

Leaching Behavior of a Fluorescent Pyoverdin Immobilized in Sol-Gel Glass

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Abstract: The leaching behavior of fluorescent siderophore, pyoverdin (iron biosensor), immobilized in acid-catalyzed sol-gel glass is examined. By varying the R value (molar ratio of water:silicon), three different formulations of pyoverdin-doped sol-gel glass pellets (R = 5.6, 8.2, 10.8) were made with tetramethyl orthosilicate (TMOS). At three different aging times (2, 4, and 6 weeks), 7-day leaching experiments were conducted on whole and ground pellets. Two different leaching solutions were used: 0.1 M acetate buffer, pH 5.0, and 1 M HCl. Pyoverdin immobilization resulted in conformation changes as suggested by the appearance of two emission peaks centered at 440 and 505 nm. As expected, pyoverdin leached more rapidly from the ground glass; 61 % of the pyoverdin leached from the ground glass within the first 20 minutes, while after 3 hours, only 54 % of the pyoverdin had leached from the whole pellets. As the sol-gel glass aged from 2 to 6 weeks, the initial fluorescence of the ground glass decreased by 34 – 46 % for the three sol-gel formulations, and the 7-day cumulative leachate decreased by 13 – 31 %. However, sol-gel with the lowest R value (5.6) retained pyoverdin better than sol-gels at higher R values. The release of pyoverdin was also characterized by the diffusional exponent (n), which ranged from 0.527 to 0.802 for the ground glass, and from 0.276 to 0.415 for the whole pellets, confirming that whole pellets retained pyoverdin better than ground glass. Taken together, the results suggest for a given sensor configuration, retention of pyoverdin can be optimized with respect to pellet size, R value and aging time.

Key Words: Biosensor, fluorescent, leaching, pyoverdin, sol-gel.

1. INTRODUCTION

Porous glass produced by the sol-gel process [1] continues to gain popularity as an immobilization matrix for biosensors. More researchers are recognizing the ease with which sol-gel glass can be made, the wide variety of molecules that can be immobilized in it, and the wide variety of biosensor applications for it [2-5]. But, for sol-gel based biosensors to become a commercial reality, not only must they be specific and sensitive for the analyte being measured, they must also be stable [6]. Depending upon the environmental conditions the biosensor is exposed to, over time there can be changes in biosensor performance. This may be due to: conformational changes of the entrapped biomolecule [7]; a decrease in sol-gel matrix pore size [4]; leaching of the biomolecule out of the sol-gel matrix [8]; or leaching of a part of the biomolecule [9].

A doped sol-gel glass can be made in many different ways by varying the following: 1. silicon precursor used (such as tetramethyl orthosilicate (TMOS) and tetraethyl orthosilicate (TEOS)); 2. additives (alcohol, surfactant, polymer, etc.); 3. pH; 4. buffer type and concentration; 5. molar ratio of water:silicon (R value); 6. acid or base catalyst; 7. at what stage components are added; 8. aging conditions (wet or dry, and temperature). All of these variables, along with the properties and concentration of the dopant, can affect the physical and chemical properties of the doped sol-gel glass.

When biomolecules or indicator molecules have been doped into TMOS and TEOS sol-gel glasses, a wide variety of leaching behaviors have been reported [8, 10, 11].

In a pioneering study, Barrero *et al.* [12] immobilized pyoverdin in a TMOS sol-gel glass to produce a sensitive ferric biosensor. Pyoverdin is a fluorescent siderophore produced by *Pseudomonas* bacteria growing in iron-low media (also spelled pyoverdine [13]). Also, Yoder and Kisaalita [14] studied the iron specificity of pyoverdin (from *P. aeruginosa*) that was immobilized in a TMOS sol-gel glass. In both of these studies no detailed leaching results were reported. Barrero *et al.* [12] reported that there was no leaching, but as the biosensor was repeatedly used the fluorescent intensity baseline kept decreasing.

The long-term goal motivating this research is the development of a high-throughput iron biosensor based on pyoverdin immobilized in sol-gel glass. Because leaching of pyoverdin was observed in preliminary experiments, the objective of this paper was to characterize the leaching of pyoverdin from acid-catalyzed TMOS sol-gel glass. This study evaluated the effect of the following four variables on the leaching of pyoverdin: 1. the size of the doped sol-gel glass (ground, sieved pieces or whole pellets); 2. three R values (5.6, 8.2, and 10.8); 3. how long the doped sol-gel glass had aged (2, 4, 6 weeks) before testing for leaching; and 4. the pH of the leaching solution (acetate buffer, pH 5.0 or hydrochloric acid, pH 0.02).

At different ages of curing, pyoverdin-doped sol-gel glass was soaked for 7 days in leaching solution. For each

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7-day leaching experiment, fluorescence emission spectra were recorded for both the immobilized pyoverdinin and leached pyoverdinin. Comparisons of the spectra and fluorescence intensity are made, and the diffusional exponent, n [15] was calculated to compare leaching rates.

2. MATERIALS AND METHODS

Fluorescence measurements (of samples in 96-well plates) were made with a FLEXstation™ benchtop scanning fluorometer (Molecular Devices Corp., Sunnyvale, CA). The fluorescence measurements were done in the bottom read mode, temp. = 25° C, PMT (photomultiplier tube) sensitivity = high, with no cutoff filters, and fluorescence was recorded in relative fluorescence units (rfu). For emission scans, the excitation (exc.) was set to 370 nm and the emission (emis.) was scanned from 390 – 600 nm, in 5 nm increments. Pyoverdinin solutions, pyoverdinin-doped sol-gel glass (ground), and the leachates from the experiments were placed in Corning Costar® 96-well plates (no. 3631, polystyrene, black with clear, flat bottom).

2.1. Solutions

Tetramethyl orthosilicate (TMOS) was 98% (Aldrich). Deionized (d.i.) water was ultrapure (Barnstead, Nanopure Infinity). Methanol was 99.9 %, HPLC grade (Fisher). The 0.1 M acetate buffer, pH 5.0, was prepared with 74.0 ml 0.2 M acetic acid + 176.0 ml 0.2 M sodium acetate + 250.0 ml d.i. water, for a total volume of 500 ml.

2.2. Pyoverdinin

Pyoverdinin from *Pseudomonas aeruginosa* ATCC 15692 was produced and purified by copper-chelate chromatography, according to the procedures of Yoder and Kisaalita [14]. Pyoverdinin from *P. aeruginosa* consists of an octapeptide, containing both D- and L-amino acids and a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline [16]. The purified pyoverdinin solution (in deionized water) was previously determined to be 6.0 mM [14]. The same stock was used for all experiments.

