Characterization of Fluorescein Isothiocyanate-Dextran Used in Vesicle Permeability Studies

Karine Andrieux, Pierre Lesieur, Sylviane Lesieur, Michel Ollivon, and Cécile Grabielle-Madelmont*

Equipe Physico-chimie des Systemes Polyphases, UMR CNRS 8612, Université Paris-Sud, 92296 Châtenay-Malabry Cedex, France

Fluorescein Isothiocyanate-dextrans of various weight average molecular masses (4400–487 000) were analyzed in buffer solution for pH, osmolarity, fluorescence intensity as a function of the polymer concentration, average molecular masses, and radii of gyration. Labeling of polymers and conformation of the polymers were characterized by high-performance gel exclusion chromatography (HPLC-GEC) and small-angle X-ray scattering. The fluorescence measurements evidence the absence of fluorescence quenching of the FITC chromophores but the existence of an inner filter effect at high polymer concentration. The conformation of the polymers in buffer is very likely of random coil type, as shown by the relationship between the radii of gyration and the weight-average molecular masses of the dextrans (Mw). The medium used to analyze the FITC-dextrans by HPLC-GEC strongly influences their elution behavior. In buffer medium, they are sieved over the TSK G4000 PW column through a water, they are separated into several species by an exclusion mechanism that depends on the number of labeled sites per dextran molecule. A Monte Carlo simulation was used to analyze the distribution of the fluorescent labels. HPLC-GEC in water could interestingly be applied to yield labeled polymers bearing a known number of functionalized groups.

Dextrans are hydrophilic polysaccharides elaborated by Leuconostoc bacteria. High molecular weight, good water solubility, low toxicity, and relative inertness characterize them. The high proportion of α-1,6 polysaccharide linkages makes them resistant to acid and alkaline hydrolysis. Fluorescein Isothiocyanate-dextrans consist of dextrans covalently linked with a fluorescent group, the fluorescein isothiocyanate. Because of their properties, FITC-dextrans are used in a wide variety of applications. They serve as markers for translational diffusion of macromolecules in the cytoplasm of cells by microinjection and for intracellular delivery by endocytosis, vesicle fusion, shock waves and electroporation. They are well-adapted for monitoring the process of vesicle fusion and vesicle formation for the characterization of polar solute transport across biological membranes and for kinetic studies on drug release from delivery systems.

FITC-dextrans are also widely used to investigate membrane permeabilization. A mechanism often encountered in this process is the formation of pores or defects in lipid bilayers. This is the case for the insertion of surfactant molecules in the vesicle membrane before micellization, for membrane perturbation induced by many toxins and antimicrobial peptides and for...

cell loading by electroporation. Formation of pores in a membrane permits the exchange of molecules across the membrane, which among other things depends on the sizes of the pores and the molecules. Determination of the size of such pores is important for understanding their structure and their formation mechanism. This determination can be done only indirectly by measuring the leakage of markers of different sizes that are able to pass through the pores. Because of their wide range of molecular masses, the FITC-dextrans allow such a determination using the same chemical species.

In the frame of a study of permeabilization of lipidic vesicles by two surfactants, one nonionic (octyl β-D-glucopyranoside), the other ionic (sodium taurocholate), FITC-dextrans were used as leakage tracers. In a previous paper, we developed a methodology based on high-performance gel exclusion chromatography (HPLC-GEC) to perform permeability studies on vesicles. In the present work, we have analyzed different physicochemical characteristics of FITC-dextrans of various average molecular masses under the conditions used for permeability experiments. Analysis of these probes focused on pH and osmolarity measurements, the characterization of their fluorescent properties as a function of the polymer concentration, the determination of their average molecular masses by HPLC-GEC, and the evaluation of their radii of gyration by small-angle X-ray scattering (SAXS). Size-exclusion chromatography is commonly used to analyze polymers, however, such an analysis has rarely been performed directly on dextrans labeled with FITC. The HPLC-GEC methodology developed in this study provides a potential tool to quantify the number of functionalized groups grafted per polymer chain and to separate labeled polymer molecules from unlabeled ones.

**EXPERIMENTAL SECTION**

**Materials.** Glucose (M = 180) and polysaccharide standards (pullulans) of 738, 5800, 12 200, 23 700, 48 000, 100 000, 186 000, 380 000, and 1 660 000 weight average molecular masses (M_w) (g/mol) were manufactured by Polymer Laboratories (Amherst, MA) and supplied by Tousart et Matignon (Paris, France). The fluorescein-isothiocyanate dextrans (FITC-dextrans) of M_w 4400, 9400, 18 900, 40 500, 69 000, 147 800 and 2 000 000 (g/mol) were purchased from Sigma (St. Louis, MO). The polysaccharide standard and the FITC-dextran characteristics provided by the supplier are given in Tables 1 and 2, respectively.

The buffer used in this study was composed of 145 mM NaCl and 10 mM HEPES, pH 7.4. All of the products were used without further purification.

**Sample Preparation.** Aqueous solutions of the standard polysaccharides and the FITC-dextrans were prepared according to the procedure given by Polymer Laboratories. After distilled water or buffer (145 mM NaCl, 10 mM HEPES, pH 7.4) was added to the polymer powder, the solution was allowed to stand 24 h, at room temperature to ensure complete swelling. The concentrations of the different samples were determined by weight, assuming solution densities equal to 1.

