasons: it is water-soluble; preliminary studies showed that it leaches readily into water from a doped sol–gel; and it is easily conjugated to the primary amine groups of PAMAM.

3.1. Fixed volume leaching of EITC and EITC-PAMAM

The TMOS monoliths containing EITC and EITC-PAMAM were prepared, aged, and then soaked in pH 6.2 buffer. The absorbance of the gels measured as a function of soaking time is plotted in Fig. 1. An exponential loss of EITC, due to leaching from the gel into the buffer was observed. After 8000 min of soaking, only ca. 8% of the EITC originally present in the gels after aging was retained. In contrast, no loss of EITC-PAMAM was observed over the same time period. It is clear that covalent attachment of EITC to PAMAM quantitatively eliminates dye leaching. The small increase in absorbance during the initial stage of the experiment is due to differences in the molar absorptivity of EITC dissolved in water and methanol.

Upon immersion of the gel into buffer, the methanol produced from TMOS hydrolysis is replaced by water in the pores of the gel.

The retention of EITC-PAMAM in a hydrated TMOS monolith evident in Fig. 1 contrasts sharply with results obtained previously for other dye-macromolecule conjugates entrapped in sol–gels. Skrdla et al. [29] reported that attaching fluorescein to a dextran carrier significantly decreased the extent of dye leaching in comparison to monoliths doped with unmodified fluorescein. However, even conjugation to a 70,000 MW dextran was not sufficient to prevent leaching; in that case, 34% of the fluorescein leached from the gel after soaking for 7560 min. Note that the conditions used to prepare monoliths in this study were identical to those employed by Skrdla et al. [26]; thus, the respective microstructures are expected to be very similar.

The difference in the retention of sol–gel encapsulated dyes attached to dextran and PAMAM is likely due to structural differences between the polymers. The PAMAM is an inherently globular macromolecule.
[33,34], whereas dextrans are primarily linear, conformationally flexible polymers [36]. The lack of an organized secondary structure in water appears to permit sufficient diffusion of a polymeric dextran within the pores of a TMOS gel to cause substantial loss via leaching.

3.2. Role of electrostatic interactions in EITC-PAMAM retention

Conjecture regarding the mechanism of PAMAM retention in a TMOS monolith prompted further experimentation. The $pK_a$ for silanol groups at a silica–water interface is reported to be in the range of 4–7 [37–39]. The reported $pK_a$ values for the primary and tertiary amine groups in PAMAM are 6.85 and 3.86, respectively [33]. It was, therefore, hypothesized that electrostatic interactions between PAMAM and the pore walls of the gel contributed significantly to the degree of dendrimer retention when the external solution was buffered at pH 6.2. To test this possibility, leaching studies were performed at pH 1.5, at which the silanol groups in the gel should be fully protonated.

The experiment was conducted as follows: after aging, sol–gel monoliths containing EITC and EITC-PAMAM were soaked in pH 6.2 buffer for 24 h, then soaked in pH 1.5 buffer for the subsequent 24 h. Finally, the gels were soaked again in pH 6.2 buffer for another 24 h. Absorbance spectra were recorded at 0, 24, 48, and 72 h. Spectra for a gel doped with EITC-PAMAM and subjected to this leaching regimen are shown in Fig. 2. No decrease in EITC-PAMAM absorbance occurred over the initial 24 h, consistent with the data plotted in Fig. 1. After soaking in pH 1.5 buffer for 24 h, the absorbance declined approximately 40%. This decline is due to the drop in molar absorptivity that accompanies protonation of EITC. After soaking the gel for another 24 h in pH 6.2 buffer, the absorbance quantitatively recovered to its original (post-age) value. Parallel experiments were performed on EITC-doped gels. An exponential loss of dye was observed; after the 72 h, only ca. 9% of the dye originally present in the gels after aging was retained (data not shown).

Employing an ion exchange reaction to remove EITC-PAMAM from sol–gels was also investigated.

![Absorbance spectra of a TMOS monolith containing EITC-PAMAM. Spectra were acquired at 24 h intervals during exposure to the following leaching regimen: (A) after aging, 0h elapsed; (B) after soaking for 24h in pH 6.2, 0.020M phosphate buffer, 24h total immersion time; (C) after soaking for 24h in pH 1.5, 0.020M phosphate buffer, 48h total immersion time; and (D) after soaking for 24h in pH 6.2, 0.020M phosphate buffer, 72h total immersion time.](image-url)
After aging, sol–gels containing EITC-PAMAM were soaked for 4 h in pH 6.2 phosphate buffer, having an ionic strength of 0.0233 M. The gels were then soaked for 97 h in pH 6.2 phosphate buffer containing 0.50 M KCl, which raised ionic strength to 0.523 M. Based on measurements of absorbance performed periodically over the duration of the experiment (data not shown), leaching of EITC-PAMAM from the gels was undetectable.

The results of the leaching experiments performed at pH 1.5 and in the presence of 0.50 M KCl showed that neither protonation of the silanols in a TMOS monolith or a 22-fold increase in the ionic strength of the leaching solution, both of which should decrease the electrostatic attraction between PAMAM and the pore walls, is sufficient to cause detectable loss of encapsulated EITC-PAMAM. These results provide strong evidence that the quantitative retention of PAMAM entrapped in a sol–gel is attributable to steric confinement of the molecules to the pores. In other words, electrostatic adsorption to surface silanol groups is not a significant factor in the retention of EITC-PAMAM evident in Fig. 1.

4. Conclusion

Leaching of a water soluble dye of low molecular weight from a hydrated TMOS monolith can be arrested by covalent attachment of the dye to a macromolecular carrier. However, to quantitatively eliminate leaching, the carrier should be a macromolecule that possesses an organized, stable secondary structure. An inherently globular dendrimer, such as a generation 4 PAMAM dendrimer, meets these requirements. It is important to note that these results also apply to dried gels. Due to inherent shrinkage upon drying, the pore size distribution in dried sol–gel (i.e. a xerogel) is inherently smaller than in a gel that has been maintained in a hydrated state [40]. Thus, if conjugation to PAMAM prevents dye leaching from a hydrated TMOS monolith, the same strategy will also prevent leaching from a xerogel.

A small globular protein (e.g. myoglobin) may also be useful as a macromolecular carrier to prevent dye leaching from a doped sol–gel. However, exposure to denaturing conditions can cause a protein to unfold, producing a random coil conformation. Analogous to the behavior observed with polymeric dextrans, a polypeptide in a random coil conformation may be susceptible to leaching from a sol–gel. Since the globular conformation of a PAMAM dendrimer is stable to many conditions that cause protein denaturation, it is, therefore, a better choice as a macromolecular carrier for dye encapsulation.

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References