2.3. Preparation of Pyoverdinin-Doped Sol-Gel Glass

Three formulations of sol-gel glass pellets were made with a single-step, acid-catalyzed procedure, very similar to that used by Yoder and Kisaalita [14]. With continuous stirring, in the order listed, the following solutions were added to a 100 ml glass beaker: 9.6 ml methanol, 7.0 ml 2 N nitric acid, 0 – 6.0 ml deionized water, 65.1 – 80.0 µl of purified pyoverdinin solution, and 9.6 ml TMOS. The amount of added water (0, 3.0, or 6.0 ml) was varied to achieve R values of 5.6, 8.2, 10.8, in the three formulations, hereafter referred to as A, B, C, respectively. The amount of pyoverdinin added to each formulation (A, B, C) was varied (65.1, 72.8, 80.0 µl, respectively) so that the ratio of pyoverdinin to the total volume of sol-gel solution was constant. The solution was stirred for an additional minute, and then 80 µl of the sol-gel solution was placed into each microwell of a 96-well plate (Falcon®, #3072, polystyrene, clear, flat bottom) and the lid was sealed onto the plate with tape. After 7 days, the tape was removed, and after 12 days the lid was tipped to allow more rapid drying. The plate was weighed every few days to

see when the sol-gel pellets had reached constant weight. Preparation and aging of the pellets was done at room temperature (ca. 22° C). Control sol-gel glass pellets, without pyoverdinin, were also made for each formula, with deionized water being used in place of the pyoverdinin solution.

Leaching experiments, each 7 days long, were conducted at approximately 2, 4, and 6 weeks after the sol-gel pellets were made on day 0. The 2-week experiments were started on day 17, the 4-week experiments were started on day 31, and the 6-week experiments were started on day 45.

2.4. Spectra of Pyoverdinin Solutions

Solutions of 5.0 µM pyoverdinin were made in both 0.1 M acetate buffer (pH 5.0) and 1.0 M HCl. For each pyoverdinin solution, 8 microwells of a Costar 96-well plate were filled with 200 µl, and the emission (exc. 370 nm) and excitation spectra (emis. 455 nm) were measured and averaged for the 8 microwells. Spectra of control solutions (acetate buffer and HCl without pyoverdinin) were measured in the same way. The control spectra were subtracted from the spectra for pyoverdinin solutions, and plots were made of these corrected spectra.

2.5. Leaching Experiment with Ground and Sieved Sol-Gel Glass

In preliminary experiments, pyoverdinin-doped sol-gel glass was ground and placed in the wells of a 96-well (clear bottom) plate with acetate buffer (pH 5.0). The addition of iron resulted in fluorescence quenching of the immobilized pyoverdinin. Approximately 0.0156 g of the ground sol-gel glass was enough to fully cover the bottom of each well, and by sieving it between fine mesh screens the variation in fluorescence measurements was kept low.

For the 3 formulations of pyoverdinin-doped sol-gel glass and the 3 control sol-gel glasses, at the aging times of 2, 4, and 6 weeks, the following protocol was followed.

Sol-gel pellets were weighed and then ground between 2 glass beakers. The pellets were placed in a 250 ml Pyrex® (heavy duty) beaker, and a 180 ml Pyrex® (tall form) beaker was placed inside this to crush the pellets with hand pressure. The ground glass was sieved between 1.0 mm and 0.50 mm nylon screen cloth (Small Parts Inc., Miami Lakes, FL) and then 8 portions of approximately 0.0156 g each were placed into 8 wells of a flat bottom, 96-well plate (Costar®, black with clear bottom).

To each of these 8 wells, 200 µl of 0.1 M acetate buffer (pH 5.0) was added at time = 0. At 3 minutes, the initial fluorescence of the glass was measured (exc.370 nm, emis. scanned from 390 – 600 nm). At 10 minutes, the buffer solution (hereafter called leachate) was transferred to empty wells in the same 96-well plate. Fresh acetate buffer (200 µl per well) was added to the glass and the fluorescence was immediately measured before any more pyoverdinin could leach out. The fluorescence of the leachate was then measured. This same procedure was followed at times 20 min., 1 hr., 3 hrs., 10 hrs., 24 hrs., 3 days, and 7 days on the same glass samples. This protocol was also followed for the control sol-gel glasses (without pyoverdinin) to have background fluorescence measurements.

2.6. Leaching Experiment with whole Pellets of Sol-Gel Glass

For the 3 formulations of pyoverdin-doped sol-gel glass and the 3 control sol-gel glasses, at the aging times of 2, 4, and 6 weeks, the following protocol was followed. About 0.234 g (between 10½ – 13½ pellets) of whole glass pellets were weighed and placed in a 15 ml centrifuge tube (Corning, #430052, polypropylene). At time = 0, 3.0 ml of 0.1 M acetate buffer (pH 5.0) was added to each tube. This ratio of 0.234 g glass:3.0 ml solution was the same ratio as that used for ground sol-gel glasses (0.0156 g glass:200 µl solution). At 10 min., the leachate was removed and transferred to a separate polypropylene tube, and 3.0 ml of fresh buffer was added to the glass pellets. Two portions of 200 µl of the leachate were transferred to wells in a 96-well plate and the fluorescence was measured. This same procedure was followed at times 20 min., 1 hr., 3 hrs., 10 hrs., 24 hrs., 3 days, and 7 days. Preliminary experiments demonstrated the presence of scattering artifacts when whole glass pellets were placed in the wells of a 96-well plate. Therefore, the fluorescence of the whole glass pellets was not measured.

2.7. Leaching Experiments with 1 M HCl

Barrero *et al.* [12] used 1 M HCl to remove iron bound to pyoverdin immobilized in sol-gel glass. Yoder and Kisaalita [14] also used this method to regenerate their iron biosensor so it could be repeatedly used for iron quenching assays. To study the effect of HCl on the leaching of pyoverdin, after the sol-gel glass had aged 4 weeks, leaching experiments with ground sol-gel glass and with whole pellets were conducted as previously described, but with 1.0 M HCl as the leaching solution.

2.8. Data Analysis for the Leaching Experiments with Ground Sol-Gel Glass

Data reduction for the emission spectra (exc. 370 nm) of both the immobilized pyoverdin and the leachates consisted of first selecting the emission at 450 nm, as this was near the maximum emission peak for all readings. Then, to correct for differences in the aged pellets and differences in the control sol-gel glasses, an adjusted fluorescence value (described below) was calculated.