**pH and Osmolarity Measurements.** The pH and the osmolarity of the FITC-dextrans in solutions in buffer were measured on the same samples. The pH measurements were performed with a pH-meter (TS4N, Tacussel Electronique) and a glass microelectrode (diameter = 4 mm, Bioblock Scientific). The osmolarity measurements were carried out with an osmometer (Roebling, Bioblock Scientific) calibrated with NaCl solutions.

**Fluorescence Measurements.** The fluorescence experiments were performed on an X-format (reference, two emissions at 90°, 1 transmission) spectrofluorimeter Fluorolog FL411 (Spex Instruments, Jobin Yvon, Longjumeau, France) equipped with four photomultipliers and connected to a computer. The maximum excitation (λ_ex) and maximum emission (λ_em) wavelengths of the FITC-dextrans were 493 and 514 nm, respectively. The emission (λ_em = 493 nm, 500 ≤ λ_em ≤ 580 nm) and excitation (λ_ex = 580 nm, 400 ≤ λ_ex ≤ 500 nm) spectra were recorded at 25 °C, using a quartz cell (optical pathway = 1 cm). The fluorescence intensity was normalized by the apparatus constants.

The sample OD (log I/I_0) was determined in two ways: (1) by measuring the intensity of light transmitted by the samples (I_0) via the transmission photomultiplier of the fluorimeter, I being the intensity of the light transmitted without sample cell; and (2) by measuring the OD values of the samples previously analyzed by fluorescence at 493 and 516 nm with a Perkin-Elmer Lambda 2 double-beam spectrophotometer.

**High Performance Gel Exclusion Chromatography (HPLC-GEC).** HPLC-GEC analysis was performed using a procedure previously developed. A 30 × 0.75 cm TSK-G4000 PW column supplied by Toyo Soda, (Tokyo, Japan) was used. The column was preceded by a 2-μm filter (Rheodyne, CA). The HPLC apparatus was equipped with a Hitachi pump (model L-6000) and a precision injection valve (Rheodyne). The eluent was distilled water or aqueous buffer, and the flow-rate was 1 mL/min. A differential refractometer (R401 Water Associates) was used for measuring the elution parameters. The HPLC-GEC elution parameters of the polysaccharide standards are given in Table 1. The polydispersity index defined as M_w/M_n was obtained by HPLC-GEC on the TSK-G4000 PW column using buffer or pure water as mobile phase.

**Table 1. Weight-Average Molecular Mass, Polydispersity, and HPLC-GEC Elution Parameters of the Polysaccharide Standards**

<table>
<thead>
<tr>
<th>Standards</th>
<th>M_w M_n</th>
<th>K_d buffer</th>
<th>K_d water</th>
</tr>
</thead>
<tbody>
<tr>
<td>738</td>
<td>1.00</td>
<td>0.938</td>
<td>0.933</td>
</tr>
<tr>
<td>5 800</td>
<td>1.07</td>
<td>0.842</td>
<td>0.836</td>
</tr>
<tr>
<td>12 200</td>
<td>1.06</td>
<td>0.728</td>
<td>0.745</td>
</tr>
<tr>
<td>23 700</td>
<td>1.07</td>
<td>0.623</td>
<td>0.670</td>
</tr>
<tr>
<td>48 000</td>
<td>1.09</td>
<td>0.504</td>
<td>0.562</td>
</tr>
<tr>
<td>100 000</td>
<td>1.10</td>
<td>0.369</td>
<td>0.452</td>
</tr>
<tr>
<td>186 000</td>
<td>1.13</td>
<td>0.243</td>
<td>0.329</td>
</tr>
<tr>
<td>380 000</td>
<td>1.12</td>
<td>0.236</td>
<td>0.236</td>
</tr>
<tr>
<td>1 660 000</td>
<td>1.19</td>
<td>0.084</td>
<td>0.210</td>
</tr>
</tbody>
</table>

**Note:**

- Polydispersity index defined as M_w/M_n provided by the supplier.
- Experimental K_d values obtained by HPLC-GEC on the TSK-G4000 PW column using buffer or pure water as mobile phase.

References:

Table 2. Weight-Average Molecular Mass, Polydispersity, Degree of Substitution, HPLC-GEC Elution Parameters, and Radius of Gyration of the FITC-Dextrans

<table>
<thead>
<tr>
<th>FITC-dextrans</th>
<th>$\overline{M}_w$/$\overline{M}_n$</th>
<th>DS$_g$</th>
<th>FITC/gluc mol/mol, initial</th>
<th>$K_d$</th>
<th>$K_w$</th>
<th>$M_{w,HPLC}$</th>
<th>$M_{w,HPLC}$</th>
<th>$M_{w,Gluc}$</th>
<th>$R_g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 400</td>
<td>$&lt;1.50$</td>
<td>0.004</td>
<td>0.842</td>
<td>0.850</td>
<td>4 052</td>
<td>3 408</td>
<td>0.035</td>
<td>0.855</td>
<td>2.0</td>
</tr>
<tr>
<td>9 400</td>
<td>$&lt;1.25$</td>
<td>0.008</td>
<td>0.804</td>
<td>0.711</td>
<td>22 726</td>
<td>17 994</td>
<td>0.015</td>
<td>1.572</td>
<td>3.4</td>
</tr>
<tr>
<td>18 900</td>
<td>$&lt;1.25$</td>
<td>0.009</td>
<td>0.566</td>
<td>0.650</td>
<td>36 675</td>
<td>26 893</td>
<td>0.004</td>
<td>0.844</td>
<td>4.8</td>
</tr>
<tr>
<td>40 500</td>
<td>$&lt;1.25$</td>
<td>0.012</td>
<td>0.457</td>
<td>0.375</td>
<td>57 320</td>
<td>110 910</td>
<td>0.006</td>
<td>2.374</td>
<td>6.3</td>
</tr>
<tr>
<td>69 000</td>
<td>$&lt;1.25$</td>
<td>0.010</td>
<td>0.323</td>
<td>0.235</td>
<td>84 700</td>
<td></td>
<td>0.014</td>
<td>3.284</td>
<td>7.7</td>
</tr>
<tr>
<td>147 800</td>
<td>$&lt;1.35$</td>
<td>0.004</td>
<td>0.233</td>
<td>0.185</td>
<td>11 910</td>
<td></td>
<td>0.004</td>
<td>10.822</td>
<td></td>
</tr>
<tr>
<td>487 000</td>
<td>$&lt;1.35$</td>
<td>0.004</td>
<td>0.123</td>
<td>0.115</td>
<td>14 700</td>
<td></td>
<td>0.004</td>
<td>10.822</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Polydispersity index and $^b$degree of substitution defined as mol FITC/mol glucose provided by the supplier. $^c$ Experimental $K_d$ values obtained by HPLC-GEC on the TSK-G4000 PW column in aqueous buffer and pure water media. $^d$ Apparent $M_w$ calculated from the calibration curves obtained in buffer (eq 4) and water (eq 5) (see text). $^e$ Weight-average molecular mass by the supplier. $^f$ Degree of substitution related to effective labeled chains of the FITC-dextrans, calculated from DS$_g$ and the fraction of the dextrans bearing the fluorophores determined by HPLC-GEC. $^g$ These $K_d$ and $M_w$ values correspond to the elution peaks numbered 1 in Figure 4.

Results

Sample Detection. Samples of 50 μL were injected. For the analysis of the FITC-dextran 9400 in aqueous medium, a fraction collector was used to recover fractions along the different elution peaks.

The polysaccharide standards ($M_w/M_n \leq 1.19$) in distilled water and buffer solutions (0.5 mg/mL) were used to calibrate the TSK-G4000 PW column in both media. The FITC-dextrans were analyzed under the same conditions. The column parameter $K_d$ was calculated from the relation:

$$K_d = (V_e - V_0)/(V_t - V_0)$$

where $V_e$ is the sample elution volume and $V_0$ and $V_t$ are the void and total volumes of the column, respectively.

$V_e$ was taken at the intercept of the baseline with the half-height tangent to the left side of the peak corresponding to the elution of the FITC-dextran of the highest molecular mass (different levels of labeling = 2 000 000). The $V_0$ values were 41.0 mL with pure water and 5.25 mL with aqueous buffer as eluent, respectively. Elution volumes ($V_e$, $V_t$) corresponding to the maximum of the chromatograms were determined by the intercept of the half-height tangents to the symmetrical HPLC peaks. $V_t$ determined from the elution of glucose was 9.98 mL in pure water and 9.93 mL in aqueous buffer, respectively.

The areas of the chromatograms were obtained from refractive index recordings to avoid the drawbacks encountered in quantification by fluorescence detection (i.e., inner filter effect, see text). They were determined by using PeakFit software (Jandel Scientific, Germany). The distributions of the fluorescent labels over the FITC-dextran populations were calculated from the degree of substitution of the FITC-dextrans (Table 2, DS$_g$) by applying the Monte Carlo simulation of labeling. Briefly, a large number of molecules (i.e., 5000 molecules, to get a precision of three digits) were progressively labeled in this model by using the random function of a TurboBasic program. The total number of the possible sites for the labeling were calculated taking into account the label index (Table 2, DS$_g$) given by the supplier (e.g., 0.004 for the FITC-dextran 4400 lead to 543 sites of labeling distributed over 5000 dextran molecules). The label position was calculated on the basis of independent probabilities of each site for labeling. After label random distribution, the number (n) of labels per dextran molecule was counted. Three n values correponding to 0, 1, and n ≥ 2 labels per dextran molecule were considered. The dextran molecules labeled with two FITC groups and over were gathered in a single population n ≥ 2 because these molecules cannot be separated on the TSK-G4000 PW column.