Although the 3 formulations of pyoverdin-doped pellets were made with the same ratio of pyoverdin to total volume of sol-gel solution, because the aged pellets dried different amounts, the ratio of pyoverdin to weight of dried sol-gel glass varied slightly between the 3 formulations. Also, the spectra of the control sol-gel glasses showed some background fluorescence and scattering due to the sol-gel matrix. Thus, to properly compare the fluorescence (exc./emis. 370/450 nm) measurements (in relative fluorescence units, rfu) the fluorescence per nanomole of immobilized pyoverdin (here called adjusted fluorescence) was calculated as follows:

$$FL_{adj} = (FL_{pvd} - FL_c) / (M_{pvd} \times W_t) \quad (1)$$

where: FL_{adj} is the adjusted fluorescence (rfu per nanomole immobilized pyoverdin); FL_{pvd} is the fluorescence (rfu) of pyoverdin-doped sol-gel glass; FL_c is the fluorescence (rfu) of control sol-gel glass; M_{pvd} is the nanomoles pyoverdin per gram of sol-gel glass; W_t is the weight (g) of pyoverdin-doped sol-gel glass.

FL_{pvd} , FL_c , and W_t were mean values per microwell, each calculated from 8 microwells, and the corresponding standard deviations were input into an equation to calculate the standard deviation of the adjusted fluorescence. Mean values of adjusted fluorescence (exc./emis. 370/450 nm) were calculated for: 1) the initial fluorescence of the pyoverdin-doped sol-gel glass; 2) the fluorescence of the pyoverdin-doped sol-gel glass after 7 days of leaching; and 3) the cumulative (over 7 days) fluorescence of the leachates. These means were compared with multiple t-tests (calculated in Microsoft Excel). Means that were not significantly different ($\alpha = 0.05$) were given the same letter designation.

2.9. Data Analysis for Whole Pellet Leachates

The fluorescence (exc./emis. 370/450 nm) values for the whole pellet leachates were adjusted as described for the ground sol-gel glass, except W_t in equation (1) was the weight of sol-gel glass per 200 µl buffer. This allowed direct comparison to the (adjusted) fluorescence values determined for the ground sol-gel glass experiments.

2.10. Leaching rate Determination

The following simple equation was developed by Ritger and Peppas [15] to describe the solute (drug) release from non-swallowable, controlled release polymers:

$$M_t/M_\infty = k t^n \quad (2)$$

where M_t is the mass of drug released at time t , M_∞ is the mass of drug released as time approaches infinity, k is a constant (which depends on the characteristics of the polymer matrix and the doped drug), and n is the diffusional exponent. When this equation is applied to data of the first 60% of the fractional release (M_t/M_∞), the value of n indicates if the transport is Fickian diffusion or not [15].

For this data analysis, it was assumed that the 7-day cumulative fluorescence corresponded to the M_∞ value. The 7-day cumulative fluorescence (adjusted, exc./emis. 370/450 nm) of the leachate solutions was plotted as the log (cumulative fluorescence) vs. log (leaching time) for all sol-gel formulations, for all age periods. Units of leaching time were in minutes (10 – 10,080 min.) so that the log values (1.0 – 4.0) would be positive for all data points. Values of n were determined from the slope of the linear regression line, using the data of the first 60% of the 7-day cumulative fluorescence.

3. RESULTS

3.1. Size of Pellets

The final pellet weight (week 6) was 98% of the weight by day 17 (2 weeks). At 6 weeks of age, the pellet weights (from a total of 30 to 40 pellets) and dimensions (height × diameter, averages of 10 pellet samples) were: A, 0.0218 g, 1.02 × 3.80 mm; B, 0.0194 g, 0.97 × 3.70 mm; C, 0.0176 g, 0.96 × 3.57 mm.

3.2. Spectra for Pyoverdin, Immobilized Pyoverdin, and Leachates

If all the pyoverdin leached out of a microwell portion (0.0156 g) of the ground pyoverdin-doped sol-gel glass into 200 µl of acetate buffer, the concentration of pyoverdin

would be 5.0 μM . The fluorescence emission spectrum (exc. 370 nm) for a 5.0 μM solution of pyoverdinin in 0.1 M acetate buffer, pH 5.0 (Fig. 1a) shows there was one peak centered at 456 nm. The emission spectrum (exc. 370 nm) for a 5.0 μM solution of pyoverdinin in 1 M HCl (Fig. 1b) also exhibited one peak, of greater fluorescent intensity, that was blue shifted to 436 nm. The maximum excitation wavelength (emis. 455 nm) for pyoverdinin in acetate buffer was 389 nm (Fig. 1c), and for pyoverdinin in 1 M HCl there was a double excitation peak at 367 and 378 nm (Fig. 1d). At an excitation of 370 nm, the emission at 450 nm was about 20% higher for pyoverdinin in HCl than for pyoverdinin in acetate buffer, pH 5.0.

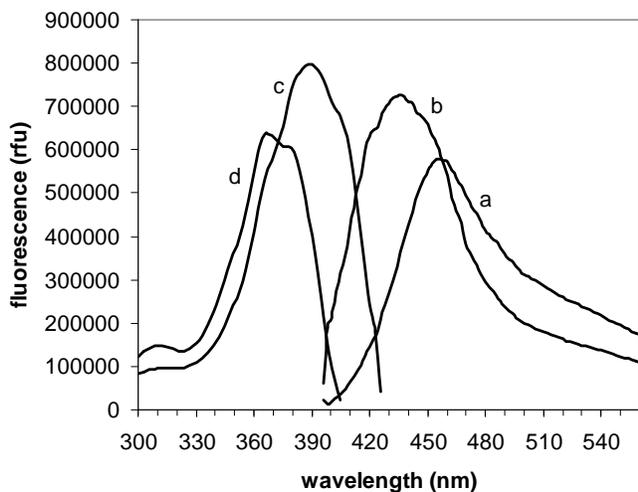
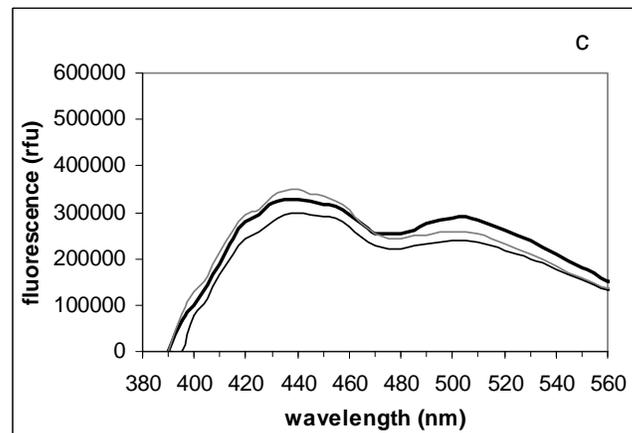
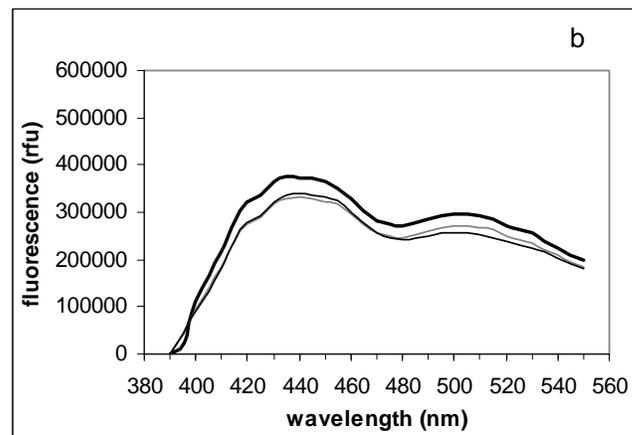
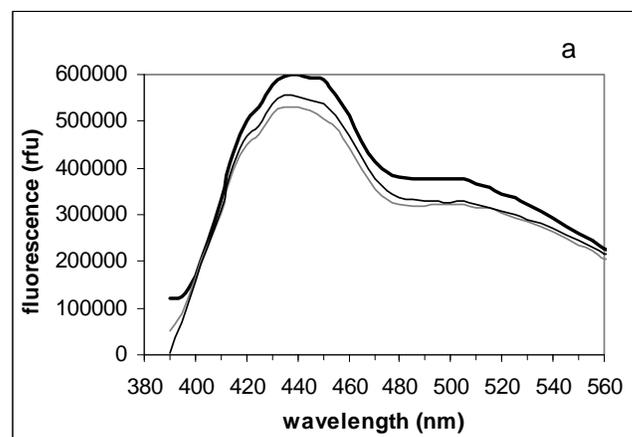