Small-Angle X-ray Scattering (SAXS). Small-angle X-ray scattering was used to determine the radius of gyration of the FITC-dextrans in buffer. The measurements were carried out at 25 °C at station D-22 of the DCI synchrotron at LURE (Orsay, France). The monochromatic wavelength (λ) of the focused X-ray beam was 1.377 Å. A sample-to-detector distance of 1774 mm was used to analyze the polymer solution at small angles (0 ≤ q ≤ 0.1 Å$^{-1}$), where q is the scattering vector related to the scattering angle 2θ by the relation: $q = 4\pi \sin \theta/\lambda$. The X-ray data were recorded on a 1024-channel Xe/ethane-filled linear detector. A thermostated holder equipped with a flow glass capillary (external diameter < 1.5 mm, wall thickness of 0.01 mm, GLAS, Millér, Berlin, Germany) connected to a syringe through a catheter, was used for the experiments. The glass capillary was loaded with the polymer solution via the syringe. This setup allows use of the same glass capillary for all of the experiments so that the intensity is measured in the same experimental conditions for all the samples. This makes possible subtractions of the scattering spectra.

Results

pH and Osmolarity Measurements. Because the fluorescence intensity of the FITC group is sensitive to pH variations, we controlled that the buffer used in this study ensures the pH stability of the fluorescent polymer solutions in a wide dilution range (88 to 10$^{-6}$ mg/mL).

The osmolarity of the FITC-dextran 4400 solutions (3.4 × 10$^{-6}$ to 88 mg/mL; i.e., 7.7 × 10$^{-7}$ to 20 mM) in the buffer used for the encapsulation was measured. Because the osmolarity of a solution is proportional to the number of the particles dissolved in the solvent, increasing values of the osmolarity were obtained with increasing polymer concentration. A linear regression of the osmolarity vs. polymer concentration was carried out.

References


Analytical Chemistry, Vol. 74, No. 20, October 15, 2002 5219
experimental values gives the following equation:

\[ N = 2.40[\text{FITC-dextran 4400}]_{\text{mM}} + 283 \]  

(2)

where \( N \) is the number of milliosmoles in the medium and \([\text{FITC-dextran 4400}]_{\text{mM}}\) is the polymer concentration expressed as millimolar.

This relation shows that the osmolarity remains in the order of magnitude of that of the aqueous buffer for dilute polymer solutions. The result is that the encapsulation of the FITC-dextrans into lipidic vesicles does not affect the osmolarity of the internal aqueous medium of the vesicles, as compared to that of the external buffer for a polymer concentration < 1 mM. Beyond this concentration threshold, the NaCl concentration of the buffer used for the encapsulation must be adjusted to prevent osmotic breaking of the liposomes. The effect of the osmolarity of the encapsulated dextran solution on the integrity of the vesicles is all the more important when the molecular weight of the polymers is lower. Indeed, the number of the dextran molecules corresponding to an equivalent weight concentration strongly decreases with increasing \( M_w \). For example, the osmolarity of an 81 mg/mL (0.55 mM) solution of the FITC-dextran 147 800 is very similar to that of the buffer, which is in agreement with the above equation.

**Fluorescence Measurements.** The fluorescent behavior of the FITC-dextrans were studied in buffer medium with two fluorescent dextrans (FITC-dextrans 4400 and 147 800) having the same FITC/glucose molar ratio (0.004) (Table 2). The insets of Figure 1 show the evolution of the wavelength at the maximum of the experimental emission spectra (\( \lambda_{\text{em, max}} \)) versus the FITC-dextran concentration. The \( \lambda_{\text{em, max}} \) remains constant at 516 nm up to a polymer concentration of 0.033 mg/mL, thereafter being continuously shifted to higher values. The dependence of the fluorescence intensity on the polymer concentration was studied at 516 nm so as to follow the fluorescence intensity in the same state of energy for the excited probe. The experimental (\( I_{\text{em, exp}} \)) emission fluorescence intensities at 516 nm are reported versus the polymer concentration in Figure 1a,b (solid circles) for the FITC-dextrans 4400 and 147 800, respectively. The fluorescence intensity linearly increases with increasing polymer concentration as long as the \( \lambda_{\text{max, exp}} \) remains constant (Figure 1 insets), that is, up to 0.033 mg/mL. Beyond this concentration, the curve of the fluorescence intensity presents a maximum around 0.33 mg/mL and then continuously decreases, in conjunction with the shift observed for the \( \lambda_{\text{max, exp}} \).

To take into account the absorption and scattering of light generated by the polymer, the fluorescence intensities were corrected by the OD of the samples at both the excitation and emission wavelengths (see Experimental Section), by using the relationship given by Lakowicz:

\[ I_{\text{corr}} = I_F \times 10^{(\text{OD}_{\text{exc}} + \text{OD}_{\text{em}})/2} \]  

(3)

where \( I_F \), \( \text{OD}_{\text{exc}} \), and \( \text{OD}_{\text{em}} \) are the experimental fluorescence intensity and the optical densities at \( \lambda_{\text{exc}} = 493 \) nm and \( \lambda_{\text{em}} = 516 \) nm, respectively.

Figure 1 (open markers) shows that the linear range of the corrected (\( I_{\text{em,corr}} \)) emission fluorescence intensities is expanded up to a polymer concentration of ~2 mg/mL, that is, to a concentration 60 times higher than without correction. Note that both methods used to determine the sample OD give the same corrections in our experimental conditions (see Experimental Section). Beyond this limit, the OD values measured for the dextran solutions are very high, and Lakowicz’s relationship gives overestimated values for the corrected intensities. We have therefore applied eq 3 only in the polymer concentration range, which gives a linear increase of the fluorescence intensity.

Excitation and emission spectra of FITC-dextran solutions with concentrations ranging from \( 10^{-6} \) to 10 mg/mL were recorded. As illustration, Figure 2 shows the experimental spectra (dashed curves) obtained with the FITC-dextran 147 800 in buffer solutions at dilute (0.03 mg/mL) (Figure 2a,c) and high (1.20 mg/mL) concentrations (Figure 2b,d). The absorption spectra, measured with the double-beam spectrophotometer are also reported (Figure

![Figure 1](image1.png)

**Figure 1.** Dependence of the fluorescence intensity of the emission spectra at \( \lambda = 516 \) nm on the concentration of the FITC-dextran 4400 (a) and 147 800 (b) in buffer solutions: (○) experimental fluorescence intensity (\( I_{\text{em, exp}} \)) normalized as above and corrected by the sample OD at \( \lambda = 493 \) and 516 nm measured with a Perkin-Elmer Lambda 2 double-beam spectrophotometer, (○) fluorescence intensity normalized as above and corrected by the sample transmission at \( \lambda = 493 \) and 516 nm measured via the X format setup of the fluorimeter. The corrected fluorescence (\( I_{\text{em, corr}} \)) intensities were calculated by using eq 3. The graph is presented with a log–log scale. Insets a and b: evolution of the wavelength at the apparent maximum of the experimental emission spectra (\( \lambda_{\text{em, max}} \)) versus the concentration of the FITC-dextran 4400 (○) and 147 800 (○).
The FITC-dextran molecular mass. Peaks 1 are observed in the high-V_c region (>7 mL) for \( M_w \) ranging from 4400 to 69 000, and their \( V_c \) max and areas decrease with increasing molecular mass. Because the peak area measured by RI is directly related to the amount of the polymer eluted, it is clear that the proportion of this polymer fraction continuously decreases with increasing \( M_w \) and is negligible for the FITC-dextran 69 000 (Figure 4e). The peak area of this polymer population follows a decreasing exponential law as a function of \( M_w \), and tends to zero from \( M_w \) > 69 000. The \( K_d \) values of peaks 1 and the corresponding \( M_w/HPLC \) calculated from eq 4 are given in Table 2. These \( K_d \)s fit the selectivity curve obtained with the polysaccharide standards (Figure 3b), indicating that the column sieves a fraction of the fluorescent polymers according to their size, as for the standards. Peaks 2 (Figure 4a–c) of weak amplitude occur only for the FITC-dextran 4400, 9400 and 18 900, and are shifted toward smaller \( V_c \) as the size of the fluorescent polymer studied except the FITC-dextran 487 000, and peaks 4 appear from FITC-dextran 18 900, both alternatively existing as a major peak or as a shoulder. Their respective \( V_c \)s remain constant, but the peak areas are \( M_w \)-dependent. \( K_d \)s corresponding to each elution peak are reported in Figure 3.

**Small-Angle X-ray Scattering (SAXS).** The buffer solution used to prepare the samples was first analyzed prior to the polymer samples. The FITC-dextran solutions in the 1 to 20 mg/mL concentration range, were prepared in buffer as described above. For each fluorescent polymer, the solutions were analyzed at various concentrations in order to determine the concentration for which the particles are not in interaction while the scattering signal remains the more intense. The scattering intensities were normalized with respect to synchrotron beam decay, acquisition times, and sample transmission. The scattering pattern of the

![Figure 2](Image 1)

**Figure 2.** Fluorescence of FITC-dextran 147 800 in buffer solution at concentrations of 0.03 (a,c) and 1.20 (b,d) mg/mL. Excitation spectra (a,b) (\( \lambda_{em} = 580 \text{ nm} \), \( \lambda_{ex} = 400–500 \text{ nm} \)): (---) experimental spectra, (--) spectra corrected by OD according to eq 3 (see text). (---) Absorption spectra recorded on the Perkin-Elmer Lambda 2 double-beam spectrophotometer. Emission spectra (c,d) (\( \lambda_{exc} = 493 \text{ nm} \), \( \lambda_{em} = 490–580 \text{ nm} \)): (---) experimental spectra, (--) spectra corrected as above.

2a,b, dash–dot curves). For the same reason as above, the experimental fluorescence spectra were corrected at each wavelength recorded by applying eq 3 (Figure 2 solid lines).