Fig. (1). Fluorescence spectra of free 5.0 μM pyoverdinin solutions. Emission spectra (excitation 370 nm): **a)** pyoverdinin in 0.1 M acetate buffer, **b)** pyoverdinin in 1.0 M HCl. Excitation spectra (emission 455 nm): **c)** pyoverdinin in 0.1 M acetate buffer, **d)** pyoverdinin in 1.0 M HCl.

In comparison, the fluorescence spectra for pyoverdinin immobilized in the 3 sol-gel formulations, for all initial readings of 2-week, 4-week, and 6-week-old glass, in 0.1 M acetate buffer, showed 2 emission peaks (Fig. 2). The larger peak was centered at about 440 nm and the smaller one was at 505 nm, but as the sol-gel glasses age the height of these peaks (initial glass reading) decreased. After these glasses soaked for 10 min. and the first leachate was removed, the spectra for the immobilized pyoverdinin had only one fluorescent peak, which changed from 455 nm at 2 weeks (not shown), to 460-470 nm at 4 weeks (Fig. 3b), to 470-480 nm at 6 weeks (not shown). Similar results were obtained with all sol-gel formulations. At leaching times greater than 10 min. the fluorescent peak of the immobilized pyoverdinin remained about the same but continued to decrease in height. For all three sol-gel formulations, the spectra of the 10 min. leachates had 2 fluorescence peaks, centered at about 450 nm and 505 nm (Fig. 3c). The spectra for the 2nd leachate samples (20 min.) only had one fluorescent peak, centered at about 460 nm at 2 weeks (not shown) and 4 weeks (Fig. 3d), and at 475 nm at 6 weeks (not shown). Leachate samples taken after 20 min. also had just one peak, centered at about 460 – 475 nm, but the height kept decreasing over the 7 days of leaching.



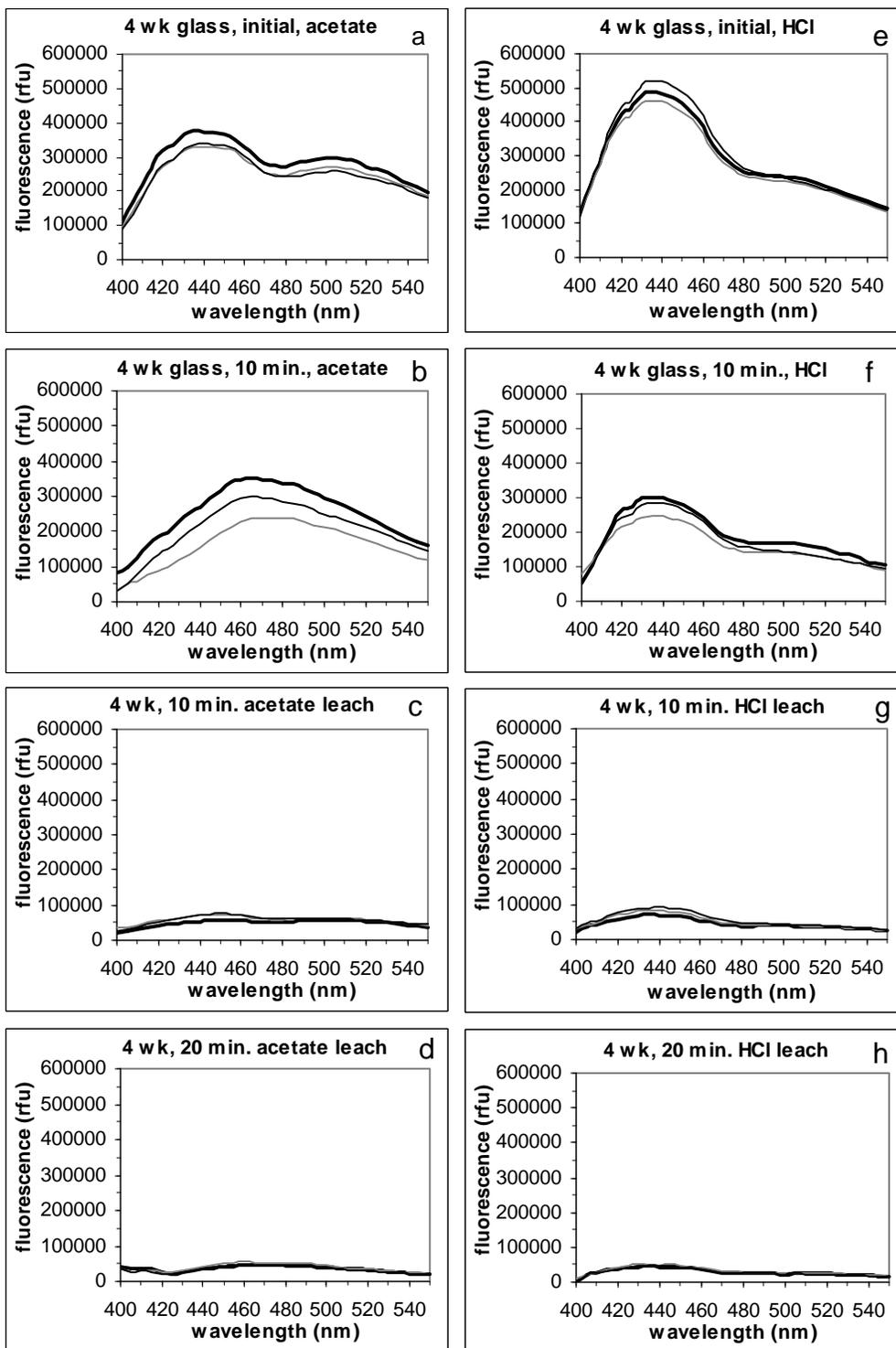
Legend: sol-gel A --, sol-gel B --, sol-gel C --.
*adjusted fluorescence, excitation 370 nm.