**HPLC-GEC Experiments.** The TSK-G4000 PW column was calibrated with polysaccharides of molecular masses of very low polydispersity (Table 1). These standards fit the molecular mass range of the FITC-dextran used. The aqueous buffer and pure water elute the polysaccharide standards through a single elution peak. Examples of chromatograms are shown in the Figure 3 insets. The selectivity curves of the column expressed as log \( M_w \) versus \( K_d \) are reported in Figure 3a for aqueous buffer and Figure 3b for pure water as the mobile phases, respectively. By fitting the experimental values (open circles, solid line Figure 3a; open squares, solid line Figure 3b) to a polynomial of the third degree, the experimental values (open circles, solid line Figure 3a; open squares, solid line Figure 3b) to a polynomial of the third degree, two equations were determined:

\[
\text{in buffer (coefficient correlation, 0.995)} (4)
\]

\[
\text{in pure water (coefficient correlation, 0.999)} (5)
\]

The FITC-dextran were chromatographed under the same conditions as those used for the standards. They were eluted through a single symmetrical peak in the buffer used as eluent (Figure 3a, inset). The evolution of the \( K_d \) values as a function of the FITC-dextrans \( M_w \) (Table 2) follows the calibration curve of the column obtained in the same buffer (Figure 3a). In contrast, the elution profiles of the fluorescent dextrans is completely different in water medium. As shown in Figure 4a–g, several major peaks (numbered 1–4) are detected, depending on the
buffer was subtracted from those of the polymer solutions to obtain the scattering intensity of the macromolecules alone. The normalized scattering profiles (intensity (I) versus q) of the FITC-dextrans 40 500, 69 000 and 147 800, are reported in Figure 5.

**DISCUSSION**

FITC-dextrans served as fluorescent markers for a number of fundamental researches, for example, in membrane permeabilization or in polar solute transport across biological membranes. Their characterization under the conditions encountered in such studies is therefore of interest. Membrane permeabilization is often followed by the release of a water-soluble probe from the inner aqueous compartment of the vesicles. This needs encapsulation of the marker, which must be achieved without osmotic shocks, to preserve the vesicle integrity. Measurements of the osmolarity of the FITC-dextrans as a function of the polymer concentration show that the FITC-dextran solutions at a polymer concentration lower than 1 mM are isoosmolar with the buffer. For higher polymer concentrations, the NaCl concentration of the buffer used for the encapsulation must be changed to match the osmolarity of the polymer solutions.

**Fluorescent Behavior of the FITC-Dextran Solutions.**

The fluorescence properties of the FITC-dextran solutions have been examined over a wide concentration range. Figure 1 shows that the experimental emission fluorescence intensity \( I_{em, exp} \) is no longer proportional to the polymer concentration above 0.033 mg/mL, higher concentrations leading to decreased fluorescence intensity. This concentration effect can result either from fluorescence quenching or an inner filter effect. This last causes an apparent quenching as a result of high optical densities, including absorption and light scattering of the samples. The corrected \( I_{em, cor} \) emission fluorescence intensities (Figure 1, open markers) clearly indicates that the apparent decrease observed in the experimental fluorescence intensities (Figure 1, solid circles) beyond the concentration of 0.33 mg/mL comes from an inner filter effect. Spectra of Figure 2 further illustrate this point. For the dilute concentration (Figure 2a,c), the profiles of the experimental (dashed curves) and corrected (solid-line curves) excitation spectra (Figure 2a) are similar to that of the absorption spectrum (dash-dot curves) and the maximum intensities of the three spectra are observed at the same wavelength. It is the same for the experimental and corrected emission spectra. (Figure 2c). Only the corrected fluorescence intensities are slightly amplified.

For the high concentration, the wavelength maximum of the experimental excitation \( \lambda_{exc} \) and emission \( \lambda_{em} \) (Figure 2b,d) are observed at 465 and 525 nm, respectively. Once the spectra are corrected, the fluorescence characteristics become similar to that observed for the dilute dextran solution regarding both the spectrum profile and \( \lambda_{max} \). In parallel, the experimental excitation and emission fluorescence intensities are strongly enhanced. The same absorption UV—vis spectrum is recorded at the low and high polymer concentrations (Figure 2a,b, dash—dot curves), indicating that there is no interaction between the chromophore groups of the FITC-dextran, and then self-quenching cannot exist. Indeed, interactions between the chromophores would shift the absorption spectrum. All of these results clearly demonstrate that the attenuation of the experimental fluorescence intensities of the FITC-dextran solutions as well as the apparent shift of the experimental fluorescence emission spectra \( \lambda_{em} \) (Figure 1) are essentially due to an inner filter effect. This result is in agreement with the degrees of substitution of the FITC-dextrans studied (0.003—0.009) (Table 2), which are too weak to produce quenching effects.

The fluorescence behaviors of the FITC-dextrans show that when they are encapsulated within vesicles for measuring a subsequent release, no fluorescence extinction will be obtained. This indicates independently measuring the fluorescence intensity of the internal medium and that of the external aqueous medium of the vesicles during the leakage process, which can be easily performed by HPLC-GEC. Note that quantitative measurements require correction for the inner filter effect of the probe at high release content. However, beyond a dextran concentration limit of 2 mg/mL, correction using Lakowicz’s relationship is no longer valid.