Fig. (2). Fluorescence* spectra of a) 2-week-old, b) 4-week-old, and c) 6-week-old ground pyoverdinin-doped sol-gel glass, when initially placed in acetate buffer.

The spectra for the 4-week-old pyoverdinin-doped sol-gel glass soaked in 1 M HCl were different from the spectra seen with acetate buffer. The spectra of the initial readings of the pyoverdinin-doped glass showed a main peak at about 436 nm and just a low shoulder at 505 nm, for all three sol-gel formulations (Fig. 3e). After the first leachate (10 min.) was removed, the spectra of the immobilized pyoverdinin showed the same peaks (Fig. 3f), but the peak heights decreased over

7 days of leaching. The spectra of the first HCl leachate sample (10 min.) had a main peak at 436 nm and a low shoulder at 505 nm (Fig. 3g). The subsequent leachates (over 7 days) exhibited these same peaks but at lower heights, as seen in the 20 min. HCl leachate (Fig. 3h).

The fluorescence spectra of the leachates obtained for the whole pellet experiments (not shown) were almost the same as those for the ground pellets except that the peak heights were lower. Also, the spectra of the 10 min. leachates (acetate buffer and HCl) for the 4-week-old whole pellets

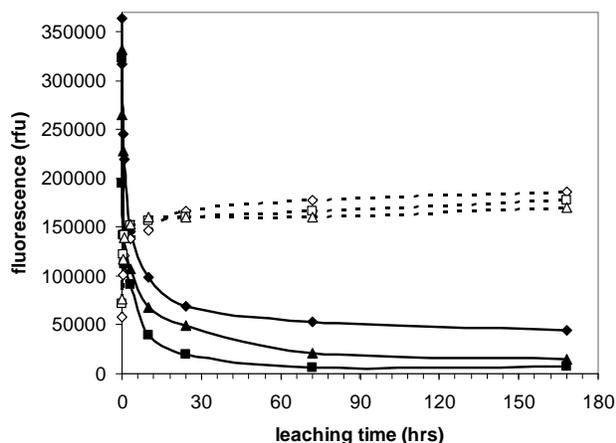


Legend: sol-gel A --, sol-gel B --, sol-gel C --.

Fig. (3). Fluorescence* spectra of 4-week-old pyoverdin-doped sol-gel glass. **a)** glass initially in acetate buffer, **b)** glass after 10 min of leaching, **c)** 10 min acetate leachate, **d)** 20 min acetate leachate, **e)** glass initially in HCl, **f)** glass after 10 min of HCl leaching, **g)** 10 min HCl leachate, **h)** 20 min HCl leachate (*adj. fluor., exc. 370 nm).

showed a wider variation among sol-gel formulas, with much less leaching of pyoverdine from sol-gel A.

Values of the adjusted fluorescence (exc/emis 370/450 nm) were plotted for both the ground immobilized pyoverdine and the cumulative fluorescence of the leachates, over 7 days (168 hrs.). Representative curves are shown for 4-week-old pyoverdine-doped sol-gel glass in acetate buffer in Fig. (4).



Legend: glass A \blacklozenge —, glass B \blacksquare —, glass C \blacktriangle —, leach A \diamond ---, leach B \square ---, leach C \triangle ---

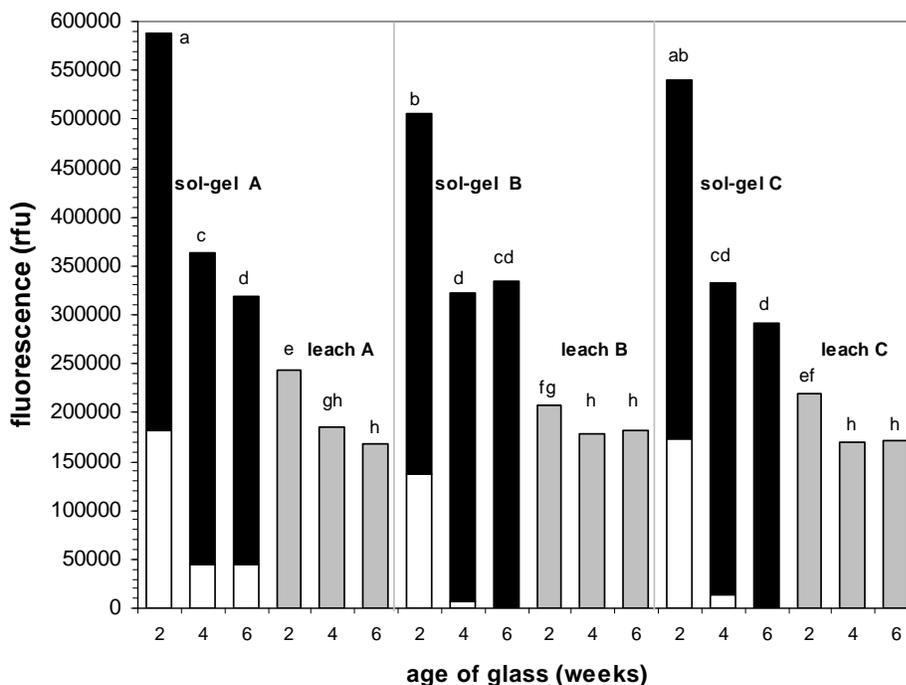
Fig. (4). Fluorescence (adjusted, exc/emis 370/450 nm) of 4-week-old ground sol-gel glass and the cumulative leachate, over 7 days (168 hours) of leaching with acetate buffer.

3.3. Differences in Fluorescence of Immobilized Pyoverdine and Leachates

The data for all sol-gel formulations at all age periods (as shown in the representative curves of Fig. 4) was reduced to three fluorescence (adjusted, exc/emis 370/450 nm) values: 1. the initial fluorescence of the immobilized pyoverdine, 2. the fluorescence of the immobilized pyoverdine after leaching for 7 days, and 3. the 7-day cumulative fluorescence of the leachate. A bar chart shows these values for ground sol-gel glasses in acetate buffer (Fig. 5). The initial fluorescence of the immobilized pyoverdine is indicated by the top of the black bar, and the fluorescence remaining after 7 days of leaching is indicated by the bottom of the black bar. The 7-day (cumulative) fluorescence of the leachates is indicated by the top of the gray bars. Bars in this chart (Fig. 5) labeled with the same (lower case) letter were not significantly different ($\alpha = 0.05$), as determined from the multiple t-tests.