**Characterization by HPLC-GEC.** The elution behavior of the polysaccharide standards (Figure 3) differs according to the mobile phase used. Indeed, the standards lie on different calibration curves with buffer and pure water. This result is clearly shown.
In Figure 3b, where the selectivity curve obtained in the presence of buffer is also reported (dashed curve). The two curves cross at a $K_d$ centered at 0.8 and give close values only in a narrow $K_d$ range (corresponding to $738 \leq M_w \leq 12200$). For higher $M_w$, the $K_d$ values are smaller when the mobile phase is buffer: the higher the mass of the polysaccharide standard, the larger the difference in $K_d$. It follows that the $K_d$ values obtained in buffer medium correspond to larger particles than those in pure water. This difference was ascribed to salt solution’s being a better solvent for dextran than pure water.\(^3\) Thus, the polysaccharide molecules may swell more in buffer solution, without the limiting
defect of $M_w$, explaining the increase in the gap observed between both calibration curves with increasing $M_w$. These results agree with the observations of Antonini et al.\(^3\) These authors have shown on a sample of native dextran that the $M_w$ measured by sedimentation and viscosity experiments remained unchanged in pure water solution and aqueous solutions containing NaCl up to 4 M, whereas the radius of gyration determined by light scattering measurements increased significantly in salt solutions. This has implications on the molecular volume of the polymer in buffer solution.

HPLC-GEC reveals that the FITC-dextrans elute over the TSK-G4000 PW column through a single population in buffer medium (Figure 3a), indicating a uniform distribution of molecular weights. Conversely, separation into several species (Figures 3b and 4), occurs when the mobile phase is pure water and only the fraction corresponding to the highest $K_d$ is sieved according to the standard selectivity curve.

The different swelling observed for the standards as a function of the solvent should also occur for the FITC-dextrans. However, this does not explain the elution behavior in pure water medium. In contrast to the standards, which are neutral molecules, the FITC groups chemically linked to the dextran molecules are negatively charged at pH 7.4.31 It has been shown that the ionic strength of the eluent markedly influences the elution behavior of charged species.40 Lowering the ionic strength of the solvent leads to a decrease in the retention time of the anionic solutes that tend to elute near the exclusion limit. This is what is observed for the elution of a fraction of the FITC-dextran solutions in pure water medium (Figures 3b and 4).

According to the FITC/glucose monomer ratios of the FITC-dextrans, which span from 0.003 to 0.008 (Table 2, DS1), only some glucose units are linked to FITC molecules. Therefore, the fluorescent polymers are likely composed of a mixture of neutral dextran molecules free of fluorophore and charged ones owing to the FITC groups in a ratio depending on $M_w$. It follows that the fluorophore-bearing and neutral molecules elute differently through the TSK-G4000 PW column in water medium. It was assumed that the fraction of the polymer free of chromophores would be eluted at the $K_d$ corresponding to the selectivity curve and, therefore, would behave as the polysaccharide standards. Conversely, the other elution peaks observed at smaller $K_d$ values would be related to the fluorescent dextran molecules according to the number of the FITC groups linked per dextran molecule.

To verify this hypothesis, the FITC-dextran 9400 was injected onto the column at a low flow rate (0.5 mL/min) with pure water as mobile phase (Figure 4b). Fractions were collected all along the chromatogram, and their respective fluorescence spectra were recorded. The evolution of the maximum fluorescence intensity of each fraction versus $V_e$ is reported in Figure 4b (dotted line). The fluorescence of the fractions collected along elution peak 1 is very weak, indicating the quasiabsence of fluorescent chromophores on the dextran molecules, whereas the fluorescence of the fractions along the peaks at smaller $K_d$ values is much higher, showing that these fractions contain FITC-labeled chains. This confirms that the dextran molecules bearing FITC groups elute independently of the nonfluorescent dextrans. The values of the $M_w$ corresponding to the fraction of the unlabeled dextrans (Figure 4, peaks 1) calculated from eq 5 (Table 2) remains relatively close to those obtained in buffer medium, indicating a similar distribution of the molecular weights for the labeled and unlabeled dextran molecules. The elution mechanism of the labeled dextran molecules in pure water is not governed by the molecule size and then by $M_w$, but directly related to the charges of the chromophore. Their lower elution volumes may be due to the fact that the charged molecules are partially excluded from the pores of the gel. Repulsive forces between the chromophores may also change the conformation of the charged dextran molecules.

Differences in elution behavior have also been observed for dextrans that are either unlabeled or labeled with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) when they are chromatographed on silica gel in pure water.41 By coupling RI and fluorescence detections, the authors have shown that for the labeled polymer, the fraction containing the fluorescent probe was eluted in the $V_0$ region, whereas the unlabeled molecules were detected separately at the $V_e$ corresponding to the parent dextran. These two peaks were resolved in a single one, when 0.05 M phosphate buffer was used as the mobile phase.

The observed exclusion mechanism may come from electrostatic repulsion between the negatively charged FITC groups of the fluorescent dextrans and the negatively charged OH groups.
Radius of Gyration of the FITC-Dextrans in Buffer by SAXS. Existence of pores in a membrane is easily visualized via permeability measurements. The use of probes with increasing \( M_w \), such as the FITC-dextrans, gives information on the nature of the pores (transient or permanent) and their subsequent growth under stress conditions, such as time, temperature, and addition of foreign molecules. The point of interest is to have access to the mean diameter of the pores created in the membrane. The radius of gyration \( R_g \) of the FITC-dextrans can give a good evaluation of the pore size. Because swelling of polymers is solvent-dependent, the values of \( R_g \) must be obtained in the solvent used in the study.

\[ R_g \text{ can be determined from X-ray scattering of the particles at small angles by using Guinier's equation,} \]

\[ I = I_\infty + I_0 \exp(-1/3 (qR_g)^2) \]  

where \( I \) is the scattering intensity of the particles, \( I_\infty \) the intensity when \( q \rightarrow \infty \), \( I_0 \) the intensity when \( q \rightarrow 0 \), \( q \) is the scattering vector. Equation 6 allows determination of \( R_g \) without assuming the particle shape.