3.4. Whole Pellet Experiments with Acetate Buffer

The 7-day cumulative fluorescence of the acetate buffer leachates from whole pellets of pyoverdine-doped sol-gel glass did not vary much. The adjusted fluorescence (exc/emis 370/450 nm) per nmole immobilized pyoverdine (per 200 μ l leaching solution) for the 3 sol-gel formulations, at 2, 4, and 6 wks (respectively) were: A, 266770, 222044, 222174; B, 217112, 239764, 200694; C, 233979, 263192, 204039. The 7-day cumulative fluorescence of the leachates from ground glass (Fig. 5) were between 4 - 35 % lower than the corresponding fluorescence values for whole pellets (listed above).



*adjusted, exc/emis 370/450 nm.

Fig. (5). The fluorescence* of ground pyoverdine-doped sol-gel glass initially (top of black bar) and after 7 days of leaching (bottom of black bar), and the 7-day cumulative fluorescence of the leachates (top of gray bars). Data for 2-week-, 4-week-, and 6-week-old ground glasses, using 0.1 M acetate buffer, for 3 sol-gel formulations (A, B, C). Bars labeled with the same (lower case) letter are not significantly different ($\alpha = 0.05$).

3.5. Leaching with 1 M HCl

Fluorescence values for 4-week-old ground pyoverdinin-doped sol-gel glass and the 7-day (cumulative) leachates, with 1 M HCl leaching solution, are presented (Fig. 6) in the same manner as in Fig. (5). The 7-day (cumulative) fluorescence of the HCl leachates from 4-week-old whole pellets is shown on the right side of Fig. (6). There was 17 – 22 % less leaching from the ground glass than from the whole pellets.

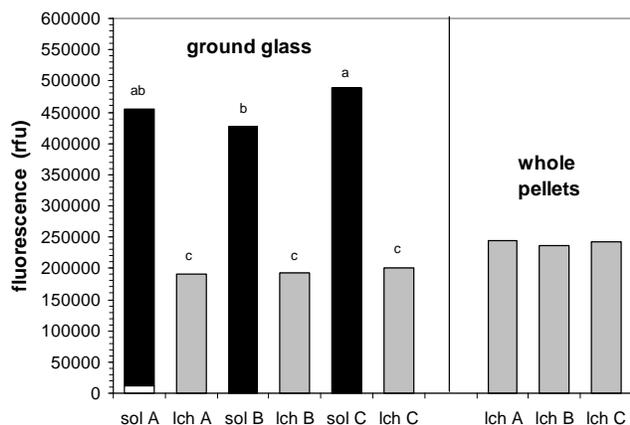


Fig. (6). Initial fluorescence* of pyoverdinin-doped sol-gel glass initially (top of black bar), and after 7 days of leaching (bottom of black bar), and the 7-day cumulative fluorescence of the leachates (top of gray bars). Bars labeled with the same (lower case) letter are not significantly different ($\alpha = 0.05$). X-axis labels: sol – sol-gel glass, Ich = leachate from ground sol-gel glass. *adjusted exc/emis 370/450 nm.

3.6. Leaching rate Determination

A representative plot of log (cumulative fluorescence) vs. log (leaching time) shows the pyoverdinin released (leached) from both ground and whole pellets of 6-week-old pyoverdinin-doped sol-gel glass (Fig. 7). For all formulations of the ground glass, at all ages, the initial release rate (10 - 20 min.) was much faster than for the remaining (1 hr. - 168 hrs.) leaching time. For the whole pellets, the initial rate of release was only slightly faster than the remaining leaching time.

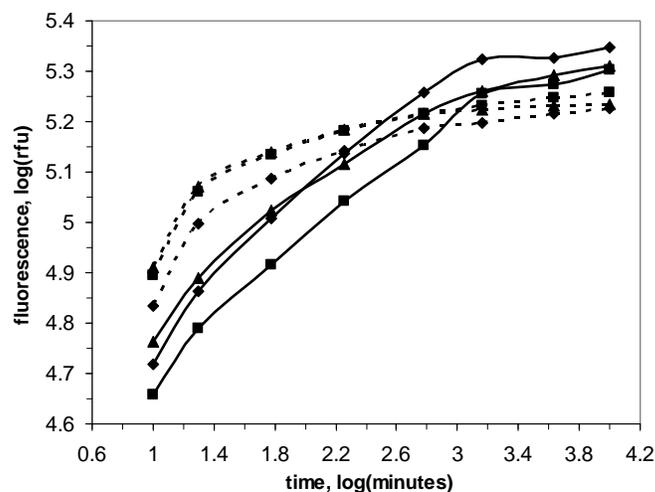
Applying equation (2) to the first 60% of the 7-day cumulative fluorescence (M_{∞} value) resulted in only being able to include leaching data of 10 min. and 20 min. for the ground glass. For the whole pellets, data up to and including 3 hours was still within 60% of the 7-day cumulative fluorescence. Values of n that were calculated for the pyoverdinin released (acetate leaching) from ground glass were much higher than values of n for whole pellets (Fig. 8). And, values of n for the pyoverdinin released by HCl leaching (at 4 weeks) were also higher for ground glass (A, 0.643; B, 0.685; C, 0.586) than for whole pellets (A, 0.387; B, 0.376; C, 0.361).

4. DISCUSSION

4.1. Influence of Immobilization Upon Pyoverdinin Spectra

As shown in Fig. (1), the single excitation peak at 389 nm and the single emission peak at 456 nm for pyoverdinin in acetate buffer (pH 5.0) and the double excitation peaks at

367 and 378 nm for pyoverdinin in 1 M HCl (pH 0.02) were consistent with previous results as described below. For example, using a solution of pyoverdinin (from *P. aeruginosa*), Cox and Adams [17] reported exc./emis. maxima of 400/460 at neutral pH, and double excitation peaks of 360 and 380 nm in acidic solution. And, these are similar to the spectra for other pyoverdins [18, 19].



Legend: whole pellets A \blacklozenge —, whole pellets B \blacksquare —, whole pellets C \blacktriangle —, ground A \blacklozenge ---, ground B \blacksquare ---, ground C \blacktriangle ---

Fig. (7). Cumulative fluorescence (exc/emis 370/450 nm) of the acetate buffer leachate over 7 days of leaching, for both ground and whole pellets of 6-week-old pyoverdinin-doped sol-gel glass. [Data points are at 10 min, 20 min, 1 hr, 3 hrs, 10 hrs, 24 hrs, 3 days, and 7 days].