The normalized scattering profiles (intensity \( I \) versus \( q \) ) (Figure 5, cross), of the FITC-dextrans were fitted by eq 6 (Figure 5, thick curve). The parameters \( R_g \), \( I_\infty \), and \( I_0 \) were adjusted to obtain a calculated curve that best fits the experimental data. Guinier's equation yields calculated intensities very close to the experimental ones up to \( q = 4 \times 10^{-2} \text{Å}^{-1} \) (Figure 5). The values of \( R_g \) determined by SAXS, are indicated in Table 2. The evolution of \( R_g \) versus FITC-dextran molecular masses determined by HPLC-CEG \((M_w,HPLC)\) (Figure 6) shows that \( R_g \) progressively increases with increasing molecular masses. This evolution verifies the proportionality of \( R_g \) versus the square root of \( M_w,HPLC \) as follows:

\[ R_g \text{ (nm) = 0.025} \ M_w,HPLC^{0.5} \ R = 0.969 \]  

This relationship indicates that the FITC-dextrans in buffer solution behave as linear flexible polymers in the Flory \( \theta \) solvent.\(^{44,45}\) Thus, the polymer chain conformation is very likely of random coil type, that is, each polymer chain can be considered as a small globular particle in buffer medium. The conformity of the relationship 7 with a solute shape expected for flexible coils shows that \( M_w,HPLC \) is representative of the average molecular masses of the FITC-dextrans. Equation 7 also shows that the wide range of the molecular masses of the fluorescent polymers analyzed corresponds in fact to a relatively limited \( R_g \) range.

For comparison, the unlabeled polysaccharide standard \( M_\theta = 48 \text{,}000 \) was analyzed in buffer solution with the same procedure. The \( R_g \) value of this standard (5.15 nm) lies on the curve fitted with the experimental values obtained for the fluorescent polymers (Figure 6), indicating that the FITC groups linked to some glucose units of the dextran molecules do not significantly modify the polymer swelling in buffer medium.

\[ \text{(43) Guinier, A. Théorie et technique de la radiocristallographie, Dunod: Paris, 1964; Chapter 14, pp} 637–669. \]

\[ \text{(44) Candau, S. J. In Surfactant Solutions. New Methods of Investigation; Zana, R., Ed.; Marcel Dekker: New York, 1987; Chapter} 3. \]

\[ \text{(45) Hagel, L. In Protein Purification, Principles, High-Resolution Methods, and Applications; Janson, J. C.; Ryden, L. Eds.; Wiley-Liss: New York, 1998; Chapter} 3, pp} 79–143. \]
CONCLUSION

The present work focused on different physicochemical characteristics of the FITC-dextrans, which are of interest when these polymers are used as release markers, especially in permeability studies. The fluorescence properties of the polymer solutions in buffer medium reveal the existence of an inner filter effect caused by the optical densities of the polymer solutions at high concentrations. This effect should be taken into account for quantitative measurements. The medium used to analyze the FITC-dextrans by HPLC-GEC on the TSK-G4000 PW column strongly influences their elution behavior. The buffer medium is appropriate to determine their average molecular masses, in contrast to pure water, which elutes the polymers in several species. HPLC-GEC in water demonstrates that the FITC-dextrans are composed of a mixture of nonfluorescent molecules and fluorescent ones bearing different levels of labeling. Separation of the different fluorescent populations follows an exclusion mechanism that depends on the number of labeled sites per dextran molecule. The elution behavior observed for the FITC-dextrans in water medium can also be expected for polymers labeled with any ionic fluorescent groups. Thus, HPLC-GEC in aqueous medium offers an interesting preparative way to produce monodisperse fluorescent polymers bearing a known chromophore number. In addition, this procedure could be applied to other functionalized polymers randomly grafted with charged derivatives or bearing reacting sites. Well-defined polymers with a known grafting rate or number of reacting sites offer potential applications in the field of permeability studies, drug targeting, and molecular tracing of living species as well as in chemical synthesis.

In conclusion, determination of the average molecular masses of charged dextrans by HPLC-GEC requires analysis in a medium that suppresses ionic effects either between the charges themselves or between the charges and the gel filtration. On the other hand, for analysis of the number of chromophores, it is necessary to use pure water as mobile phase to observe the charge effect. The characterization of the polymer conformation must be carried out in the medium used for the study, because the polymer shape is strongly dependent on the solvent. From the radii of gyration of the FITC-dextrans obtained by SAXS, it has been shown that each polymer chain can be considered to be a small globular particle in buffer medium.

ACKNOWLEDGMENT

Dr. K. Andrieux thanks the I.F.S.B.M. (Institut de Formation Supérieure Biomédicale) for its financial support. We thank Dr. Yoshio Kato (Toyo Soda, Japan) for the gift of the TSK G4000 PW column.

Received for review February 22, 2002. Accepted July 26, 2002.

AC020119L