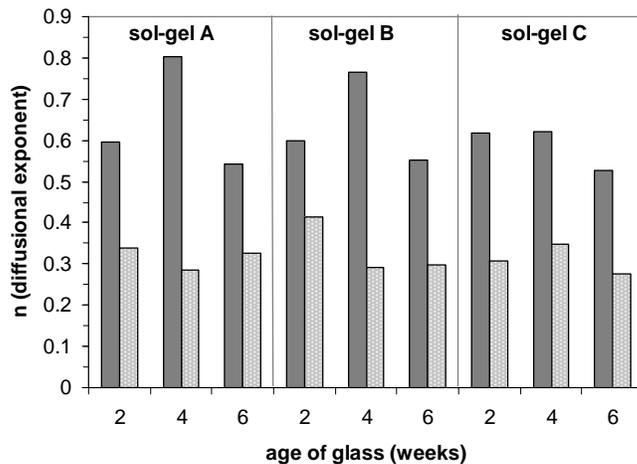


Fig. (8). Diffusional exponent, n , for both ground (dark) and whole (light) pellets of 2-week-, 4-week-, and 6-week-old pyoverdinin-doped sol-gel glass in 0.1 M acetate buffer, pH 5.0.

In this study, there was a change in the spectra of pyoverdinin upon immobilization. The maximum excitation was blue shifted 19 nm, from 389 nm (solution) to 370 nm (immobilized), and the emission maximum was blue shifted 16 nm, from 456 nm (solution) to 440 nm (immobilized, initial reading). The Stokes shift changed only slightly, from 67 nm (solution) to 70 nm (immobilized). Barrero *et al.* [12]

reported that pyoverdin (in solution, from *P. fluorescens*) had exc./emis. maxima at 390/465 nm, while pyoverdin immobilized in sol-gel glass had exc./emis. maxima at 330/405 nm.

The source of the emission peak at 505 nm, seen in both the immobilized pyoverdin and in the initial (10 min.) leachates is uncertain. However, Keeling-Tucker and Brennan [20] noted that the emission of a fluorescent molecule doped into sol-gel glass can be complex because of the variety of interactions that the excited molecule can have with the local microenvironment. These interactions include collisions with solvent and other molecules, rotational motions, solvent dipolar relaxation, tautomerization, and protonation. The formation of an excimer or exciplex can also lead to the appearance of another emission peak at a longer wavelength [21].

It appears that only a fraction of the immobilized pyoverdin exhibited a peak at 505 nm, because after 10 min. of leaching the peak at 505 nm was absent in both the sol-gel glass (Fig. 3b) and the leachates (Fig. 3d), but the peak at approx. 455 – 470 nm remained. Others have also seen changes in biomolecules when immobilized in sol-gel glass. When Ferrer *et al.* [9] immobilized horseradish peroxidase in an acid-catalyzed TMOS sol-gel glass, they found that the small (616.5 Da) heme group leached out while none of the larger protein (44 kDa) did.

4.2. Influence of Aging

For 2-week-, 4-week-, and 6-week-old sol-gel glasses, the initial fluorescence of the immobilized pyoverdin (Fig. 2) showed the same 2 peaks (440, 505 nm). But, the initial fluorescence intensity decreased by 36 – 38 % from 2 weeks to 4 weeks, for all three R values of sol-gel glass (Fig. 5). This suggested that the conformation of the pyoverdin molecule had changed, causing a change in the fluorescent yield. As the pellets aged from 2 weeks to 4 weeks, the amount of pyoverdin that leached out decreased by 14 – 24 %. As seen by the difference between the top and bottom of the black bars in Fig. (5), there was a large decrease (68 – 100 %) in fluorescence of immobilized pyoverdin after 7 days of leaching, for all R values and all ages. And, although the cumulative leachate was highest at 2 weeks (Fig. 5), the same 2-week old sol-gel exhibited significantly higher pyoverdin fluorescence after the 7 days of leaching (as shown by the bottom of the black bars in Fig. 5). The practical implication for biosensor development is that a short 2 week aging period may be preferable based on the sol-gel capacity to retain pyoverdin after seven days of leaching.

Decreases (over 6 weeks) in the fluorescent intensity of the immobilized pyoverdin and in the cumulative leachate may be explained by a reduction in pore size as the sol-gel ages. From 2 weeks to 6 weeks, the average weight of the sol-gel pellets had dropped by 0.005 – 0.001 g per pellet, indicating that they were still shrinking in size, and thus the pores were also shrinking. As a sol-gel glass ages beyond the gel point, there can be changes that continue for months at room temperature [1]. These changes include more polymerization due to condensation reactions, shrinkage of the gel network (syneresis), and changes in porosity. Gill [2] believes that pore collapse (shrinkage in pores as the sol-gel ages) is the main cause of the decline in activity of encapsu-

lated biomolecules. Flora and Brennan [7] found that human serum albumin that was immobilized in acid-catalyzed TMOS sol-gel glass had only 15 % binding ability after the sol-gel had aged for 2 months.

4.3. Influence of R Value

The influence of R value (from 5.6 – 10.8) upon the leaching behavior was minimal. In some instances, sol-gels A and B were different, as in the initial fluorescence and cumulative leachate of 2-week-old glass (Fig. 5), but in other cases sol-gels A and B were not significantly different, as in the HCl leaching of 4-week-old glass (Fig. 6). At the end of each 7-day leaching experiment, there was a large decrease in the fluorescence of immobilized pyoverdin for all 3 sol-gel formulations, as seen by the span from top to bottom of the black bars in Fig. (5). As the sol-gel glasses aged from 2 weeks to 6 weeks, the decrease in fluorescence became less, especially for sol-gel A. For the 6-week-old ground glasses, after 7 days of leaching, only in sol-gel A was there still some pyoverdin left. Thus, sol-gel A (R value 5.6) would be the best for pyoverdin retention. Using an R value even lower than 5.6 could possibly result in greater retention of the pyoverdin. However, it is possible that the relationship between R value and leaching behavior may not be linear.

Nonlinear leaching behavior has been described with TEOS sol-gel glasses. Kortessuo *et al.* [22] reported that an increase in the R value from 6 to 28 increased both the release rate and the total amount of a doped drug released from acid-catalyzed TEOS sol-gel glass. But, when others studied acid-catalyzed TEOS sol-gel glass and the range of R values was between 2 to 6, an increase in R value resulted in a decrease in leaching or porosity [23-25]. While an increase in R value can increase hydrolysis [1], the concentrations of other components, such as solvent, must also be considered. When the ratio of alcohol:TEOS was held constant, a change in the R value resulted in a non-linear change in the time to gel [26]. Starting at low R values (< 1), as the R value increased the time to gel decreased, until a certain R value (dependent on the alcohol:TEOS ratio) was reached. Then, as the R value increased the time to gel increased.

4.4. Influence of Leaching Solution pH

The pyoverdin molecule, perhaps because of the alternating L- and D-amino acids in its structure, is not degraded (like other peptides are) by enzymes such as trypsin and pepsin [27], and it is resistant to denaturation by alcohol and strong acids. These properties allow a high concentration of methanol to be used in the sol-gel formulations and for 1 M HCl to be used as a regenerating solution to remove iron that binds to pyoverdin when performing an iron assay.

While the largest value of initial fluorescence for the 4-week-old ground glass in acetate buffer was for sol-gel A (Fig. 5), when the leaching solution was 1 M HCl the largest value of initial fluorescence was for sol-gel C (Fig. 6). The initial fluorescence with HCl was 24.7, 32.4, and 47.0 % higher, for sol-gels A, B, C (respectively) than with acetate buffer. Some of this increase was expected, as the fluorescence (at exc/emis 370/450 nm) of pyoverdin in 1 M HCl was 20% greater than the fluorescence of pyoverdin in acetate buffer (Fig. 1). The amount of HCl leachate from the ground glass was not significantly different ($\alpha = 0.05$) for the

3 sol-gel formulations, and there was no difference in the HCl leachates from the 3 formulations of whole pellets (Fig. 6). Thus, in terms of reducing the leaching due to 1 M HCl, the choice of R value is not important. Because the fluorescence of pyoverdin is greater in 1 M HCl than in acetate buffer, and the HCl leachates from the ground glasses (Fig. 6) were only slightly greater than the comparable (4 week) acetate buffer leachates (Fig. 5), it is concluded that there is no difference in the amount of leaching from HCl versus acetate buffer leachates.

Albrecht-Gary *et al.* [28] determined that the dissociation of the ferric-pyoverdin complex is complete at pH below 1.5. Thus, when sol-gel-immobilized pyoverdin is being used as a ferric ion biosensor, even 0.1 M HCl (pH 1.0) should be effective in removing bound iron from the pyoverdin (regeneration). Although the cumulative leachate due to 1 M HCl was not different from the leachate due to acetate buffer, there might be less degradation of the sol-gel glass matrix with a milder (less acidic) regeneration. Kortessuo *et al.* [21] found that over the pH range of 1-5, the greatest degradation of TEOS sol-gel glass was at pH 1.

4.5. Release Mechanism

Although the ground glasses had a greater initial leaching rate (Fig. 7) and larger n (Fig. 8) than the whole pellets, it is unclear why the leachates from the ground glasses (Fig. 5) were between 4 – 35 % less than the leachates from whole pellets (not shown). The average aspect ratio (diameter:thickness) of all the whole pellets was calculated to be 3.75. From the data of Ritger and Peppas [15] for tablets, this aspect ratio coincides with a diffusional exponent of 0.451. Thus, since the calculated values of n for the whole pellets were less than 0.451 (Fig. 8), the leaching from the whole pellets deviated from Fickian diffusion. In Non-Fickian diffusion, the media penetration is in the same range as the chemical specie diffusion [21], which is undesirable if the objective is to retain the diffusing chemical. The ratio of the weight of sol-gel glass to the volume of leaching solution was constant between the ground and whole pellet experiments. But, the ratio of the surface area of the sol-gel glass to the volume of leaching solution was not. With the ground glasses, the dimensions were smaller and the surface area exposed to the leaching solution was greater, so a faster leaching rate was predicted.

The initial rate of release (between 10 and 20 min.) of pyoverdin from the ground sol-gel glasses was very fast while the rate of release for the remaining 7 days was very slow. This was observed for all 3 ages, with both HCl and acetate buffer leaching, and is shown by the representative data for 6-week-old glass (Fig. 7). This agreed well with Ritger and Peppas [15], who found that a particle size distribution (such as the ground sol-gel glass) had a much faster release at early times and a much slower release at longer times, compared to the release from a monodisperse sample. And, the breadth and shape of a particle size distribution can affect the values of n . Kortessuo *et al.* [20] also reported an initial burst in the release of a doped drug from small, acid-catalyzed, TEOS sol-gel rods (made at different R values), and the fastest release was from the smallest sol-gel rods. For some unknown reason the values of n for 4-week-old

ground sol-gel glasses A and B are higher than the other values of n (Fig. 8).

For pyoverdin-doped sol-gel glass to be used as an iron biosensor in 96-well plates, the sol-gel particle size must be smaller than the whole pellets (3.7 mm diameter \times 1 mm height) to avoid scattering artifacts. However, larger pellets of pyoverdin-doped sol-gel glass (3.4 mm diameter \times 3.6 mm height) have been successfully used in a custom-made flow cell designed the Perkin Elmer LS50B Luminescence Spectrometer [14]. If ground pyoverdin-doped sol-gel glass (of the R values used) was washed for 20 minutes before being used in an iron assay, the remaining activity would at least be more stable over several days than that of whole pellets.

CONCLUSIONS

While pyoverdin has been successfully doped into sol-gel glasses to produce iron biosensors by others, this study shows that there can be considerable leaching of pyoverdin from acid-catalyzed TMOS sol-gel glass. Specifically, the results from this study support the following conclusions:

1. At all R values, compared to the emission spectra of pyoverdin in solution, there was a change in the emission spectra (two peaks) of both immobilized pyoverdin and the first leachates. Subsequent spectra of immobilized pyoverdin and leachates only exhibited one peak. Thus, immobilization resulted in a conformational change in at least a fraction of the pyoverdin.
2. As the sol-gel glasses aged (from 2-6 weeks) there was a reduction in the initial fluorescence of the immobilized pyoverdin and a reduction in the 7-day (cumulative) leaching. And, these changes were the greatest between 2 weeks and 4 weeks.
3. The leaching behavior of pyoverdin from the ground sol-gel glass was different from that of the whole pellets. For the ground glass, 61 % of the leaching occurred within the first 20 minutes and then the rate slowed. For the whole pellets, 54 % of the leaching occurred within the first 3 hours, and the leaching rate was fairly constant over the 7 days of leaching. Although the ratio of pyoverdin-doped sol-gel glass to leaching solution was kept constant, the 7-day (cumulative) leachate for the ground glass was 4-35 % less than the leachate from whole pellets.
4. When immobilized pyoverdin was placed in 1 M HCl, there was a change in the emission spectra, and these same changes were seen in the HCl leachates. For the 4-week-old sol-gel glasses there was no difference in the amount of pyoverdin leached due to the pH of the leaching solution.
5. Differences in leaching due to R value (5.6 to 10.8) were minor compared to the differences in leaching due to aging. In certain respects sol-gel A was different from sol-gels B and C, but in other respects sol-gel C was different from sol-gels A and B. For 4-week-old and 6-week-old glasses, only sol-gel A still contained pyoverdin after 7 days of leaching, suggesting that it would be more suited for use in the iron biosensor, other factors being equal.

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